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Research Article

Antibiotic use during pregnancy is linked to offspring gut microbial dysbiosis, barrier disruption, and altered immunity along the gut–lung axis

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Antibiotic use during pregnancy is associated with increased asthma risk in children. Since approximately 25% of women use antibiotics during pregnancy, it is important to identify the pathways involved in this phenomenon. We investigate how mother-to-offspring transfer of antibiotic-induced gut microbial dysbiosis influences immune system development along the gut–lung axis. Using a mouse model of maternal antibiotic exposure during pregnancy, we immunophenotyped offspring in early life and after asthma

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induction. In early life, prenatal-antibiotic exposed offspring exhibited gut microbial dysbiosis, intestinal inflammation (increased fecal lipocalin-2 and IgA), and dysregulated intestinal ILC3 subtypes. Intestinal barrier dysfunction in the offspring was indicated by a FITC-dextran intestinal permeability assay and circulating lipopolysaccharide. This was accompanied by increased T-helper (Th)17 cell percentages in the offspring's blood and lungs in both early life and after allergy induction. Lung tissue additionally showed increased percentages of RORγt T-regulatory (Treg) cells at both time points. Our investigation of the gut–lung axis identifies early-life gut dysbiosis, intestinal inflammation, and barrier dysfunction as a possible developmental programming event promoting increased expression of RORγt in blood and lung CD4$^+$ T cells that may contribute to increased asthma risk.

Keywords: asthma · antibiotics · pregnancy · Th17 cell · gut–lung axis

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Antibiotic use during pregnancy and subsequent alterations to the maternal and offspring gut microbiomes, are associated with increased risk for the development of childhood asthma [1]. During infancy, the gut microbiota is established via a sequential process [2–4], and interactions between immune cells and intestine colonizing microbes are instrumental to educate the developing immune system [5, 6]. This early-life process of host–commensal interaction is critical for mucosal immunity and homeostasis, and subsequently, perturbations to the system can have long-term effects on immune function [7, 8]. Human studies have shown significant differences in the early-life gut microbiota composition between asthmatic and nonasthmatic children [2, 9], and germ-free mice have prominent immune system defects [10] and increased asthma severity compared to WT mice [11]. Collectively, these findings contribute to the Developmental Origins of Health and Disease Hypothesis, which identifies critical developmental "windows of opportunity" during the pre- and postnatal time periods in which programming of immune cell subsets can influence disease susceptibility later in life. Though epidemiological [12, 13] and proof-of-concept mouse models [14, 15] provide strong evidence for the association between antibiotic use during pregnancy, gut microbial dysbiosis, and increased offspring asthma susceptibility, the key players and pathways in this process are still yet to be elucidated. Since 25–40% of pregnant women use antibiotics [16, 17], it is increasingly important to understand these underlying mechanisms.

Considering the gut–lung axis, the intestinal microbiota is thought to have a far-reaching systemic influence that can also impact pulmonary immunity [18, 19]. Studies show that antibiotic-induced, gut bacterial dysbiosis transferred from the mother can induce intestinal inflammation and barrier dysfunction in murine progeny [20], allowing bacterial constituents such as lipopolysaccharide (LPS), access to the circulation [21]. There are several ways that the microbiota can influence intestinal inflammation, including dysregulation of short-chain fatty acid (SCFA) production, secretory immunoglobulin A (sIgA) concentrations, and innate lymphoid cell (ILC) function. Taken together, these candidate immune components can all also contribute to barrier dysfunction. SCFAs are anti-inflammatory metabolites produced by the microbiota during dietary fibre fermentation, and microbial dysbiosis results in decreased SCFA production due to the elimination of particular bacterial clades. Reduced concentrations of fecal acetate, propionate, and butyrate in early life are associated with barrier dysfunction [22] and increased asthma risk [2, 23]. Additionally, mouse models have shown that acetate can influence sIgA concentrations in the small intestine, which acts as a first line of defense at the mucosal surface by binding to commensal bacteria [24]. Dysregulation of sIgA binding specificity can disrupt barrier function [25] and was observed to precede asthma development in children [26].

In addition to soluble molecules, gut immune cells can also contribute to gut permeability in the inflamed intestine during microbial dysbiosis. Of particular interest in this regard are the ILCs, the innate counterparts of T cells that participate in the establishment of mucosal immunity [27]. Intestinal ILC populations in mice increase significantly 2–4 weeks after birth, corresponding to the rise in intestinal microbiota diversity that occurs during the weaning process [28]. In the case of the gut–lung axis, type 2 and 3 ILCs (ILC2 and ILC3) are of interest due to their respective roles in allergy and gut epithelial homeostasis [29]. Though ILCs are normally tissue resident, recent evidence shows that under inflammatory conditions, ILC2 subsets may migrate from the intestine to the lungs [28, 30]. In addition to this, ILC3s have been recently shown to "patrol" the intestinal barrier during times of inflammation, and cessation this movement was associated with intestinal barrier damage [31]. The mesenteric LNs provide a robust connection between the intestine and the circulation, and we propose that disruption of intestinal barrier function in early life may play a key role in susceptibility to allergic asthma [21, 22].

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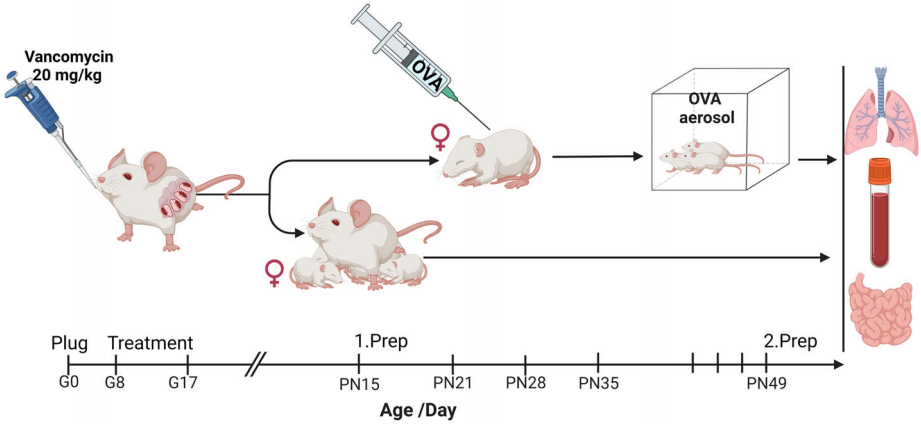

Figure 1. Experimental design. Pregnant mice were treated orally, daily from G8 to G17 with 20 mg/kg vancomycin plus Ora-Sweet syrup. Control mothers were treated orally with water and Ora-Sweet. At PN15, a group of pregnant mice and their pups were sacrificed for analysis of breast milk and offspring immune system development. At weaning (PN21), female offspring were subjected to an experimental asthma protocol consisting of subcutaneous sensitization with OVA at offspring age 21, 28, and 35, followed by asthma provocation with a 20-min daily OVA aerosol challenge at offspring age 46, 47, and 48. G = Gestation day, PN = Postnatal day, OVA = Ovalbumin.

Though there is much evidence that gut dysbiosis alters the developing mucosal immune system, there is still a paucity of information regarding how maternal antibiotic use during pregnancy influences offspring immunity and asthma susceptibility. To study this phenomenon, we recently developed a mouse model in which maternal treatment with the antibiotic vancomycin during pregnancy resulted in increased allergic asthma severity in the offspring [15]. The objective of this research is to use our newly developed mouse model to investigate how the transfer of antibiotic-induced maternal gut dysbiosis to the next generation influences gut permeability and immune system development in the progeny along the gut–lung axis. We hope to delineate important pathways that may be involved in the increased asthma susceptibility observed in our model. Further, we aim to assess the persistence of these immune changes by examining the influence of this early-life programming on susceptibility to allergic asthma in adult mice.

Results

Experimental design

We previously established a model in which treatment of pregnant mice with vancomycin resulted in increased asthma severity in the offspring [15]. For the present study, we treated pregnant mothers with vancomycin from gestation day (G)8-17, then conducted immunophenotyping of the offspring gut–lung axis both in early life (postnatal day-PN15) and after asthma induction PN49, shown in Fig. 1.

Vancomycin during pregnancy, offspring gut dysbiosis, and decreased fecal SCFA concentrations

To temporally assess changes in the maternal and offspring gut microbiota and SCFA concentrations after treatment with vancomycin during pregnancy, we collected fecal samples at the following time points: Maternal (before mating, at the end of antibiotic treatment at gestation day (G)17, and at weaning postnatal day (PN)21, offspring (at weaning at PN21, and after allergic asthma induction at PN49). The top 16 abundant clades identified by 16S rRNA sequencing showed that G17 pregnant mice had a marked gut dysbiosis on the last day of antibiotic treatment when compared with control pregnant animals. This was evidenced by a significantly increased abundance of the genera *Parabacteroides*, *Escherichia*, and *Sutterella*. G17 antibiotic-treated mothers also exhibited significantly decreased proportions in unclassified members of the families Lachnospiraceae and Rikenellaceae and the genera *Oscillospira, Odoribacter*, and *Prevotella*, shown in Fig. 2A and Supporting Information Table S1.

Analysis at weaning (PN21) confirmed the transgenerational transmission of gut bacterial dysbiosis, as the gut microbiota resembled each other in prenatal-control as well as prenatal-antibiotic mother-offspring groups. In antibiotic-treated mothers and offspring at PN21, we observed increased proportions of the genera *Akkermansia* and *Sutterella* compared to controls. The PN21 offspring also displayed increased proportions of *Bacteroides* and *Escherichia*. Proportions of Lachnospiraceae, *Oscillospira*, *Odoribacter*, and *Prevotella* decreased in mothers from G17 to PN21, and these decreases were also observed in their offspring. PN21 prenatal-antibiotic offspring also exhibited decreased proportions of Rikenellaceae and *Lactobacillus* (Fig. 2B and Supporting Information Table S1). Finally, prenatal-antibiotic adult allergic offspring exhibited gut dysbiosis until PN49. This was characterized by increased proportions of *Akkermansia*, *Bacteroides, Parabacteroides*, and *Sutterella*, and reduced *Odoribacter* and *Prevotella* compared to PN49 prenatal-control allergic offspring (Fig. 2C and Supporting Information Table S1). In addition to changes to the top 16 clades of the maternal and offspring gut microbiota, there were also several significant changes observed in less abundant bacterial groups including increased proportions of the genus *Blautia* (PN21 and PN49), Supporting Information Table S1. Decreased proportions of less abundant clades included the genera *Bilophila* (G17), *Butyricicoccus, Coprobacillus*, and *Dorea* (G17 and PN21), *Candidatus* (G17 and PN49) and *Clostridium, Coprococcus*, *Dehalobacterium*, *and Ruminococcus* (all time points), shown in Supporting Information Table S1.

Maternal antibiotic treatment during pregnancy also reduced the gut microbiota α-diversity (Shannon index) in both mothers

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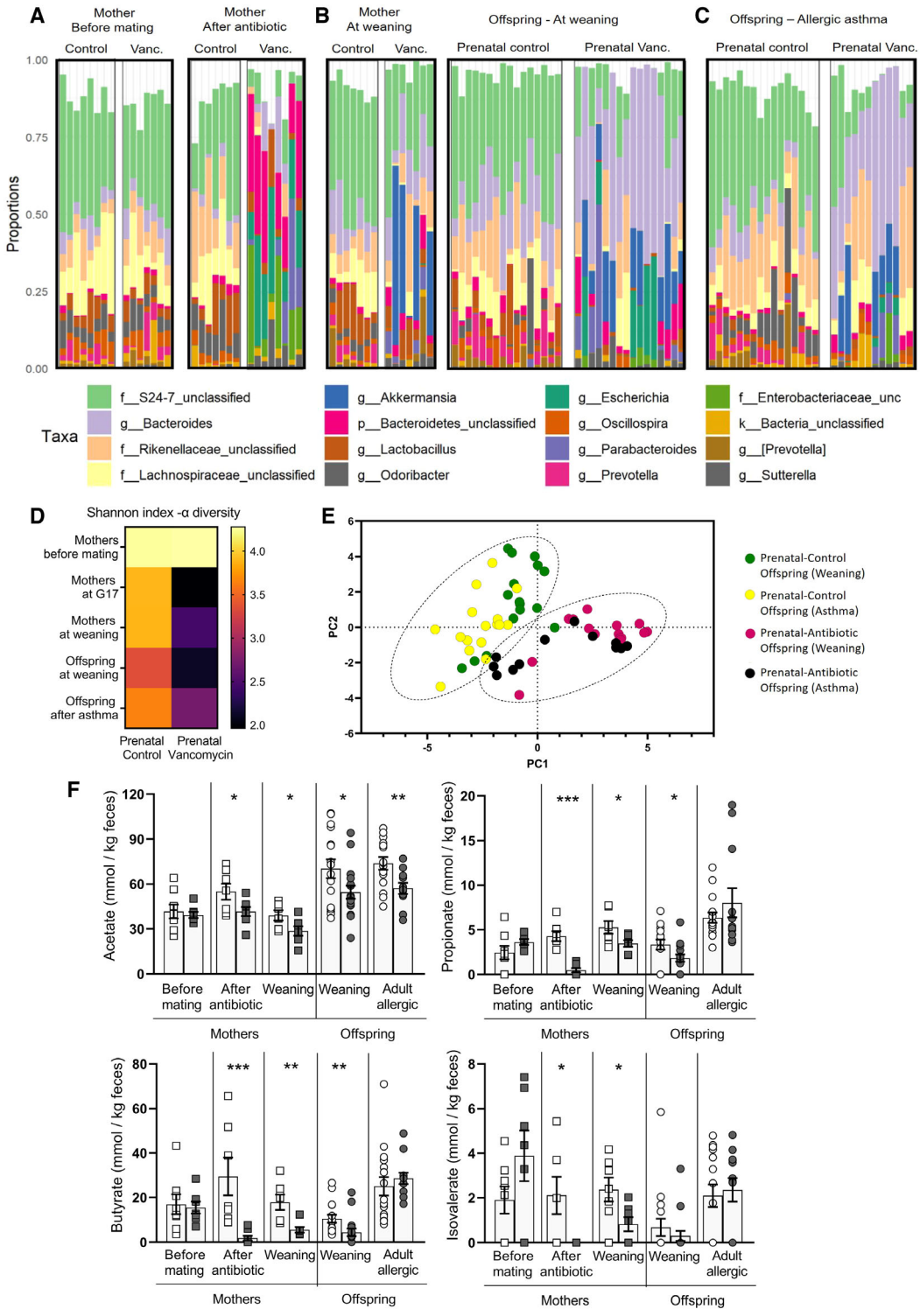

Figure 2. Antibiotic treatment during pregnancy alters the maternal and offspring gut microbiomes as well as fecal SCFA concentrations. Fecal samples were collected from: Mothers (n = 7) before mating, after antibiotic treatment at G17, and at weaning at PN21; Offspring (n = 16) at weaning PN21 and after allergic asthma induction at PN49 (prenatal-control n = 16, prenatal-antibiotic n = 12). **(a-c)** taxonomic bar plots of 16S rRNA gene frequencies in feces. White space on top of a bar represents lower-abundance taxa. Classification: k - kingdom; p - phylum; f - family; g - genus. Each bar represents one mouse. **(d)** Shannon index (α-diversity) of the fecal gut microbiota from mothers and offspring. **(e)** Principal component analysis comparing fecal bacterial communities in prenatal-control and prenatal-antibiotic offspring at weaning and after allergic asthma induction. **(f)** Short-chain fatty acid concentrations in maternal and offspring feces: acetate, propionate, butyrate, and isovalerate. Means ± SEM are shown. Data shown is pooled from two replicated experiments showing the same trends and significance is represented by $^{*}P < .05$, $^{**}p < .01$, $^{***}p < .001$. Student's *t*-test or Mann–Whitney test of antibiotic exposed animals compared against control animals at each specific time point.

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and their offspring at all time points, shown in Fig. 2D. Principal component analysis of the offspring at weaning and after allergy induction revealed that prenatal-control offspring grouped together regardless of age and asthma induction; likewise, prenatal-antibiotic offspring grouped together at both of these time points. Antibiotic treatment during pregnancy was more of a determining factor for gut microbiome composition than asthma induction, showing a large shift in community composition when compared to prenatal-control offspring (Fig. 2E).

Finally, we sought to investigate if the antibiotic-induced changes to the intestinal bacterial community were associated with temporal changes in fecal SCFA concentrations. We showed that antibiotic treatment during pregnancy resulted in significantly reduced acetate, propionate, butyrate, and isovalerate in the feces of G17 antibiotic-treated mothers when compared with control mothers, shown in Fig. 2F. These decreased SCFA concentrations persisted until weaning at PN21 and were also observed in the offspring (with the exception of isovalerate). In adult allergic offspring at PN49, though propionate and butyrate levels in prenatal-antibiotic offspring returned to similar levels as prenatal-control offspring, acetate still remained significantly decreased in the antibiotic exposed group (Fig. 2F). In summary, maternal antibiotic treatment during pregnancy resulted in a low diversity, dysbiotic maternal gut microbiota that was transferred to the offspring. This was associated with significant reductions in fecal SCFA concentrations in the mothers and offspring that persisted until weaning and, in the case of acetate, into adulthood.

PN15 prenatal-antibiotic offspring have increased IgA, intestinal inflammation, and decreased weight

During the postnatal period, a dysbiotic maternal gut microbiota could influence maternal intestinal adaptations to lactation as well as breast milk composition, with possible effects on offspring weight, gut health, and subsequent immune system development. The maternal intestine adapts to the demands of lactation by increasing in length and area for nutrient absorption [32], thus, we first assessed if the dysbiotic maternal gut microbiota in our model was associated with changes to intestinal growth of lactating mothers at PN15. As shown in Fig. 3A and B, we observed no differences in intestinal length or villi-crypt length in prenatal-control versus prenatal-antibiotic treated mothers. Next, to investigate if maternal gut microbial dysbiosis was associated with changes in immunological components in the breast milk, we obtained milk from lactating mothers at PN15. Measurement of 23 cytokines in PN15 milk revealed that of the detectable cytokines (IL-1α, eotaxin, G-SCF, KC, RANTES, and TNF-α) no differences were observed in milk from lactating mothers, shown in Fig. 3C. Additionally, examination of SCFAs revealed that though acetate, propionate, and butyrate were measurable in the milk, there were no differences between prenatal-control and prenatal-antibiotic mothers, Fig. 3D.

Finding no changes in breast milk cytokines or SCFAs, we next examined if vancomycin treatment during pregnancy could influence maternal or offspring IgA concentrations. As shown in Fig. 3E, prenatal-antibiotic mothers had significantly higher serum IgA and milk sIgA concentrations compared to prenatal-control mothers. Further, we observed increased serum IgA concentrations and increased fecal sIgA concentrations in prenatal-antibiotic PN15 offspring (Fig. 3F). Intestinal inflammation at PN15 was evidenced by significantly increased fecal lipocalin-2 concentrations compared to controls, shown in Fig. 3G. Finally, we observed decreased weight in PN15 offspring from antibiotic-treated mothers as compared with the control group (Fig. 3H). These results show that maternal antibiotic use during pregnancy and the ensuing gut dysbiosis transferred to the offspring is associated with increased IgA concentrations and intestinal inflammation in both PN15 mothers and their offspring, as well as decreased offspring weight, compared to control animals.

Prenatal-antibiotic offspring have gut dysbiosis, barrier disruption, and changes in ILC3 subtypes

As intestinal inflammation could influence mucosal immune system development, we next immunophenotyped the offspring's small intestinal lamina propria (SI-LP), both in early life and after asthma induction. Flow cytometric analysis showed no changes in the absolute number of SI-LP CD45$^+$ intestinal leukocytes, however, increased percentages of these cells were observed in both PN15 and PN49 prenatal-antibiotic offspring compared with prenatal-control offspring, shown in Fig. 4A. Examination of Th cells revealed that CD4$^+$ T-cell percentages were increased in PN15 prenatal-antibiotic offspring and decreased in PN49 adult allergic offspring, (Fig. 4B). Though maternal antibiotic treatment did not influence the T-bet$^+$ or RORγt$^+$ percentages at PN15, GATA3$^+$ CD4 T cells were significantly decreased in prenatal-antibiotic offspring in comparison to controls (Fig. 4C). Examination of CD4$^+$ T-cell transcription factors in adult allergic offspring revealed that prenatal-antibiotic allergic offspring SI-LP had global reductions in T-bet, GATA3, and RORγt percentages. Reductions in the RORγt populations were observed in both RORγt$^+$ FoxP3$^-$ Th17 cells and RORγt$^+$ FoxP3$^+$ CD25$^+$ Tregs (Fig. 4C).

We additionally assessed SI-LP ILC populations, due to their importance in the maintenance of gut homeostasis and the establishment of gut lymphoid structures [33, 34]. ILC analysis revealed a reduction in total ILC percentages at both PN15 and PN49 in prenatal-antibiotic offspring. Though percentages of ILC subtypes ILC1 and ILC3 were not affected in general, ILC2 percentages were slightly increased in the small intestine of PN49 prenatal-antibiotic allergic offspring, Fig. 4D. Subpopulation analysis of SI-LP ILC3s showed significantly decreased percentages of NCR+ ILC3s (CCR6$^-$ T-bet$^+$), accompanied by significantly increased percentages of NCR- ILC3s (CCR6$^+$ T-bet$^-$), shown in Fig. 4E, mimicking disproportions already observed in inflammatory bowel diseases [35].

Finally, to assess if the intestinal inflammation observed in prenatal-antibiotic PN15 offspring was associated with changes in

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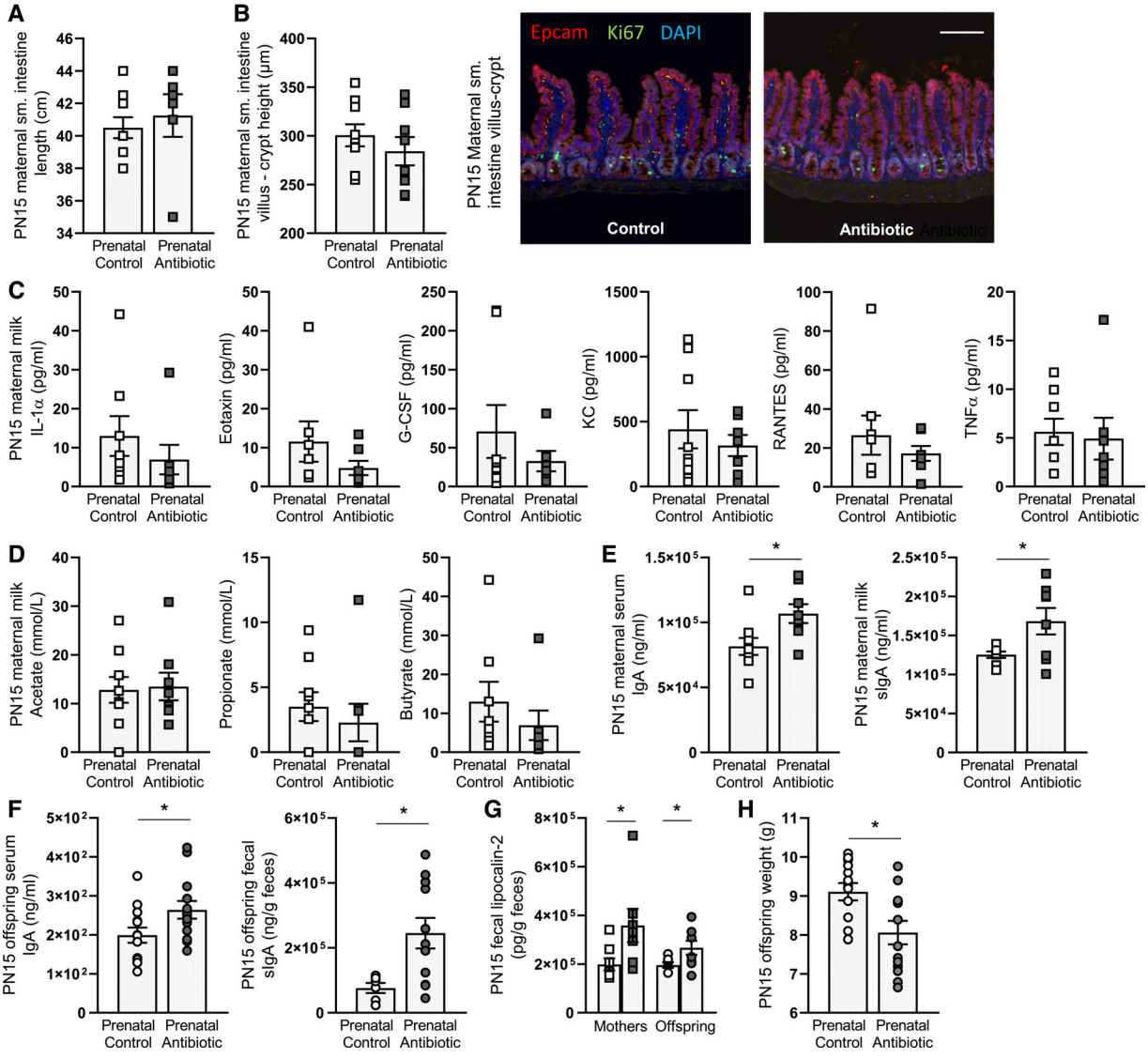

Figure 3. Antibiotic treatment during pregnancy is associated with increased IgA concentrations, intestinal inflammation, and decreased offspring weight during lactation at PN15. (**a**) Maternal small intestine length during lactation. (**b**) Maternal small intestine villus–crypt length during lactation and representative Epcam, Ki67 and DAPI staining. Scale bar = 100 μm. (**c**) Measurable cytokines in breastmilk from control- and antibiotic-treated mothers: IL-1α, eotaxin, G-CSF, KC, RANTES, and TNF-α. (**d**) SCFA concentrations in maternal milk from control and antibiotic-treated animals: Acetate, propionate, and butyrate. (**e**) IgA concentrations in maternal serum and milk (sIgA) from control and antibiotic treated animals. (**f**) Serum IgA and fecal sIgA concentrations of PN15 prenatal-control and prenatal-antibiotic offspring. (**g**) Fecal lipocalin-2 concentrations from control and antibiotic mothers and their offspring. (**h**) Weight of PN15 offspring. **a-e** Mothers: Prenatal-Control n = 9, Prenatal-Antibiotic n = 8. (**f and h**) Offspring: prenatal-control and prenatal-antibiotic n = 13. **g** All groups n = 8. Means ± SEM are shown. Data shown is pooled from two replicated experiments showing the same trends and significance. Significance is represented by $^*p < .05$. Student's *t*-test or Mann–Whitney test of antibiotic exposed compared against control at each specific time point.

gut barrier integrity, we next performed a FITC-dextran intestinal permeability assay. As shown in Fig. 4F, PN15 prenatal-antibiotic offspring serum contained significantly higher concentrations of FITC-dextran, indicating barrier disruption in these animals compared to prenatal-control animals. Allergic offspring were also tested in this manner, and though there was a trend towards increased serum FITC-dextran in the serum of allergic offspring from antibiotic-treated mothers, it was not significant (Fig. 4F). Taken together, these results show that early-life gut microbial dysbiosis is associated with increased inflammatory markers on SI-LP ILC3 subsets, accompanied by a significant reduction in gut barrier integrity in prenatal-antibiotic offspring in early life.

Measurable endotoxin and increased Th17-cell percentages in prenatal-antibiotic offspring blood

Since maternal vancomycin treatment led to the expansion of Gram-negative bacterial clades, increased intestinal inflammation, and reduced gut barrier integrity, we next assessed

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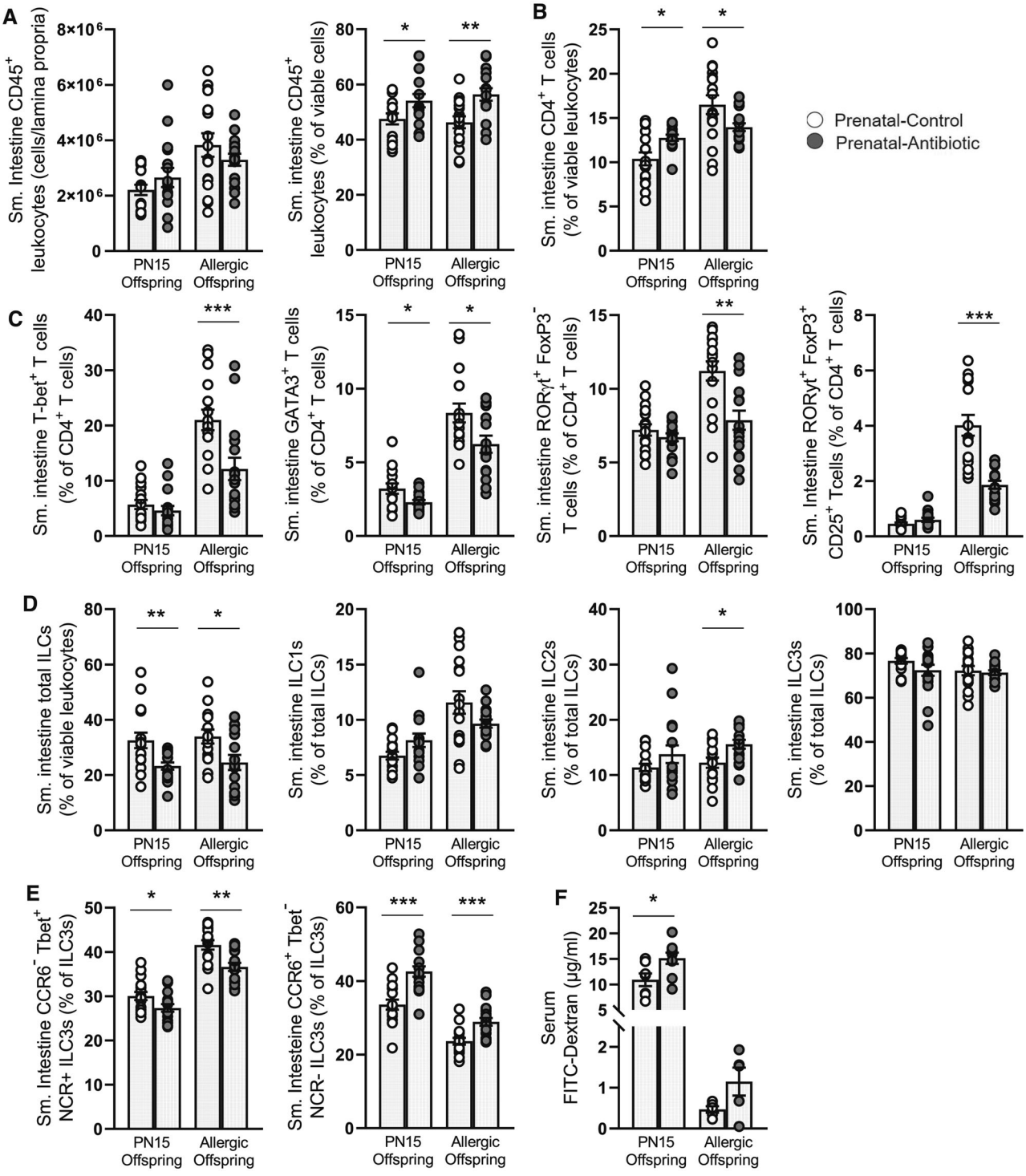

Figure 4. Antibiotic use during pregnancy is associated with significant changes to ILC3 subsets in the SI-LP and gut barrier disruption in early life. Comparison of prenatal-control versus prenatal-antibiotic offspring in early life at PN15 and after allergy induction at PN49. (**a**) Absolute number and percentage of $CD45^+$ leukocytes. (**b**) Percentages of: $CD4^+$ T cells (from viable leukocytes). (**c**) T-bet^+, $GATA3^+$, $ROR\gamma t^+$ $FoxP3^-$ (Th17) cells and $ROR\gamma t^+$ $FoxP3^+$ $CD25^+$ ($ROR\gamma t$ Tregs) (from $CD4^+$ T cells). (**d**) Percentages of: total ILCs (from viable leukocytes) and ILC1s, ILC2s, and ILC3s (from total ILCs). (**e**) CCR^- T-bet^+, NCR+ ILC3s (percent of ILC3s), CCR^+ T-bet^-, NCR- ILC3s (percent of ILC3). (**f**) Serum FITC concentration as measured by a FITC-dextran intestinal permeability assay. (**a-e**) All groups n = 16. (**f**) PN15 Prenatal-control and PN15 Prenatal-antibiotic n = 8, allergic offspring all groups n = 5 Mean ± SEM are shown. Data shown is pooled from two replicated experiments showing the same trends and significance. Significance is represented by $^*p < .05$, $^{**}p < .01$, $^{***}p < .001$. Student's *t*-test or Mann–Whitney test of antibiotic exposed compared against control at each specific time point.

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the possibility that microbial compounds, such as LPS, might have been disseminated to the circulatory system. As shown in Fig. 5A, prenatal-antibiotic G17 mothers and PN15 (but not PN49) offspring had significant concentrations of LPS in the blood when compared with prenatal-control samples. As this observed LPS increase could also influence blood immune cell populations, we next performed a flow cytometric analysis of lymphoid and myeloid cell types in the blood. Examination of circulating immune cell populations revealed fewer leukocytes in prenatal-antibiotic offspring at PN15, although no differences were observed in CD45 cell percentages. These differences resolved in adult offspring after asthma induction at P49, Fig. 5B. Myeloid cell analysis revealed no differences in the percentage of Ly6C^{hi} monocytes, Ly6G^{+} neutrophils, or SiglecF^{+} eosinophils (Fig. 5C), however, MFI analysis revealed significantly increased expression of eosinophil activation markers SiglecF and CD11b in prenatal-antibiotic offspring blood, both during early life and after allergy induction (Fig. 5D). Finally, supporting the fact that LPS is a known inducer of the transcription factor RORγt and the differentiation of naïve CD4^{+} T cells into Th17 cells [36], transcription factor analysis revealed though there were no changes in the total CD4^{+} T-cell population (Fig. 5E) or the T-bet and GATA3 subpopulations (Fig. 5F), we observed a marked increase in Th17 cells in the blood of prenatal-antibiotic offspring compared with prenatal-control offspring at both PN15 and PN49 (Fig. 5F). No changes were observed in the CD4^{+} FoxP3^{+} CD25^{+} population at any time point and RORγt Tregs were not detectable in the blood of any group (Supporting Information Figure S1). This analysis demonstrates that offspring from antibiotic-treated mothers have circulating LPS in early life, which is in turn associated with a sustained increase in Th17 cell percentages in the blood of offspring from early life until adulthood.

Prenatal-antibiotic offspring lungs exhibit increased RORγt T cells and more severe asthma

To complete our assessment of the gut–lung axis, we immunophenotyped lung tissue from offspring at both the early life (PN15) and allergic adult (PN49) time points. As shown in Fig. 6A, lung tissue from PN15 prenatal-antibiotic offspring had significantly higher leukocyte numbers compared with prenatal-control offspring. No differences were seen in absolute leukocyte numbers in allergic animals or leukocyte cell percentages in any of the groups. Examination of lung tissue ILCs revealed that though there were no differences in total ILC percentages at PN15 or PN49, prenatal-antibiotic allergic offspring had significantly increased ILC2 percentages (Fig. 6B). Analysis of myeloid cells revealed that only neutrophils were significantly reduced in prenatal-antibiotic offspring at PN15. As expected, allergic offspring from antibiotic-treated mothers had increased lung tissue macrophages and eosinophil percentages compared to allergic offspring from control mothers, indicating a more severe asthma phenotype (Fig. 6C). Finally, examination of T-cell populations in the lung tissue revealed a significant increase in both Th17 and RORγt^{+} Treg percentages in the offspring lung tissue, both in early life and after allergy induction. No differences were seen in CD4^{+}, T-bet^{+}, or GATA3^{+} T-cell percentages (Fig. 6D). Confirming our previous observations of increased asthma severity in prenatal-antibiotic offspring [15], we also demonstrate increased airway reactivity (Supporting Information Fig. S2A), increased eosinophils, and lymphocytes in the bronchoalveolar lavage (BAL) (Supporting Information Fig. S2B) as well as increased serum total IgG, OVA-specific IgG1, and OVA-specific IgE in prenatal-antibiotic offspring compared to their prenatal-control counterparts (Supporting Information Fig. S2C).

Discussion

Using a mouse model, we show that antibiotic use during pregnancy resulted in maternal gut microbial dysbiosis and decreased SCFA concentrations that were also observed in the next generation. Offspring gut dysbiosis in early life was associated with decreased fecal SCFA concentrations as well as increased IgA and lipocalin-2 concentrations, which is indicative of intestinal inflammation [25, 37]. Decreased gut barrier integrity in PN15 prenatal-antibiotic offspring was shown using both a FITC-dextran permeability assay and measurement of significant levels of LPS in offspring serum. Finally, increased CD4^{+} RORγt^{+} T-cell percentages were found in offspring blood and lung tissue in both early life and after allergy induction. We propose that the dysbiotic gut microbiota, intestinal inflammation, and disruptions to intestinal integrity that we observed during early life, reveal a potential gut–lung axis pathway that merits further investigation with regard to the offspring asthma severity previously observed in this model [15].

During the postnatal time period, the maternal gut microbiota, breast milk sIgA, and SCFAs play a major role shaping bacterial populations in the neonatal intestine. Breast milk sIgA promotes intestinal homeostasis in the progeny by directly binding to bacteria [38], and it was shown in 12-month-old children that decreased binding to *Bacteroides* and *E. coli* in the gut was associated with increased childhood asthma risk [26]. Increased fecal sIgA concentrations are a marker of intestinal inflammation and barrier dysfunction [25], and in our model, we observed increased fecal sIgA in the progeny of antibiotic-treated mothers. With regard to intestinal integrity, mouse studies demonstrate that disruption of IgA was associated with Proteobacteria overgrowth and inflammation [39], as well as translocation of bacteria across the intestinal epithelium in nursing mice [40]. We also observed Proteobacteria overgrowth and increased IgA in the neonatal intestine, accompanied by increased fecal lipocalin-2 concentrations suggesting intestinal inflammation. Furthermore, sIgA binding to commensal bacteria is promoted by the SCFA acetate [24, 41] and we also observed decreased fecal acetate in our prenatal-antibiotic offspring. Since evidence suggests that mice with low SCFA concentrations have impaired antibody responses [42], examination of IgA-commensal bacteria binding specificity warrants further study. Taking into account this information, we propose that the

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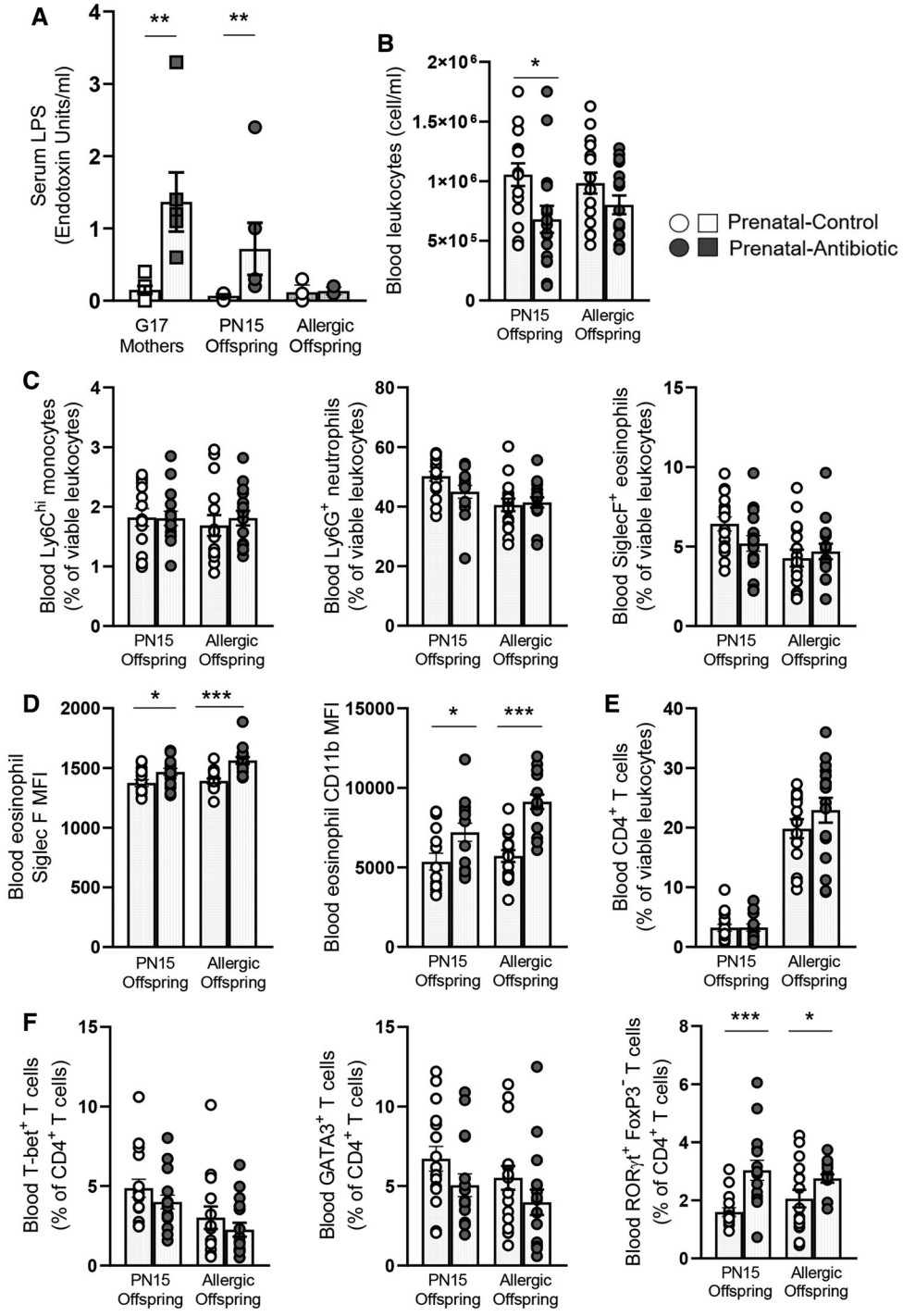

Figure 5. Antibiotic use during pregnancy is associated with increased serum LPS concentrations and increased RORγt T-cell percentages in the blood of prenatal-antibiotic offspring. Comparison of prenatal-control versus prenatal-antibiotic offspring in early life at PN15 and after allergy induction at PN49. (**a**) Maternal and offspring serum LPS measurements. (**b**) Absolute numbers of leukocytes. (**c**) Percentages of Ly6C^{hi} monocytes, Ly6G^{+} neutrophils and SiglecF^{+} eosinophils (from viable leukocytes). (**d**) MFI of SiglecF and CD11b on eosinophils. (**e**) Percentages of: CD4^{+} T cells (from viable leukocytes). (**f**) T-bet^{+}, GATA3^{+}, and RORγt^{+} FoxP3^{-} (Th17) cells (from CD4^{+} T cells). (**a**) Prenatal-control and prenatal-antibiotic mothers and offspring n = 6. (**b-f**) Prenatal-control offspring and prenatal-antibiotic offspring n = 16. Mean ± SEM are shown. Data shown is pooled from two replicated experiments showing the same trends. Significance is represented by $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$. Student's *t*-test or Mann–Whitney test of antibiotic exposed compared against control at each specific time point.

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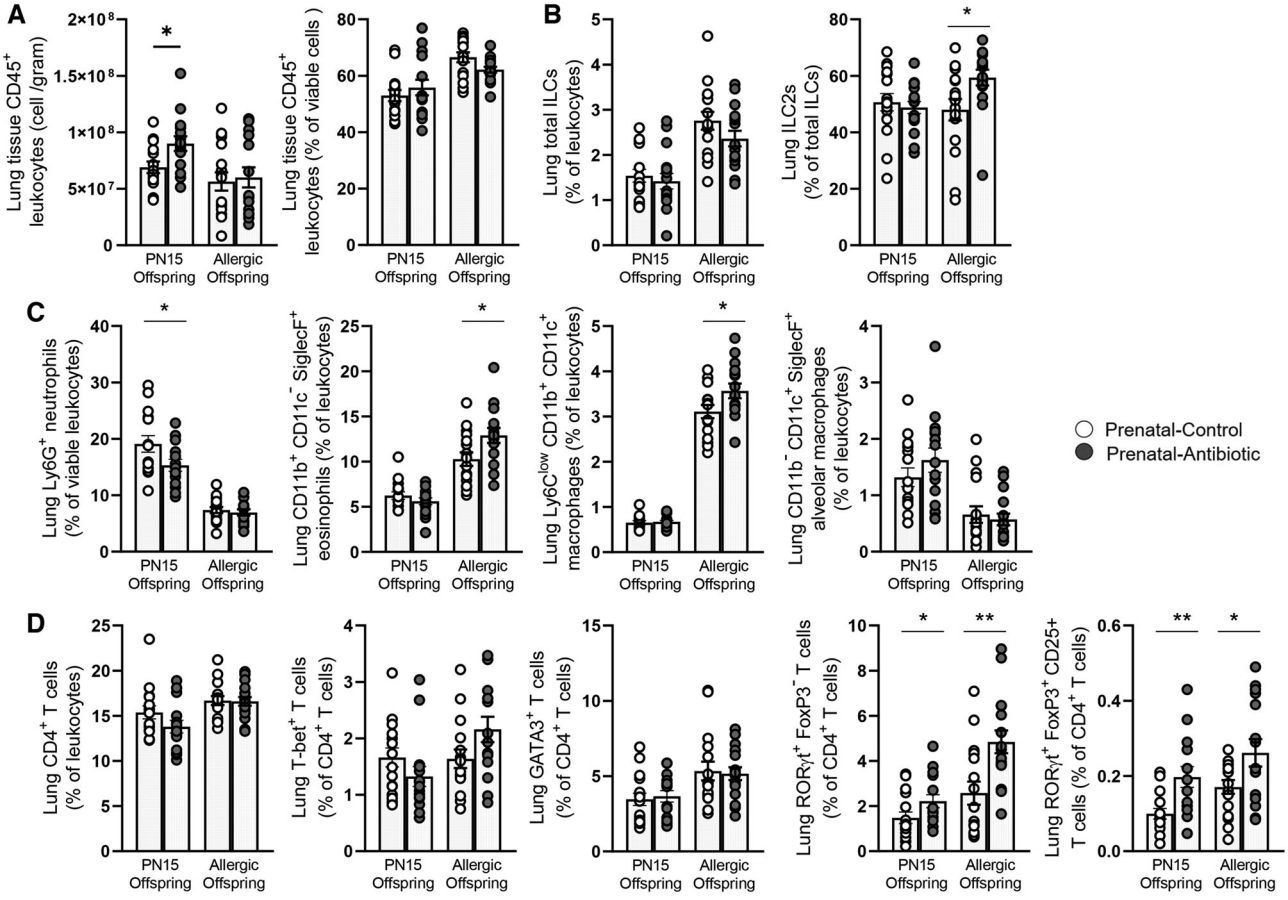

Figure 6. Antibiotic use during pregnancy is associated with altered lung tissue immune cell populations in PN15 and adult allergic offspring. **(a)** Absolute numbers and percentages of $CD45^+$ leukocytes. **(b)** Total ILC percentages (% of leukocytes), ILC2 percentages (% of total ILCs). **(c)** Percentages of neutrophils, eosinophils, macrophages, and alveolar macrophages (from viable leukocytes). **(d)** Percentages of: $CD4^+$ T cells (from viable leukocytes), as well as T-bet^+, $GATA3^+$, $ROR\gamma t^+$ $FoxP3^-$ (Th17) cells, and $ROR\gamma t^+$ $FoxP3^+$ $CD25^+$ ($ROR\gamma t$ Tregs) (from $CD4^+$ T cells). **(a-d)** Prenatal-control n = 16, Prenatal-antibiotic n = 16. Data shown is pooled from two replicated experiments showing the same trends and significance. Mean ± SEM are shown. Significance is represented by $^*p < .05$, $^{**}p < .01$, $^{***}p < .001$. Student's *t*-test or Mann—Whitney test of antibiotic exposed compared against control at each specific time point.

increased relative abundance of Proteobacteria observed in our model combined with decreased acetate and increased sIgA concentrations contribute to intestinal inflammation in early life.

An additional interesting finding from our study was the changes in ILC3 populations observed in prenatal-antibiotic compared to prenatal-control offspring. We observed dysregulation of ILC3 subtypes, characterized by increased NCR- ILC3 ($CCR6^+$, T-bet^-) and a concurrent decrease in NCR^+ ILC3 ($CCR6^-$, T-bet^+) percentages at both time points. ILC3s are essential for maintaining mucosal homeostasis in the intestine [29], and we propose that a combination of intestinal inflammation and alteration of ILC3 subsets may have contributed to a disrupted intestinal barrier in early life. Indeed, an FITC-dextran intestinal permeability assay revealed loss of intestinal integrity in prenatal-antibiotic offspring, and further detection of LPS in the circulation of these offspring indicates the systemic dissemination of Gram-negative microbial components from the dysbiotic intestine. Increased concentrations of bacterial components in the blood have previously been associated with gut microbial dysbiosis in both humans [43] and mice [44, 45]. LPS is a strong mediator of immunity and is associated with several systemic inflammatory effects, the most prominent of which is the generation of $CD4^+$ $ROR\gamma t^+$ T cells [46, 47]. We propose that systemic LPS dissemination from a disrupted intestinal barrier in early life is an important pathway that merits further study.

In our model, examination of lung tissue from prenatal-antibiotic offspring showed increased $CD4^+$ $ROR\gamma t^+$ T cells (both Th17 and FoxP3) and decreased neutrophil percentages in early life. After allergy induction in these animals, we observed a more severe asthma phenotype, evidenced by increased eosinophil and ILC2 percentages, and significantly higher airway reactivity compared to allergic prenatal-control animals. This is in line with our previous results demonstrating more severe asthma in offspring from mothers treated with antibiotics during pregnancy [15]. Though allergic responses are primarily associated with Th2 cells, recent studies indicate that Th17 cell responses are more interconnected in the allergic process than previously thought [48]. Th17 cells are associated with increased asthma severity in human

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patients [49] and Na et al. (2018) recently demonstrated that mice deficient in either IL-17A, IL-17F, or RORγt showed attenuated Th2 and Th17 responses in a mouse model of allergic asthma [50]. It was further shown *in vitro* that blockade of RORγt limited the differentiation of human naïve CD4 T cells into Th2 or Th17 cells, indicating a close linkage between these cell types . The increased Th17 and RORγt Treg percentages observed in the blood and lung tissue of prenatal-antibiotic offspring indicate a possible, previously underestimated participation of these cell types in the establishment of lung mucosal immunity in early life and contribution to asthma exacerbation.

In summary, we show that maternal treatment with antibiotics during pregnancy resulted in gut microbial dysbiosis that was transferred to the next generation. Offspring gut microbial dysbiosis was associated with dysregulation of immunity along the gut–lung axis in early life, beginning with intestinal inflammation and gut barrier disruption, LPS dissemination to the circulation and cumulating in significantly increased percentages of Th17 cells in the blood and Th17 cells and RORγt Tregs in the lung. We propose that these processes represent a major pathway for future research regarding how antibiotics during pregnancy can contribute to increased offspring asthma susceptibility.

Materials and Methods

Animals

A 12-week-old BALB/c mice were obtained from Janvier Labs (Le Genest-Saint-Isle).and housed under specific pathogen-free conditions, five animals per cage, with a 12/12 light/dark cycle and food and water available ad libitum. The animal experiment was approved by the Berlin authorities (Landesamt für Gesundheit und Soziales-LAGeSo) and performed according to German and international regulatory guidelines.

Experimental design

Mice were mated, and the presence of a vaginal plug was denoted as gestation day G(0) of pregnancy. From G8–G17, pregnant mice received a daily oral dose of the antibiotic vancomycin (Sigma–Aldrich, 20 mg/kg body weight) mixed with Ora-Sweet syrup (Paddock Laboratories). The mixture was freshly prepared (25 μL vancomycin solution + 15 μL Ora-Sweet) and a 40 μL volume was delivered through drops from a pipet that were voluntarily consumed by the mice. Control mice were sham treated with water and Ora-Sweet. At PN15, breast milk was collected from the mothers, then mothers and pups were euthanized for organ collection. The remaining pups were weaned from their mothers at PN21 and female pups were subjected to an adjuvant-free experimental asthma protocol [51]. Sensitization was performed at offspring age 21, 28, and 35 days, by subcutaneous injection of 10 μg OVA VI (Sigma) in 200 μL PBS. Asthma exacerbation in the lungs was provoked by a 20-min daily aerosol challenge with 1% OVA V (Sigma) at offspring age 46, 47, and 48. On day 49 of the protocol, airway reactivity was measured in allergic animals, then they were euthanized for organ harvesting and analysis. The protocol is shown in Fig. 1.

Fecal intestinal microbiota, SCFA, and lipocalin-2 analyses

16S rRNA sequencing

To assess the microbial composition of the maternal and offspring gut microbiota, fecal samples were collected at the following time points: maternal (before mating, after antibiotic treatment at G17, and at weaning PN21), offspring at weaning (PN21) and after allergic asthma induction (PN49). Feces were flash frozen with liquid nitrogen and stored at −80°C until analysis. 16S rRNA sequencing was performed by Microbiome Insights Inc. as per the following procedure. A PowerSoil for KingFisher kit (MO Bio) was used to extract DNA, then 16S rRNA gene fragments from the V4 region were amplified with the following bar-coded primers (5′-3′): fwd: GTGCCAGCMGCCGCGGTAA, rev: GGACTACHVGGGTWTCTAAT. PCR amplicons were pooled and diluted to 20 ng/mL and a MiSeq 2000 bidirectional Illumina sequencing and Cluster Kit v4 (Macrogen) was used for sequencing. A TruSeq DNA Sample Prep v2 Kit (Illumina) was used for Library preparation (100 ng DNA/sample) and Qubit (Thermo Fisher Scientific) was used to quality check and quantify the library. A modified MOTHUR standard operating procedure was used to trim, quality filter, and cluster sequence data into operational taxonomic units (97% identity) [52]. Per sample, an average of 31 554 quality-filtered reads were generated, totaling 8631 operational taxonomic units that were taxonomically annotated with the SILVA database [53]. Phyloseq was used to generate global community structure comparisons [54].

Analysis of maternal and offspring fecal SCFA concentrations

Fecal samples from the previously mentioned time points were also analyzed for SCFA concentrations. Measurements were performed using an HP 5890 series II gas chromatograph (Hewlett-Packard) with an HP-20 M column and a flame ionization detector. Feces were diluted and centrifuged, then the supernatant was mixed with 1 M NaOH, 0.36 M $HClO_4$, and 2-ethylbutyric acid as an internal standard. This was then lyophilized overnight, then redissolved in 5 M formic acid plus 400 μL acetone. The resulting mixture was centrifuged, then 1 mL of supernatant was injected into the gas chromatograph.

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Lipocalin-2 analysis

PN15 fecal samples were weighed, homogenized, and resuspended in 1 mL PBS + 0.05% TWEEN20, then centrifuged at 12 000 rpm for 10 min. Supernatant was diluted 1:100 and analyzed using a lipocalin-2 ELISA (BioLegend) according to the manufacturer's instructions.

Collection of PN15 maternal milk

To allow for milk accumulation in PN15 lactating mothers, offspring were separated from their mothers 4 h prior to milk harvest. Lactating mice from control- and antibiotic-treated groups were injected intraperitoneally with 1 IU of oxytocin (Sigma–Aldrich) in 50 μL PBS to stimulate milk flow. For milk collection, mice anesthetized with ketamine and xylazine were placed on a heating pad and breast milk was collected for 20 min using a specially constructed breast pump, protocol adapted from Ref. [55]. After milk collection, mother mice were sacrificed, blood was collected from the orbital sinus and serum was frozen at −80°C.

Collection of PN15 and PN49 offspring tissues

Blood samples

Offspring were euthanized and blood samples were collected from the orbital sinus of either PN15 or PN49 allergic offspring. One blood sample was used immediately for flow cytometry (undergoing RBC lysis, followed by staining with antibodies) and one sample was centrifuged for serum isolation, then stored at −80°C.

Small intestine

Small intestine lamina propria was harvested according to a protocol adapted from Ref. [56]. In short, the small intestine was dissected (removing the Peyer's patches), cleaned, cut into pieces, and digested in Hanks balanced salt solution with collagenase D (Sigma), DNase1 (Roche), dispase (Sigma), and FBS (Sigma). Immune cells were purified on a Percoll gradient and subsequently stained and analyzed by flow cytometry as previously described [56].

Lungs

In PN49-allergic offspring, BAL was performed via a tracheal canula using 1 mL of PBS, containing a complete protease inhibitor cocktail (Roche). Both PN15 and PN49 lungs were removed and digested following the protocol from Ref. [57]. Briefly, lungs were cut into pieces and digested with collagenase and DNase 1 (Roche) in RPMI medium with FBS. After digestion and straining, cells were subjected to red cell lysis and then analyzed by flow cytometry.

Small intestine, blood, and lung flow cytometry in PN15 and PN49 offspring

Intestine staining antibodies

Isolated cells from the small intestine were stained with a Zombie aqua viability kit (BioLegend) and then with the following antibodies (CCR6, CD4, IL-17RB, CD25, FoxP3, CD45, CD127 (IL-7Rα), EOMES, GATA3, Ki-67, KLRG1, Live dead, NKp46, RORγt, ST2, T-bet, CD90.2 and lineage (CD3e, CD5, CD19, Gr1, TCRβ, TCRγδ). CD4 T cells were checked later for CD3 positivity.

Blood staining antibodies

After RBC lysis, blood leukocytes were stained with a Zombie aqua viability kit (BioLegend) and then with the following antibodies for lymphocytes (B220, CD3, CD4, CD8), myeloid cells (Ly6C, Ly6G, CD11b, CD11c, SiglecF), and transcription factors (RORγt, T-bet, GATA3, and FoxP3).

Lung staining antibodies

After staining with a viability dye, samples were stained with antibodies for myeloid cells (CD11b, CD11c, CD45, CD49b, FcεR1α, Ly6C, F4/80, Ly6G and SiglecF), ILC2s (CD45, CD90.2, GATA3, KLRG1, RORγt, ST2), and lineage (B220, CD3, CD4, CD11b, CD11c, CD19, FcεR1a, Ly6G, NK1.1, TCRβ, TCRγδ, TER119). Lungs were also stained with the previously mentioned lymphoid and transcription factor blood panels.

Stained cells were analyzed using a BD LSR Fortessa X-20 (BD Biosciences) or MACSQuant Analyzer 10 (Miltenyi Biotech). Gating strategies for all panels are located in Supporting Information Fig. S3–5.

BAL and differential cell counts in PN49 allergic offspring

BAL was centrifuged, the supernatant was stored at −80°C, and total leukocytes were counted with a Neubauer chamber. Differential cell counts were performed on cytospin preparations stained with Diff-Quick (Merz & Dade) using standard morphological criteria for cell type identification. Two hundred cells were counted per cytospin by a researcher blinded to the sample names.

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Serum and breast milk antibody measurement

Sandwich ELISA was used to measure breast milk sIgA in PN15 mothers, serum IgA in PN 15 mothers and offspring, and fecal sIgA concentrations in PN15 offspring. Additionally, total IgG, OVA-specific IgG, and OVA-specific IgE were measured in the serum of PN49 allergic offspring by ELISA (BD Bioscience).

Serum LPS (endotoxin) measurement

Serum samples from G17 mothers, PN15 offspring, and PN49 offspring were diluted 1:100 in LAL (endotoxin-free) water and heat treated for 10 min at 80°C. Endotoxin concentration was measured using a kinetic turbidimetric test by Mikrobiologisches Labor Dr. Michael Lohmeyer GmbH.

Measurement of airway reactivity

Airway reactivity was measured in allergic offspring at PN49 by using a FlexiVent ventilator (SCIREQ). Mice were anesthetized and mechanically ventilated with a tidal respiratory rate of 150/min and a tidal volume of 10 mL/kg. Assessment of lung function was performed at baseline, followed by aerosolized methacholine (Sigma–Aldrich) at increasing doses (0.625, 1.25, 2.5, 5, 10, 20, and 40 mg/mL). Airway resistance is reported as Newtonian resistance.

FITC-Dextran intestinal permeability assay

Pregnant mice were treated from G8 to G17 orally with 25 μL of either vancomycin (20 mg/kg) or water mixed with 15μL Ora-Sweet. On PN15, female offspring were separated from their mothers for 1.5 h and then gavaged with 500 mg/kg of 4 KDa FITC-Dextran (Sigma). Food and water were removed for 1.5 h, then the animals were euthanized, and blood was collected from the retro-orbital sinus. Serum samples were diluted at 1:4, loaded on a black 96-well plate, and measured with the plate reader (Infinite® 200 PRO) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Statistical analysis

Samples were tested for normal distribution using a Shapiro-Wilk normality test. Parametric or nonparametric data were analyzed using either Student's *t*-test or a Mann–Whitney U test, respectively. Graphing and statistical tests were performed using the software GraphPad Prism 9.

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Author contributions: MMA designed and conducted experiments, analyzed and interpreted data, and wrote the article; YA conducted experiments; WWM analyzed the microbiota and SCFA data; OH, CD, SH, and SG conducted flow cytometry experiments and analysis; NB and AP performed intestinal histology analysis; DL and JG performed airway reactivity analysis; MLC and GB conceived the study, provided funding, oversaw the research program and wrote the article.

Ethics approval statement: These experiments were approved by the Berlin authorities (Landesamt für Gesundheit und Soziales - LAGeSo) and were performed according to German and international regulatory guidelines.

Data availability statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Abbreviations: **BAL**: bronchoalveolar lavage · **G**: Gestation day · **PN**: Postnatal day · **SCFA**: Short-chain fatty acids · **sIgA**: secretory immunoglobulin A · **SI-LP**: Small intestine lamina propria

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