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RESEARCH ARTICLE

Innate Immunity

Maternal Administration of Probiotics Augments IL17-Committed γδ T Cells in the Newborn Lung

Yohannes Tafesse[1,2,3,4] | Arnaud Köhler[2,3] | Guillem Sanchez Sanchez[1,2,3,4] | Patricia Brito Rodrigues[4,5] | Marko Verce[4,5] | Panagiotis Vitsos[1,2,3,4] | Isoline Verdebout[1,2,3,4] | Moosa Rezwani[1,2,3,4] | Maria Papadopoulou[1,2,3,4] | Amandine Everard[4,5] | Véronique Flamand[2,3] | David Vermijlen[1,2,3,4]

[1]Department of Pharmacotherapy and Pharmaceutics, Université Libre de Bruxelles (ULB), Brussels, Belgium | [2]Institute for Medical Immunology (IMI), Université Libre de Bruxelles (ULB), Gosselies, Belgium | [3]ULB Center for Research in Immunology (U-CRI), Belgium | [4]WELBIO Department, WEL Research Institute, Wavre, Belgium | [5]Metabolism and Nutrition Research Group, Louvain Drug Research Institute, Université Catholique De Louvain, Brussels, Belgium

Correspondence: David Vermijlen (David.Vermijlen@ulb.be)

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ABSTRACT

The early life period is increasingly being recognized as a window of opportunity to shape immunity, where microbiota and related probiotics have an important impact. Innate γδ T cells are the first T cells generated in utero, populating epithelial tissues such as the lung and contributing to tissue protection through, for example, IL17 production. Here, we studied the influence of maternal microbiota and probiotic supplementation during pregnancy on innate γδ T cells in the lung and thymus of newborn mice. Detailed time-kinetic experiments showed that at birth, the murine lung T cell population was specifically dominated by IL17-committed γδ T cells expressing an invariant Vγ6Vδ1 TCR. Single-cell RNA-sequencing showed that the biased IL17-commitment of perinatal lung γδT cells is highly conserved between mice and humans. While maternal microbiota depletion with antibiotics tended to decrease the frequency of the lung Vγ6 T cells of the offspring at birth, the maternal administration of *Lacticaseibacillus rhamnosus* (*L.rhm.*), but not of *Bifidobacterium animalis* subsp. *lactis* (*B.lac.*), increased significantly their frequency, resulting in the augmentation of the IL17-commitment of the mouse lung T cell compartment. Altogether, our data indicate that the maternal microbiota contributes to the shaping of IL17-committed γδT cells in the lungs of newborns and that maternal administration of specific probiotic strains can enhance this process.

1 | Introduction

Microbial colonization of mucosal tissues during infancy plays an instrumental role in the development and education of the immune system that will influence life-long immune homeostasis [1–4]. Importantly, exposure to microbiota-derived metabolites already starts in utero, affecting the immune cell development postnatally [5–7]. In line with this, maternal antibiotic

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exposure has been shown to influence the establishment of neonatal gut microbiota [8] and impact immune-related diseases in mucosal tissues of newborns, such as the lung [9–11]. A potential mitigation strategy for perinatal dysbiosis in pregnant mothers can be through the supplementation with probiotics. Probiotics are live microorganisms that, when administered in adequate amounts, confer health benefits to the host [12–14]. Studies in mice show that bacteria strains of the genus *Lacticaseibacillus* and genus *Bifidobacterium* can help in the prevention and reduction of disease severity during respiratory tract infections [15–20]. Particularly, the administration of *Bifidobacterium animalis* subsp. *lactis* and *Lacticaseibacillus rhamnosus* have been shown to decrease lung viral load, reduce disease severity, and increase survival in influenza A virus-infected adult mice [15, 20]. More recently, maternal administration of probiotics has emerged as a potential strategy for modulating neonatal immune responses in the early life period, particularly in the context of neonatal asthma [21] and respiratory infections [18].

$\gamma\delta$ T cells are prototypic "unconventional" T cells, with several features that set them apart from their 'conventional' $\alpha\beta$ T cell counterparts [22, 23]. Unlike $\alpha\beta$ T cells, $\gamma\delta$ T cells do not significantly populate secondary lymphoid organs but are rather enriched in organs that interface with the environment, such as the lung [23]. Besides, $\gamma\delta$ T cells do not require priming by an obligatory antigen-presenting cell in secondary lymphoid organs, nor do they necessarily depend on conventional major histocompatibility complex (MHC)/antigen processing or presentation [24–26]. Not long after their discovery, $\gamma\delta$ T cells have been divided into subsets based on the type of Vγ and/or Vδ chain they express in their T cell receptor (TCR) [23]. Subsets of $\gamma\delta$ T cells have the ability to respond en masse with innate-like characteristics to infection and cellular stress [23]. These innate $\gamma\delta$ T cells are typically produced in waves in the mouse fetal thymus, as exemplified by the Vγ6 $\gamma\delta$ T cell subset that seeds the lung, among other organs [27–29]. Innate $\gamma\delta$ T cells are already functionally programmed in the thymus; for example, the mouse Vγ6 subset possesses an IL17-committed program and can thus readily produce IL17, which plays an important role in the protection against influenza infection in early life [30, 31]. Similar waves have been found recently to be generated in the human fetal thymus [32, 33], and congenital infection with cytomegalovirus or *Toxoplasma gondii* leads to profound $\gamma\delta$ T cell expansions in utero [34, 35].

IL17-committed $\gamma\delta$ T cells, especially the Vγ6 subset, are influenced by microbiota in a tissue-specific manner [36–45]. However, conflicting data exist regarding the dependency on microbiota of the homeostatic maintenance of (mainly Vγ6) IL17-committed $\gamma\delta$ T cells in the lung [36, 38, 45]. Importantly, it is not known whether the maternal microbiome or maternal administration of specific probiotics can affect the development and functional commitment of innate $\gamma\delta$ T cells, such as Vγ6 IL17-committed $\gamma\delta$ T cells in the offspring. Here, we used a mouse model to address these questions by administering antibiotics or one of two probiotic strains (*L. rhamnosus* VES 001 (*L.rhm.*) and *B. animalis* subsp. *lactis* VES 002 (*B.lac*)) to mothers during pregnancy.

2 | Results

2.1 | IL-17 Committed Vγ6 $\gamma\delta$ T Cells Dominate the Mouse Perinatal Lung T Cell Pool at Birth

First, we wanted to obtain detailed insight into the total T cell composition and $\gamma\delta$ T cell subset dynamics in the lung and thymus of newborns (Day (D) 0, D3, D7) and how these evolve into adulthood. In order to identify $\gamma\delta$ T cell subsets based on the Vγ chain they express within their TCR, we employed flow cytometry using antibodies specifically directed toward a series of Vγ chains, including a novel Vγ6 monoclonal antibody [46]. In the lung, on the first day of life, $\gamma\delta$ T cells were the dominant T cell type, comprising about 80% of the total T-cell population (Figure 1A). This composition changed rapidly in the first few postnatal days as the lung was increasingly populated by $\alpha\beta$ T cells (Figure 1A). The Vγ6 subset was the dominant $\gamma\delta$ T cell subset in the lung through the first week of life, with a peak at D3, while the Vγ4 subset took over at two weeks (Figure 1B, right panel). In other peripheral tissues (liver, spleen, intestine, skin), other $\gamma\delta$ T cell subsets dominated the $\gamma\delta$ T cell pool at birth (Figure S1). In adult mice (8 weeks), Vγ4 was the main lung $\gamma\delta$ T cell subset, followed closely by the Vγ6 subset and Vγ1 (Figure 1B; Figure S1). Strikingly, at birth, the Vγ6 subset was the most abundant T cell type in the lung, comprising about a third of the total T cell population (Figure 1B, middle panel). To verify the effector commitment of the perinatal lung Vγ6 and other $\gamma\delta$ subsets, $\gamma\delta$ T cell effector commitment was determined by cell surface expression of CD44 and CD45RB: CD44highCD45B- cells are committed to IL17 secretion (and no IFNγ) ("IL17-committed"), whereas CD44-CD45RB$^+$ cells have the potential to secrete IFNγ but not IL17 ("IFNγ-intermediate"), while the CD44$^+$CD45RBhigh combination identified cells with robust commitment to IFNγ secretion in peripheral tissues ("IFNγ-committed") [47] (Figure S2). This analysis showed that the perinatal lung Vγ6 $\gamma\delta$ T cells were highly biased toward IL17-commitment, while other $\gamma\delta$ T cell subsets showed more variable commitments (Figure S3). Vice versa, the IL17-committed cells consisted almost only of Vγ6 $\gamma\delta$ T cells, while Vγ4 cells did not contribute to this effector commitment in the perinatal lung (Figure 1C).

To gain an understanding of the de novo generation of $\gamma\delta$ T cells capable of migrating to the lung, we analyzed in detail the $\gamma\delta$ T cell subsets in the thymus. At birth (D0), $\gamma\delta$ thymocytes represented about one-third of CD3$^+$ thymocytes (Figure 1D). The domination by $\alpha\beta$ T cells further increased in the first postnatal days of life (Figure 1D). While a sizable fraction of Vγ6 thymocytes could be observed at birth, the dominant subset comprised Vγ4 cells (Figure 1E), thus contrasting with the observations in the lung (Figure 1A). This is in line with Vγ6 cells being generated before Vγ4 cells in the fetal thymus [48]. The thymic generation of the Vγ6 subset declined rapidly and subsided about a week after birth, while the Vγ4 and Vγ1 subsets took the lead (Figure 1E). At birth and through early adulthood, the Vγ4 subset was the dominant $\gamma\delta$ subset generated in the thymus, in line with previous observations [46], and contributed to the IL17-commitment of the $\gamma\delta$ thymocytes along with the main contribution of the Vγ6 subset (Figure 1F). The frequencies of the Vγ1 subset continued to increase from day 3 to early adulthood (Figure 1E). This increase

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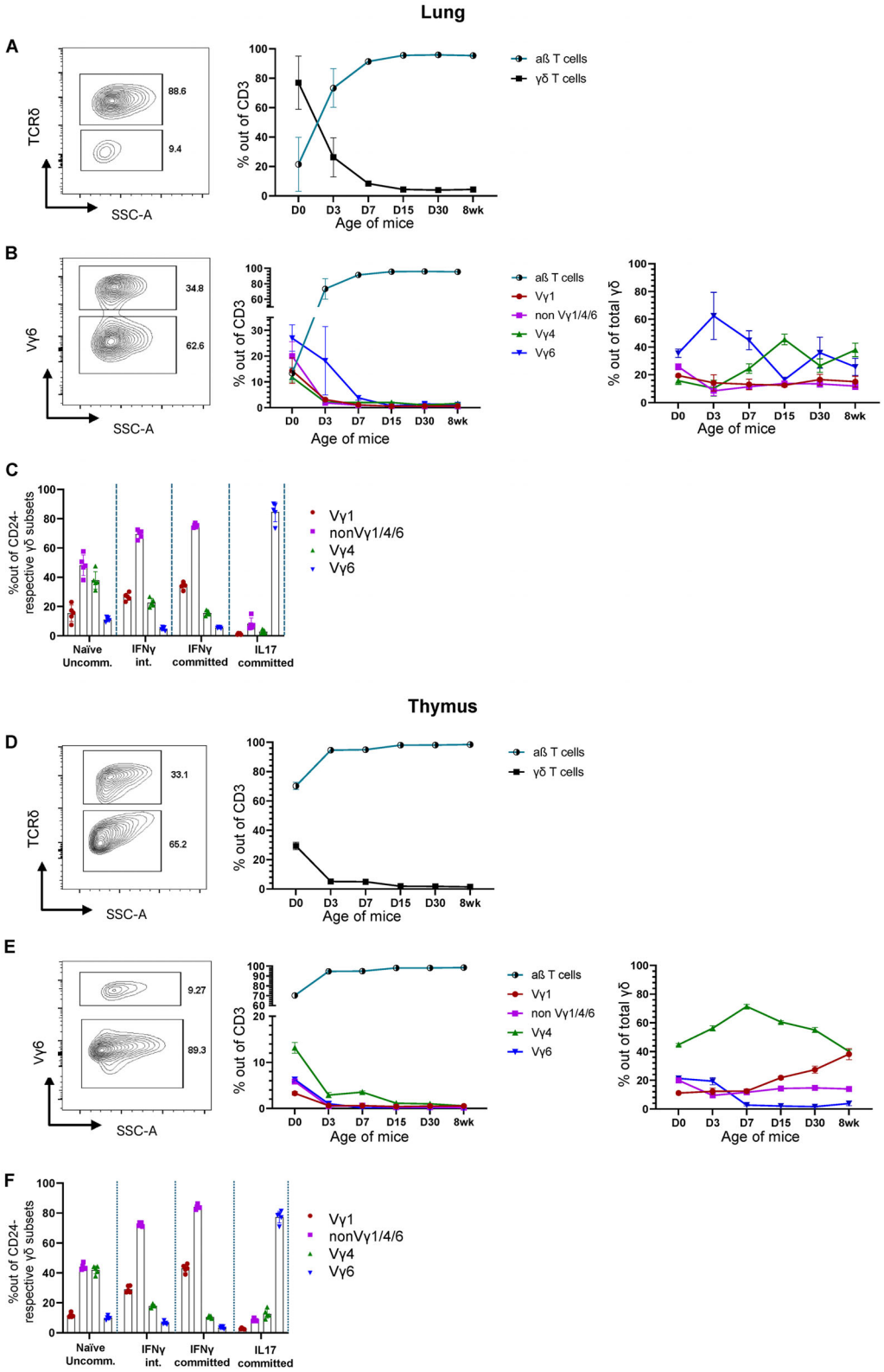


FIGURE 1 | IL17-committed Vγ6 $\gamma\delta$ T cells dominate the perinatal lung T cell pool at birth. (A) Flow cytometry plot showing total lung $\gamma\delta$ T cell percentage out of $CD3^+$ lymphocytes at day 0 on (left), lung total T cell kinetics (right). (B) Flow cytometry plot of lung Vγ6 percentage out of $CD3^+$ cells on day 0 (left), lung $\gamma\delta$ T cell subset kinetics expressed as a percentage out of $CD3^+$ lymphocytes (middle), and lung $\gamma\delta$ T cell subset kinetics expressed as a percentage out of total $\gamma\delta$ T cells (right). (C) Subset composition of lung $\gamma\delta$ effector clusters on day 0 (D) Flow cytometry plot showing total thymic $\gamma\delta$ T cell percentage out of $CD3^+$ lymphocytes at day 0 on (left), thymic total T cell kinetics (right). (E) Flow cytometry plot of thymic Vγ6 percentage out of $CD3^+$ cells on day 0 (left), thymic $\gamma\delta$ T cell subset kinetics expressed as a percentage out of $CD3^+$ lymphocytes (middle), and thymic $\gamma\delta$ T cell subset kinetics expressed as a percentage out of total $\gamma\delta$ T cells (right). (F) Subset composition of thymic $\gamma\delta$ effector clusters on day 0. D0 = Day 0, D3 = Day 3, Day 7, D15 = Day 15, D30 = Day 30, 8wk = 8 weeks of age. n = 3–5 mice per time point, error bars indicate means with standard deviation.

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in Vγ4 production in the thymus (Figure 1E) preceded their higher prevalence in the lung (Figure 1B).

In summary, at birth, the mouse lung is dominated by IL17-committed Vγ6 T cells. At later time points, Vγ6 T cells are replaced by αβ T cells and other γδ T cells, which are probably derived from postnatal thymic production.

2.2 | The IL17-Commitment of Perinatal Lung γδ T Cells Is Conserved between Mice and Humans

Next, we wondered whether IL17-committed γδ T cells could also be detected in the human lung. Therefore, we analyzed perinatal (D0) and postnatal (10 weeks) mouse lung γδ T cells and perinatal human (18–23 weeks gestation, lung developmental stage close to D0 mice [49]) and postnatal human (1.3–2 years) [50] lung γδ T cells by single-cell gene expression with parallel analysis of TRGV (Vγ) and TRDV (Vδ) usage in their TCR. After quality control and integration, the mouse lung dataset (n = 8 perinatal + 8 postnatal), had a total of 12,039 γδ T cells for further analysis (average RNA count = 6332, average feature count = 2027) and the integrated human dataset (n = 6 perinatal + 3 postnatal) had a total of 7043 γδ T cells (average RNA count = 3375, average feature count = 1406 (Figure S4A). Upon integration of D0 with adult mouse lung γδ T cells (Figure 2A, left panel) and of fetal with pediatric human lung γδ T cells (Figure 2A, right panel), 7 distinct clusters were identified in each species dataset. In both species, specific clusters were more abundant in early life (mainly cluster 1 in mouse, cluster 2 in human; note that there is no link between the cluster numbers between the two species), while other clusters were more prevalent in later life (mainly cluster 4 in mouse, cluster 3 in human) (Figure 2A). These different cluster abundancies were related, both in mouse and human, to a conserved bias toward IL17-commitment of lung γδ T cells in perinatal life, while in later life, IFNγ-commitment prevailed (Figure 2B,C; Figure S4B,C). While it is known that the TRGV and TRDV genes show poor conservation in mouse and human [23, 33], we wondered whether the IL17-committed human fetal lung γδ T cells would be highly enriched for certain Vγ or Vδ chains like the strong Vγ6 bias observed in mouse D0 IL-17-committed γδ T cells (Figure 1C; Figure S3). The strong Vγ6 bias in the mouse perinatal lung IL17-committed γδ T cells was confirmed at the single-cell RNA level (Figure 2D; Figure S4D), which consisted almost entirely of the previously described invariant CDR3γ CACWDSSGFHKVF paired with the invariant CDR3δ CGSDIGGSSWDTRQMFF (Vδ1 chain encoded by TRDV4) [23] (Figure 2D). These TRGV and TRDV biases were specific for the IL17-committed cluster (Figure 2D). In contrast, human perinatal lung IL17-committed γδ T cells possessed a more variable TRGV usage, and there was no specific enrichment of a TRGV and TRDV gene in IL17-committed γδ T cells compared with the other γδ T cells (Figure 2E).

Thus, despite the lack of conservation of TRGV and TRDV genes between mouse and human and overt differences between mouse and human lung γδ T cells at the TCR level, the bias toward IL17-commitment in mouse perinatal lung γδ T cells is strikingly conserved in humans.

2.3 | Maternal Microbiota Impacts Vγ Usage of Perinatal Lung γδ T Cells at Birth

We wanted to understand if the maternal microbiota impacts the neonatal lung and thymic γδ T cells. Particularly, we wanted to investigate if and how the neonatal γδ T cell subset composition and effector commitment profiles are affected. For this, we administered a cocktail of antibiotics in the drinking water to pregnant mice during the last week of pregnancy, coinciding with the functional development of the fetal thymus and the generation of mature and effector committed innate γδ T cells [27], to deplete their microbiota (Figure S5). Pups born from mothers that received antibiotics had a tendency to have a decreased proportion and counts of the Vγ6 and nonVγ1/4/6 γδ subset in the lung, corresponding to an increased proportion of the Vγ1 subset (Figure 3A,B, left panels; Figure S6A). There were no significant changes in the total lung (Figure 3A, right panel) and thymus γδ T cell effector commitment (Figure 3B, right panel).

In summary, our results indicate that the maternal microbiome impacts the neonatal lung Vγ γδ T cell subset composition, albeit with minimal changes in the overall effector commitment within the γδ T cell compartment.

2.4 | Maternal Administration of *L. rhamnosus* (*L.rhm.*) Promotes IL17-Committed Perinatal Lung γδ T Cells at Birth

Next, we wanted to investigate if the effect of the maternal microbiome on the neonatal γδ T cells can be further influenced/augmented by maternal administration of probiotics, with strains shown to have beneficial effects on neonatal health [15, 20, 21]. For this, we administered two different probiotic strains, *L. rhamnosus* VES 001 (*L.rhm.*) or *Bifidobacterium animalis* subsp. *lactis* VES 002 (*B.lac.*) to pregnant mothers and examined their effects on neonatal γδ T cells by comparing with control group. Maternal administration of *L.rhm.* led to increased frequencies of Vγ6 cells in the lung at birth, mainly at the expense of the nonVγ1/4/6 γδ T cell fraction (Figure 4A; Figure S6B). Importantly, this change was associated with an overall skewing toward IL17 commitment at the expense of IFNγ commitment in the γδ T cell compartment (Figure 4B). This overall skewing toward IL17 commitment was mainly due to the increase of Vγ6 γδ T cell frequencies (Figure 4A) rather than an increase in IL17-commitment within the lung Vγ6 γδ T cell subset that was already inherently highly biased toward that fate (Figure S7). There was no increase in IL17-commitment in the other lung T cell subsets (Figure S7). Of note, this effect was limited to *L.rhm.*, as such effect was not observed in the *B.lac.* group (Figure 4A,B). In fact, maternal administration of *B.lac.* led to reduced frequencies of the perinatal lung Vγ1 subset, with no major modulations of the total γδ effector commitment (Figure 4A,B; Figure S6B). In addition, the effect of *L.rhm.* on the Vγ subset composition and effector commitment in neonates was limited to the D0 lung, as such effects could not be observed in the D0 thymus (Figure 4C,D) or D3 lung (Figure S8). Of note, the administration of *L.rhm.* or *B.lac.* did not modulate the microbiome composition (α-diversity and β-diversity) of the offspring (D1, D3, D7), while the age of the offspring had a profound effect [51].

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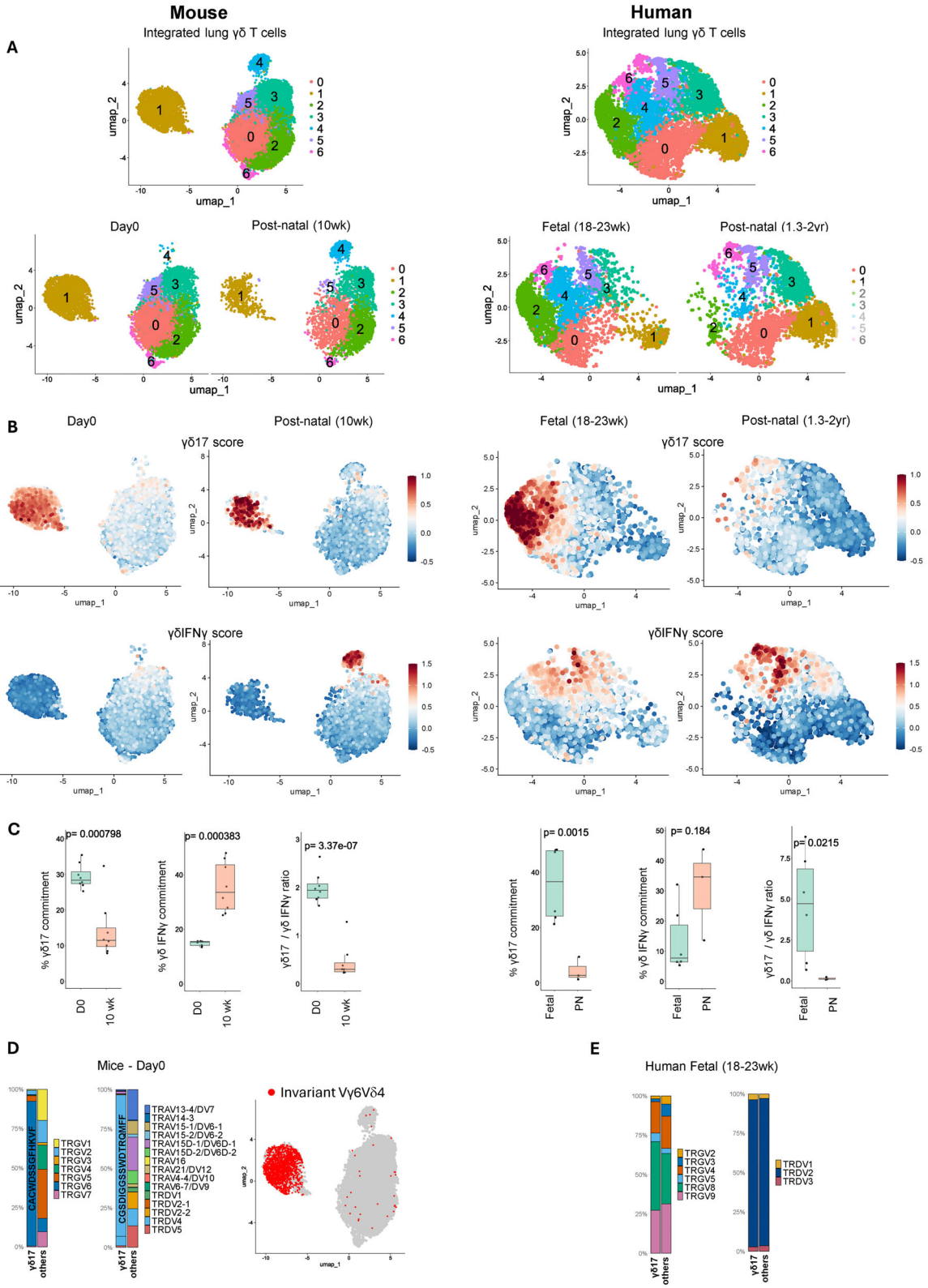


FIGURE 2 | The IL17-commitment of perinatal lung *γδ* T cells is conserved between mice and humans. (A) UMAP plots showing lung *γδ* T cell clusters—top panels (mouse left, human right). (B) UMAP plots showing gene module scores for *γδ*17-commitment and *γδ*IFN*γ*-commitment (mouse left, human right). (C) Comparisons of *γδ*17 and *γδ*IFN*γ* committed clusters per individual mouse/human subject, based on gene module scores (mouse *γδ*17 cluster 1, human *γδ*17 cluster 2, mouse *γδ*IFN*γ* clusters 3 & 4, human *γδ*IFN*γ* clusters 3 & 5). (D) Bar plots showing mouse TRGV and TRDV usages in *γδ*17-committed clusters and non-*γδ*17 clusters (others) (left panel), UMAP plot showing mouse V*γ*6V*δ*1 cells expressing invariant TRGV6 (CDR3*γ*—CACWDSSGFHKVF) paired with invariant TRDV4 (CDR3*δ*-CGSDIGGSSWDTRQMFF) (on the right). (E) Bar plots showing human TRGV and TRDV usage in *γδ*17-committed clusters and non-*γδ*17-committed clusters (others). $n = 8$ mice for Day 0; $n = 8$ mice for 10wk; $n = 6$ for human fetal (18–23 week); n = 3 for human postnatal (1.3–2 years).

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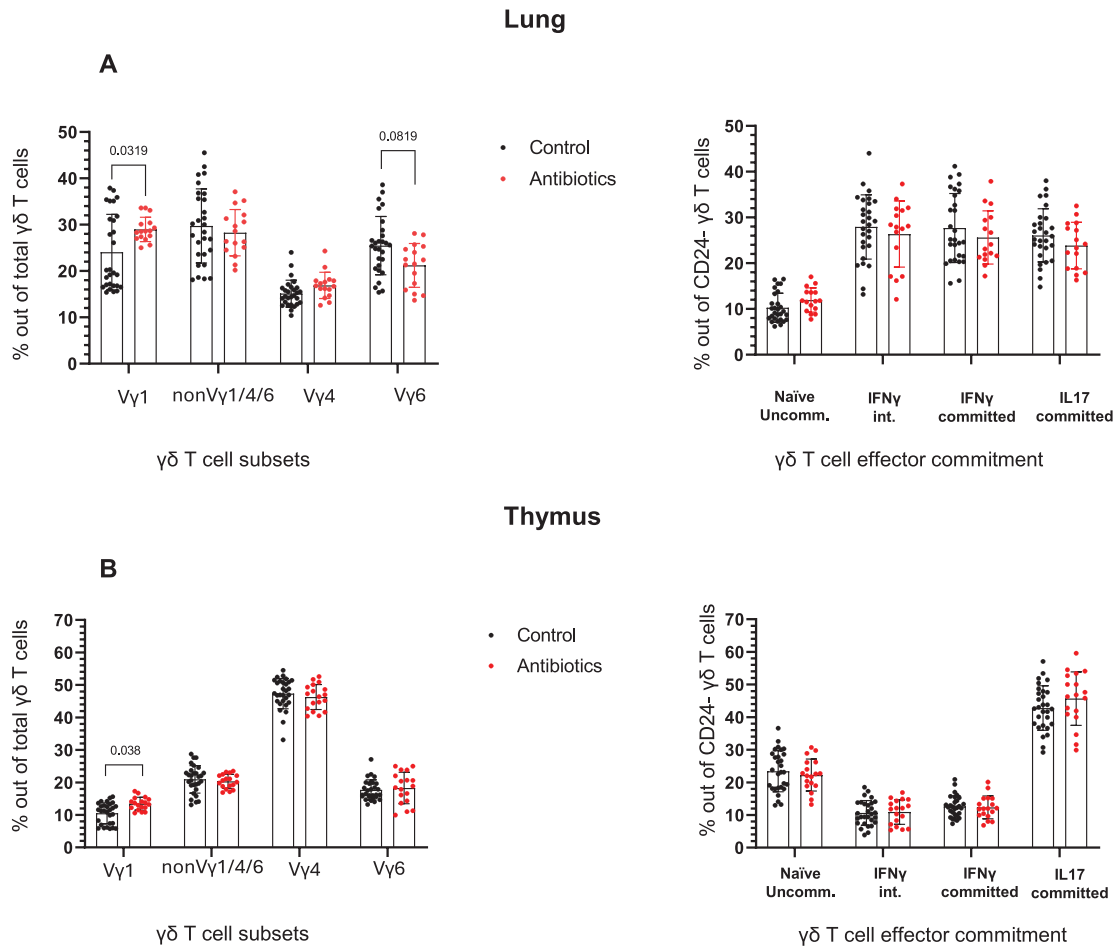


FIGURE 3 | Maternal microbiota impacts Vγ usage of perinatal lung $\gamma\delta$ T cells. (A) Lung $\gamma\delta$ T cell subsets on day 0 (left), total lung $\gamma\delta$ T cells effector commitment via CD44 and CD45RB expressed as a percentage out of the mature (CD24−) $\gamma\delta$ population (right). (B) Thymic $\gamma\delta$ T cell subsets on day 0 (left), total thymic $\gamma\delta$ t cell effector commitment via CD44 and CD45RB expressed as a percentage out of mature (CD24−) $\gamma\delta$ thymocyte population (right). Two-way ANOVA, multiple comparison with Sidak's test, $n = 16–29$ mice per group pooled from three independent experiments, error bars indicate range of min to max with mean.

In summary, *L.rhm.* but not *B.lac.* administered to the mother during late pregnancy drives further the IL17-commitment of perinatal lung $\gamma\delta$ T cells at birth mainly by increasing the prevalence of Vγ6 $\gamma\delta$ T cells.

3 | Discussion

In this study, by using a mouse model, we show that the maternal administration of the probiotic strain *L.rhm.* leads to an increase of IL17-committed Vγ6 $\gamma\delta$ T cells in the newborn lung at birth.

While it was known that the mouse lung contains a large fraction of Vγ6 T cells [28, 29], the exact prevalence and the kinetics following birth were not known. Using a recently described monoclonal antibody specific against the mouse Vγ6 chain [46], we found that Vγ6 T cells dominate the lung T cell population at birth (day 0): they are more prevalent than $\alpha\beta$ T cells and other $\gamma\delta$ T cell subsets. In addition, we showed that at this age, the Vγ6 cells are highly enriched for the previously described invariant γ chain TRGV6/TRGJ1-CACWDSSGFHKVF coupled to the invariant δ chain TRDV4/TRDD2/TRDJ2-CGSDIGGSSWDTRQMFF [23] (coding for the Vδ1 chain) and that this Vγ6Vδ1 TCR is associated with a program toward IL17-commitment. Recently, it has been described that this same invariant TCR shows polyspecificity, capable of recognizing ligands as broad as microbe-derived metabolites such as indole-3-propionic acid (IPA) and viral-derived proteins [52]. Thus, this would allow the lung T cells to recognize both compounds from bacterial and viral origin, allowing a broad reactivity before the development of $\alpha\beta$ T cell-based tissue-resident memory [53]. While the structural basis of this polyspecificity of the same invariant $\gamma\delta$ TCR still needs to be identified, it appears that such "promiscuous" recognition of antigens might be a more general characteristic of early life T cells, as mouse neonatal $\alpha\beta$ T cells are more cross-reactive to peptides than their adult counterparts [54].

Maternal administration of the probiotic strain *L.rhm.*, but not of the probiotic strain *B.lac.*, augmented the prevalence of IL17-committed Vγ6 $\gamma\delta$ T cells in the newborn lung. An opposite trend was observed by depleting the maternal microbiome using a mix of antibiotics. Others have either observed a dependency [38] [45] or not [36] on the microbiome of the prevalence of (Vγ6) $\gamma\delta$ T cells in the adult mouse lung. Of note, in the context of

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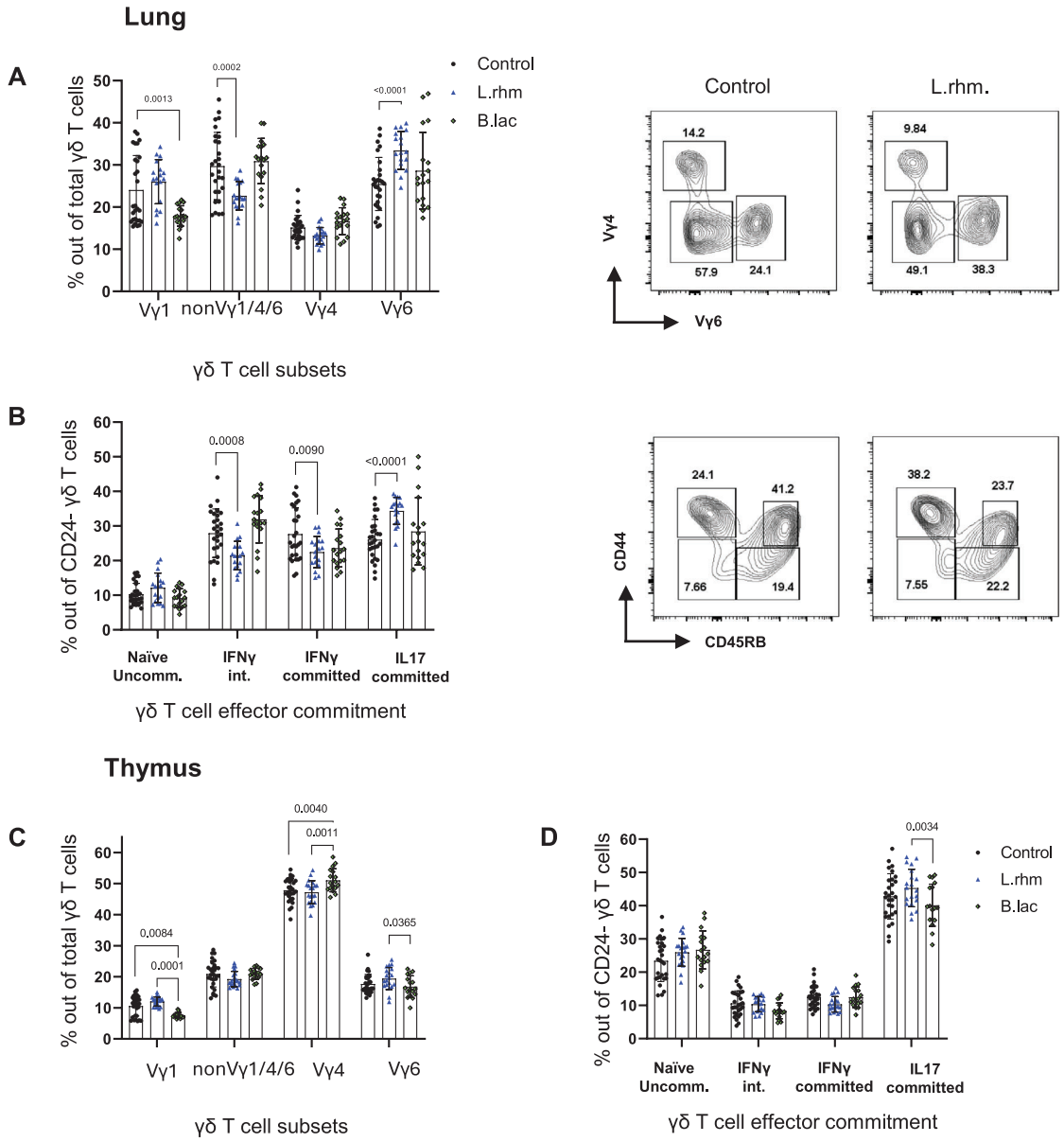


FIGURE 4 | Maternal administration of *Lacticaseibacillus rhamnosus* (L.rhm.) promotes IL17-committed perinatal lung *γδ* T cells. (A) Lung *γδ* T cell subsets on day 0 expressed as percentages out of total *γδ* population (left), flow cytometry plot showing Vγ6 subset percentage of mice from control group (middle), flow cytometry plot showing Vγ6 subset percentage of mice from L.rhm. Group (right). (B) Total lung *γδ* T cell effector commitment on day 0 (left), flow cytometry plot showing *γδ* effector cluster profile of control mice (middle), flow cytometry plot showing *γδ* effector profile of mice in L.rhm. group (right). (C) Thymus *γδ* T cell subsets on day 0 expressed as percentages out of the total *γδ* population. (D) Thymus total *γδ* T cell effector commitment on day 0, two-way-ANOVA, Tukey's multiple comparison, $n = 17–29$ mice per group, on D0, pooled from three independent experiments, error bars indicate range of min to max with mean.

lung cancer, local microbiota promotes the development of IL17-committed Vγ6Vδ1 T cells that are associated with the promotion of lung cancer development [55]. Here, we show that the fetal period can be a window of opportunity to steer the presence of IL17-committed immunity in the lung at birth by maternal administration of the probiotic strain *L.rhm*. during pregnancy. In early life, this could have a beneficial effect, as IL17-committed *γδ* T cells can protect neonatal mice against influenza infection by promoting tissue homeostasis [31], where the invariant Vγ6Vδ1 TCR can potentially react toward influenza-derived proteins [52]. Thus, the timing of the promotion of IL17-commitment might be key and could explain why, in adult mice, as we show here, the lung *γδ* T cell compartment switches toward IFNγ-committed *γδ* T cells. When this regulation is disturbed, such as in lung cancer, the IL17 axis may promote lung cancer development [55]. A similar disturbance of IL17 versus IFNγ-committed *γδ* T cells has been observed in other cancers as well [56, 57]. Further illustrating the tight regulation of IL17-commitment in the lung is that at D3 of postnatal life, no differential effect was observed on the lung Vγ6 *γδ* T cell subset following maternal administration of *L.rhm*. While no significant effect could be observed on the global microbial composition metrics of the gut microbiome of the mothers and the offspring upon maternal administration with *L.rhm*., the early postnatal spontaneous colonization with

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commensal microbes resulted in a notable increase in the offspring gut microbiota richness and evenness during the first 7 days of development [51]. This developmental increase in the gut microbiota richness observed by D3, corresponding with a peak in the proportion of the Vγ6 subset, may have neutralized the effect of maternal administration of probiotics on the frequencies of Vγ6 cells observed at D0. In addition, while clear effects were observed in the lung at birth (D0) upon maternal administration of *L.rhm*., this was not the case in the corresponding thymic, indicating that the effects were mainly mediated locally in the lung. That said, effects on embryonic thymic Vγ6 cells (either Vγ6 cell levels and/or their speed of thymic egress) cannot be excluded. On the other hand, it has to be noted that the fetal lung is much more vascularized than the thymus and comes potentially readily into contact with maternal microbial-derived metabolites [5, 58] following the maternal administration of *L.rhm*. Furthermore, while the intestinal microbiome can influence the thymic development of certain innate lymphocytes, it appears that thymic γδ T cells are not influenced [59], which is in line with our study. Other innate T lymphocytes such as iNKT and MAIT cells can also be shaped by early exposure to microbial metabolites [60–62], but here, this occurs weeks after birth, coinciding with their emergence [3]. Thus, our data indicate that fetal γδ T cells are the main cellular target to be shaped by the microbiome/probiotics in the perinatal period before the appearance of other innate lymphocytes. Of note, a recent study by Papotto and colleagues showed that γδ T cells can influence the mother-offspring interaction also via other pathways, as maternal γδ T cells can shape offspring (D15) pulmonary type 2 immunity in a microbiota-dependent manner [63].

In contrast to iNKT and MAIT cells, the TCR genes of γδ T cells are not conserved between mice and humans [23, 64]. Also, differences at the functional level are observed: while IL17 can be readily produced by mouse γδ T cells, in humans, this is usually only the case in pathological conditions such as cancer [57]. Here, we show that the γδ T cell compartments of both the human and mouse perinatal lung are biased toward IL17-commitment, despite clear differences at the TCR level. The distinct functional programming in the human fetal thymus [33] is likely at the origin of this IL17-committed population in the fetal lung. We speculate that maternal microbiota-derived factors, such as phosphorylated metabolites of the isoprenoid pathway [65], can contribute to the local expansion of IL17-committed γδ T cells in the fetal lung, similar to the mouse model. Indeed, data derived from the perinatal blood human Vγ9Vδ2 T cells indicate that fetal Vγ9Vδ2 T cells are highly responsive to microbiome-derived factors [23, 66–68].

This study has some limitations. Different models can be used to investigate the causal role of gut microbes on the host, such as antibiotics treatment or the use of germ-free mice, each one having its own limitations. We chose to use antibiotics treatment and not germ-free mice since it is well established that germ-free mice present multiple adaptations, including in the immune system, that could interfere with our investigation [69, 70]. While the exact molecular mechanism remains to be investigated, our data open the possibility of utilizing maternal-specific probiotic administration to enhance neonatal lung γδ T cell functions. Future studies investigating the effect of *L.rhm*. administered to pregnant women on γδ T cells in the offspring, for example, in cord blood, would be of particular interest to extend our experimental data.

4 | Materials and Methods

4.1 | Mice

C57BL/6 mice were acquired from ENVIGO. All mice used in our experiments were housed and bred in a specific pathogen-free (SPF) animal facility in individually ventilated cages with a controlled day-night cycle and given food and water ad libitum. All experiments were approved by the institutional Animal Care and Local Use Committee.

4.2 | Human Lung Material

Fetal lungs (gestation 18–23 weeks gestation) were obtained by Cercle Allocation Services and reviewed and approved by WCG IRB (20111833) and by the Erasme Ethical Committee (N2023/006). Pediatric lungs were obtained from deceased organ donors at the time of organ acquisition (1.3–2 years old), from which γδ T cell data (GSE240858) was downloaded and integrated with human fetal lung γδ data for downstream analysis [50].

4.3 | Maternal Antibiotic and Probiotic Administration Protocol

For maternal microbiota depletion two weeks after mating (i.e., last week of pregnancy), pregnant C57BL/6 females (10–12 weeks of age) received a cocktail of antibiotics (Ampicillin (1 g/L), Neomycin (1 g/L), Vancomycin (0.25 g/L), Streptomycin (1 g/L)) in drinking water. For probiotics administration, pregnant mothers received daily oral gavages of either 2.10^8 CFU *L. rhamnosus* VES 001 (*L.rhm*.) (SUPERBIOTICS) or 2.10^8 CFU *B. animalis* subsp. *lactis* VES 002 (*B.lac*) (SUPERBIOTICS)/100 µL of NaCl 0.9% (B.Braun) as previously described [21]. Antibiotics and daily probiotics gavages of pregnant mice were maintained until delivery.

4.4 | Flow Cytometry

Mouse lung, liver, small intestine, spleen, thymus, and skin tissues were harvested and dissociated to obtain single-cell suspensions. Thymus and spleen samples were disrupted using a Pyrex Potter tissue homogenizer (VWR; Leuven), FACS buffer (PBS/0.5% BSA/2 Mm EDTA). Lung and liver tissues were perfused with PBS, cut into small pieces with scissors, incubated in RPMI-1640 medium supplemented with recombinant Grade I DNAse I (10 U/mL, Thermo Fisher) and collagenase A (1 mg/mL, Roche) for 20 min, and dissociated using GentleMACS (Miltenyi Biotec). Intestine samples were incubated in HBSS (Hank's Balanced Salt Solution) containing Collagenase IV (0.5 mg/mL, Roche) and DNAse I (10 U/mL, Thermo Fisher) for 30 min. Skin samples were cut into small pieces and incubated in HBSS containing Collagenase IV (0.5 mg/mL, Roche), Dispase II (2 mg/mL, Sigma), and DNAse I (10 U/mL, Thermo Fisher) for 1 h and dissociated using the GentleMACS (Miltenyi Biotec). In

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lung, liver, spleen, and thymus cell suspensions, red blood cells were lysed by resuspending cell pellets in ammonium-chloride-potassium lysis buffer (ACK) for 1 min.

Human fetal lung samples were dissociated using the same protocol as mouse lung samples. The cell suspensions were subsequently cryopreserved in liquid nitrogen and stored until thawing. For extracellular staining, single-cell suspensions were stained with a mix of antibodies diluted in appropriate buffers (FACS buffer or BD brilliant stain buffer) at 4°C in the dark for 20 min. For intracellular/intranuclear staining, cells were fixed and permeabilized using the Foxp3-staining buffer kit (eBiosciences) following the one-step manufacturer protocol. Antibodies used for flow cytometry are listed in Table S1. The gating strategy for identifying γδ T cells is illustrated in Figure S2.

4.5 | Quantification of Cecal Eubacteria Load

Cecal content was harvested from mothers that received antibiotics in drinking water and control mice. Microbial DNA was extracted using Nucleospin Microbial DNA (Macherey-Nagel) kit according to the manufacturer's protocol. DNA samples were quantified using the NanoDrop spectrophotometer and stored at −20°C. These samples were used to quantify total intestinal microbiota by RT-qPCR targeting bacterial 16S gene using Lightcycler 480 SYBR Green I Master reaction mix (Roche Diagnostics) and RPL32 gene for normalization.

4.6 | Single-Cell RNA-seq (Gene Expression and γδ TCR) Libraries Construction

Mouse lung cells from individual subjects (8 fetal [Day 0] and 8 adults [10 weeks old]) were stained with barcoded hashing antibodies TotalSeq-C 5′ chemistry (Table S1), following the recommendations of the manufacturer (Biolegend), prior to staining for FACS sorting. Human lung cells from six fetal lung samples (18–23 weeks old) were individually hashed with MULTI-Seq lipid barcodes as previously described [71], followed by staining for FACS sorting. For all samples, $CD3^+$ $\gamma\delta TCR^+$ cells were sorted on FACS Aria III cell sorter (BD Biosciences), with purity of 98.3% and 82.5% out of living cells, respectively, for mouse Day 0 and 10-week lung γδ T cells.

Libraries for sc RNA and TCR sequencing were generated from 1.5-2*104 FACS-pool sorted γδ T cells using the Chromium Single Cell 5' Library Gel Bead and Construction kit as well as Chromium Single Cell V(D)J Enrichment Kit (10× Genomics) according to the user guidelines (v2 Chemistry, Single Cell V(D)J protocol number CG000330).

Single-cell TCR, gene expression, and surface protein libraries were generated according to Chromium Single Cell V(D)J protocol (10× Genomics). 2 μL of cDNA amplified and purified from GEMs ("Gel bead in Emulsion" droplets) were used to amplify γδTCR CDR3 sequences. Custom primers specific for TRDC and TRGC constant gene segments were designed for this purpose. In brief, for the first step in the enrichment of CDR3 sequences, the custom outer primer sets were followed by second target enrichment (inner) primer sets (Table S2). The remaining volume of cDNA was used for the construction of a gene expression library according to 10X Genomics protocol instructions. Multiseq hashing libraries were constructed in a separate step, as described before [71]. Agilent Bioanalyzer High Sensitivity DNA chips were used to check quality control read-outs of sc RNA-seq and sc TCR-seq libraries using a Bioanalyzer 2100 machine (Agilent Technologies). Indexed libraries were pooled and sequenced on the Illumina NovaSeq 6000 from the BRIGHTcore (Brussels Interuniversity Genomics High Throughput Core) platform.

Pediatric lung γδ T cell data GSE240858 were downloaded and integrated with human fetal lung γδ data for downstream analysis [50].

4.7 | Single-Cell RNA-seq Data Processing

Single-cell RNA sequencing was performed using 10x Genomics with 5' feature barcoding technology to multiplex cell samples from different subjects. CellRanger (v7.1.0) software from 10x Genomics was used to demultiplex and map sequencing reads against GRCm39 and GRCh38 genomes. Count matrices were loaded into R using the "read10x" function from the Seurat R package. All downstream analyses were conducted using Seurat v5.0.0 in R v4.3.2. Low-quality and doublet cells were filtered out using the minimum cutoffs nFeature_RNA ≥200, % mitochondrial gene >5%. *ScaleData* function was used to normalize and stabilize variance in the datasets regressing out percent mitochondria genes, percent ribosomal genes, cell cycle scores, nFeatureRNA, and TRDV TRGV genes. Principal component analysis (PCA) was subsequently performed using the *RunPCA* function with 30 principal components. To account for batch effects in the integration of datasets, the Harmony algorithm was applied using the *RunHarmony* function. The Harmony embeddings were extracted using the Embeddings function. Dimensionality reduction was then carried out using the *RunUMAP* function, based on the top 10–20 Harmony components to generate a two-dimensional UMAP projection for visualization. The nearest-neighbor graph was constructed with the *FindNeighbors* function, and clustering was performed using the Louvain algorithm via the *FindClusters* function at a resolution of 0.5. Visualization of cell clusters and feature expression was carried out using *DimPlot* and *FeaturePlot*, respectively. Differential gene expression analysis was performed using the *FindAllMarkers* function to identify marker genes for each cluster, filtering for genes with a minimum expression threshold of 0.25 and log fold change ≥0.25. Single-cell gene signature enrichment scores for IL17-commitment and IFNγ-commitment were calculated using the "AddModuleScore" function of Seurat using a list of genes previously described [33] (Figure S4B,C). scTCR libraries were generated using CellRanger vdj pipeline (v3.0.2) and integrated with respective gene expression data (Seurat objects). Only those cells expressing productive TCR sequences (γ and/or δ chain) were retained for data integration in the Seurat objects using the "Addmetadata" function.

Author Contributions

Yohannes Tafesse designed and performed experiments, analyzed data, and wrote the manuscript. Guillem Sanchez Sanchez performed

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experiments and analyzed data. Patricia Brito Rodrigues, Marko Verce, Amandine Everard, and Moosa Rezwani provided support for data analysis. Arnaud Köhler, Isoline Verdebout, Panagiotis Vitsos, and Maria Papadopoulou performed the experiments. Véronique Flamand supported the design of the experiment. David Vermijlen designed and supervised research, analyzed data, and wrote the manuscript. All authors approved the final version of the manuscript.

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Conflicts of Interest

VF is a co-inventor on patent applications dealing with the use of *L. rhamnosus* and *Bifidobacterium animalis* subsp. *lactis* and its components in the treatment of respiratory viral infections. AE is an inventor on patent applications dealing with the use of *A. muciniphila* and its components in the treatment of metabolic disorders. AE is also an inventor on patent applications dealing with the use of gut microbes in food reward dysregulations. The remaining authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study (scRNA-seq data and scTCR-seq data of mouse day 0 and adult (10 weeks) lung $\gamma\delta$ T cells and of fetal (18–23 weeks) human lung $\gamma\delta$ T cells) have been deposited in the GEO database under accession code GSE278178. Pediatric lung $\gamma\delta$ T cell data were downloaded from GSE240858.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

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