Maternal gut microbiome regulates immunity to RSV infection in offspring

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Development of the immune system can be influenced by diverse extrinsic and intrinsic factors that influence the risk of disease. Severe early life respiratory syncytial virus (RSV) infection is associated with persistent immune alterations. Previously, our group had shown that adult mice orally supplemented with Lactobacillus johnsonii exhibited decreased airway immunopathology following RSV infection. Here, we demonstrate that offspring of mice supplemented with L. johnsonii exhibit reduced airway mucus and Th2 cell-mediated response to RSV infection. Maternal supplementation resulted in a consistent gut microbiome in mothers and their offspring. Importantly, supplemented maternal plasma and breastmilk, and offspring plasma, exhibited decreased inflammatory metabolites. Cross-fostering studies showed that prenatal Lactobacillus exposure led to decreased Th2 cytokines and lung inflammation following RSV infection, while postnatal Lactobacillus exposure diminished goblet cell hypertrophy and mucus production in the lung in response to airway infection. These studies demonstrate that Lactobacillus modulation of the maternal microbiome and associated metabolic reprogramming enhance airway protection against RSV in neonates.

Introduction

Respiratory syncytial virus (RSV) infects nearly all infants by 2 yr of age and is the leading cause of bronchiolitis in children worldwide (Openshaw et al., 2003). RSV is especially detrimental in very young infants, whose airways are small and easily occluded. Several epidemiological studies link severe RSV infection with the later development of hyper-reactive airway disease that persists even years after the initial viral infection has resolved (Bacharier and Geha, 2000; Henderson et al., 2005; Sigurs et al., 2010). RSV infection interferes with the development of an appropriate antiviral immune response and results in altered lung pathology that may be permissive to the development of wheezing later in life (Sigurs et al., 2000; Sigurs et al., 2005). This includes the recruitment of T helper type 2 (Th2) cells to the lungs, which produce IL-4, IL-5, and IL-13, all of which are cytokines that contribute to allergic pathogenesis (Barnes, 2001; Cohn and Ray, 2000; Robinson, 2000). Neonatal RSV infection causes persistent changes in the lung such as mucus production and increased type 2 innate lymphoid cells (ILC2s) that produce IL-5 and IL-13 (Malinczak et al., 2019; Saravia et al., 2015).

Early life immune development is central to establishing appropriate responses to infectious stimuli without inducing inflammatory states with potential long-term sequelae. Adaptive immunity is evident as early as 13 wk gestation in humans (Howie et al., 1998; McGovern et al., 2017), capable of responding to commensal microbes and skewed away from inflammatory responses in utero to avoid abortive birth (Chen et al., 2020; Ghazal et al., 2013; Sharma et al., 2012). Evidence has emerged that initial immune responses are influenced by early-life microbes and their bioactive products, exerting pervasive effects on immune maturation and function (Rackaiyte et al., 2020; Torow et al., 2017). The effect of alterations to early postnatal microbiome composition and function on allergic responses is associated with changes in development of immune responses, especially during infancy, when the microbiome is initially established (Durack et al., 2018; Fujimura et al., 2016; Lynch, 2016; Marsland, 2013). Increases in a broad range of microbial- and/or mammalian-derived lipids, amino acids, and peptides with immunomodulatory potential support the growing concept that

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metabolic signaling may represent an important mechanism by which the microbiome and host interact (Li et al., 2008). Recent data have begun to build mechanistic evidence for how the gut microbiome and its bioactive metabolites may regulate inflammation and immune responses.

A number of factors contribute to the immune response to RSV, including both genetic and environmental factors. Recently, the role of the gut microbiome in dictating the immune response at distal mucosal surfaces and organs has received increased attention (Budden et al., 2017; Dang and Marsland, 2019; Maschirow et al., 2019). Previously our group had shown that adult mice orally supplemented with *Lactobacillus johnsonii* before viral infection resulted in gut microbiome restructuring (Fonseca et al., 2017; Fujimura et al., 2014). *L. johnsonii* supplementation altered the profile of circulating metabolites 48 h after RSV infection, including a range of known anti-inflammatory polysaturated fatty acids and altered dendritic cell (DC) function (Fonseca et al., 2017). In the studies presented here, we show prenatal supplementation with *L. johnsonii* regulates offspring immunity to RSV infection via metabolic reprogramming and gut microbiome modification.

**Results and discussion**

**Maternal supplementation with *L. johnsonii* regulates offspring immunity to RSV**

We have previously shown that supplementing adult mice with *L. johnsonii* before RSV infection protects against airway mucus and Th2 cytokine production (Fonseca et al., 2017; Fujimura et al., 2014). To determine whether maternal prenatal *L. johnsonii* supplementation conferred protection against RSV infection in offspring, female mice were orally supplemented daily with *L. johnsonii* (10^7 CFU per dose) for 7 d before mating, with continued supplementation twice per week until delivery. Offspring were infected with RSV at 7 d of age (Fig. 1 A), and samples were collected at 7 d after infection. Histopathologic examination indicated that mucus production and inflammatory infiltration was reduced in the lungs of mice born to *L. johnsonii*-supplemented mothers compared with neonates from PBS-supplemented mothers (Fig. 1 B). Decreased mRNA levels of the mucogenic gene Gob5 (Fig. 1 C) in lung tissue of offspring born to *L. johnsonii*-supplemented mothers confirmed this phenotype. Interestingly, these differences in the immune response are not due to changes in viral clearance, as expression of the gene for the RSV F protein is not different in offspring from PBS- or *L. johnsonii*-supplemented females (Fig. 1 D). Lung draining lymph node (LDLN) samples from neonates born to *L. johnsonii*-supplemented mothers had decreased levels of Th2 cytokines, IL-4, IL-5, and IL-13, and an increase in IFN-γ compared with responses from mice born to control, PBS-supplemented mothers (Fig. 1 E). Analysis of the inflammatory cell composition in the lung of the neonates by flow cytometry indicated decreased numbers of ILC2s in the mice born to *L. johnsonii*-supplemented mothers (Fig. 1 F), although, interestingly, we did not note any difference in eosinophil numbers (Fig. 1 G). We also measured diminished numbers of CD4⁺ T cells migrating to the lungs of these mice compared with neonates born to PBS mothers (Fig. 1 H). While we did note an increase in regulatory T cells in the infected mice compared with uninfected controls, there were no significant differences (Fig. 1 I). These data suggest that maternal prenatal supplementation with *L. johnsonii* alters neonatal immune response to RSV infection by altering the immune response and reducing mucus.

To determine the longevity of the maternal influence of supplementation, mice born to PBS control or *L. johnsonii*-supplemented females were infected at 6 wk of age (Fig. S1 A). Histopathological examination at 8 d after infection found a marked decreased inflammatory response and mucus in the lungs from adult mice born to *L. johnsonii*-supplemented females (Fig. S1 B). Corresponding to the decreased mucus, gob5 mRNA was decreased in the lungs of infected mice born to supplemented females, although not statistically significantly (Fig. S1 C). A significant decrease in IL-4, IL-5, and IL-13, and IL-17 in mice born to supplemented females, with no change in IFN-γ, was observed (Fig. S1 D). Correspondingly, a decrease of CD4⁺ T cells in the lungs of infected mice born to supplemented females compared with mice born to control females (Fig. S1 E) was observed. There was also a decrease in both CD11c⁺CD11b⁺ (Fig. S1 F) and CD11c⁺CD103⁻ DCs (Fig. S1 G). Together, these data indicate that the protection from RSV-induced pathology we found in neonates exposed to maternal *L. johnsonii* persists into maturity.

**Prenatal supplementation with *L. johnsonii* alters both maternal and offspring gut microbiota and metabolic features**

To assess the impact of maternal *L. johnsonii* supplementation on maternal and offspring gut microbiota, 16S ribosomal RNA (rRNA) profiles of cecal samples collected at 12–13 wk of age (maternal) and 14 d of age (offspring) were performed. Cecal microbiota of mothers supplemented with *L. johnsonii* exhibited a relatively consistent gut microbiota composition that was significantly distinct from that of PBS-supplemented mothers (permutational multivariate ANOVA [PERMANOVA]; R² = 0.177; P = 0.002; Fig. 2 A) and primarily characterized by expansion of specific amplicon sequence variants (ASVs) belonging to the Lachnospiraceae and Muribaculaceae (Fig. 2 B). Cecal microbiota of offspring born to *L. johnsonii*-supplemented mothers had a discrete and less variable gut microbiota compared with that of offspring from PBS-supplemented mothers (PERMANOVA; R² = 0.341; P = 0.01; Fig. 2 C). Offspring from *L. johnsonii*-supplemented mothers had an expansion of ASVs belonging to Lachnospiraceae and Muribaculaceae genera similar to *Lactobacillus*-supplemented mothers (Fig. 2 D and Table S1). Furthermore, neonates born to *L. johnsonii*-supplemented females demonstrate a stable microbiome when infected with RSV, unlike those of PBS-treated females (Fig. 2 E). Thus, *L. johnsonii* exposure has a stabilizing effect on the neonatal microbiome, even after RSV infection. While we do not see *L. johnsonii* as a predominant species, *Lactobacillus* has been shown to exhibit a founder effect, acting as low-abundance keystone species that creates an environment for bacterial communities that produce beneficial metabolites (Faust and Raes, 2012; Garcia Rodenas et al., 2016). Other probiotic bacteria may exhibit similar effects.
The link between diet and the gut microbiome as a regulator of key immune responses has been established in studies where dietary fiber intake enriches fermentative species, which produce short-chain fatty acids and promote the development of regulatory T cells (Arpaia et al., 2013; Geuking et al., 2013; Kosiewicz et al., 2014; Zeng and Chi, 2015). Furthermore, ω-3 polyunsaturated fatty acids and short-chain fatty acids reduce adhesion molecule expression and cytokine production by blocking NLRP3 and reducing inflammasome and NF-κB activation (Borthakur et al., 2008; Draper et al., 2011; Narushima et al., 2014; Teague et al., 2013). To identify metabolic features associated with prenatal L. johnsonii versus PBS supplementation, maternal plasma as well as plasma and ingested breast-milk from offspring were assessed using untargeted liquid...
chromatography mass spectrometry (Metabolon, Inc.). Following supplementation with *L. johnsonii*, a total of 48 maternal plasma metabolites significantly differed compared with PBS controls (Fig. S2 A and Table S2). Plasma of *L. johnsonii*–supplemented mothers was depleted of several proinflammatory fatty acid (palmitate, arachidonate, and linoleate), glycerophosphocholines, and a range of monohydroxy fatty acids, monoacylglycerols (1-dihomo-linoenylglycerol and 2-oleoylglycerol), sphingolipids, ceramides, lysolipids, and histamine. Increases in the amino acids N-acetyl tyrosine and hydroxyproline, TCA cycle intermediates (*α*-ketoglutarate, and malate), and evidence of reductive sugar metabolism (increased ribotol, galactitol, and arabitol), 2-aminoctanoate, a mitochondrial fatty acid, and myo-inositol were also observed (Table S2) in these animals. Metabolic profiles were assessed at 7 d of age (before RSV infection), and a total of 139 metabolites exhibited significant differences in relative concentration between offspring of *L. johnsonii*– and PBS-supplemented mothers (Fig. S2 B and Table S3).
We collected ingested milk from the stomachs of naive pre-weaned pups, resuspended the content in sterile PBS, and analyzed it by mass spectrometry for metabolites. The results detected 129 metabolites significantly different in the milk from *L. johnsonii*-supplemented mothers compared with the milk of the PBS-supplemented mothers (27 up and 102 down; Fig. S2 C and Table S4). A comparison of ingested breastmilk metabolites indicated parallel reductions in glycerophosphocholine-linked fatty acids, ceramides, sphingolipids, and lysolipids in addition to other metabolites in milk of offspring born to *L. johnsonii* compared with PBS-supplemented mothers (Table S4). 9 metabolites were significantly altered in both the plasma of *L. johnsonii*-supplemented mothers and their offspring, 6 overlapped between maternal plasma and ingested milk, and 15 were shared between ingested milk and offspring plasma (Table S5). Interestingly, we detected the down-regulation of 2-oleoylglycerol in all groups and samples in the *L. johnsonii*-supplemented group. Finally, we compared the metabolic profile of plasma of the offspring and milk (Table S5) and detected 4 shared metabolites up-regulated and 11 down-regulated. In particular, we detected down-regulation of 9,10-dihydroxyoctadecenoic acid (DiHOMA), a linoleic acid metabolite, and guanosine in plasma and milk samples from the *L. johnsonii*-supplemented group. Published studies from our group have shown that high concentrations of DiHOMA metabolites in infant feces relate to increased risk of atopy and asthma development in childhood (Fujimura et al., 2016), supporting previous studies associating DiHOMA in the airways of allergen-provoked asthmatic patients compared with healthy patients (Larsson et al., 2014; Lundström et al., 2012). Our studies had previously identified that *L. johnsonii* supplementation of adult mice led to increases in plasma polyunsaturated fatty acids (Fonseca et al., 2017), which were not observed in the present studies, perhaps due to the timing and/or fact that we are looking during pregnancy and in the offspring. Products that are found in both mothers’ and offspring plasma, such as 2-aminocanoate, that may represent an increase in fatty acid metabolism (Nieman et al., 2018), may be due to microbiome changes and induced by milk consumption. Thus, metabolic factors (host and microbiome-derived) are likely influenced by both the developing commensal microbiome and milk-derived products (Grier et al., 2017; Oliphant and Allen-Vercoe, 2019). Collectively, these data indicate that prenatal *L. johnsonii* supplementation leads to profound reprogramming of maternal circulating lipid metabolites and altered energy biogenesis during pregnancy, features of which are transmitted in breastmilk and evident in the circulating metabolite profile of the offspring of these animals.

**RSV lung pathology and immune responses are differentially altered pre- and postnatally by maternal supplementation with *L. johnsonii***

To understand if RSV-protective immunity observed in offspring of *L. johnsonii*-supplemented mothers was mediated via a prenatal (in utero) or postnatal (breast milk consumption) mechanism, a cross-fostering experiment was performed. Offspring from PBS-supplemented mothers were cross-fostered to a *L. johnsonii*-supplemented mother (PBS-LJ) and vice versa (LJ-PBS), as well as control PBS-PBS and LJ-LJ groups (Fig. 3 A). We observed that cross-fostered controls (PBS-PBS) had increased mucus in the lung (periodic acid–Schiff stain [PAS]) compared with the LJ-LJ group, findings that were validated by quantitative PCR (qPCR)–based expression of mucus-related genes in these animals (Fig. 3, B–D) and consistent with our initial observations (Fig. 1). Of note, mice born to PBS mothers but raised by *L. johnsonii* mothers (PBS-LJ) showed an effect similar to the LJ-LJ group following RSV infection, with decreased production of mucus in the lungs. However, mice born to *L. johnsonii*-supplemented mothers but raised by PBS-supplemented mothers had similar levels of mucus in the lungs to those of mice born to and raised by PBS mothers (Fig. 3 B). These histological observations were confirmed by qPCR-based expression of gob5 (Fig. 3 C) and muc5ac (Fig. 3 D) in the lungs of RSV-infected offspring. As noted in Fig. 1, there were no differences in the expression of the RSV F gene (Fig. 3 E). These results suggest that prenatal *L. johnsonii* exposure is associated with reductions in RSV-induced airway mucin responses following RSV infection of offspring postnatally, possibly via breast milk, and that these responses are independent of viral clearance.

Cross-fostered mice were analyzed for cytokine production and cellular infiltration following RSV infection. We found that mice born to *L. johnsonii*-supplemented mothers (LJ-LJ and LJ-PBS) had significantly diminished production of Th2 cytokines IL-4, IL-5, and IL-13 from restimulated lymph node cells compared with those from PBS-supplemented mothers (PBS-PBS and PBS-LJ; Fig. 4, A–C). Interestingly, the mice that were born to PBS-supplemented mothers and cross-fostered by *L. johnsonii*-supplemented mothers (PBS-LJ) had similar Th2 cytokine production to the PBS-PBS control group. Analysis of the inflammatory infiltration in the lungs of the offspring identified that mice born from mothers supplemented by *L. johnsonii* had significantly decreased numbers of ILC2s in the lungs, independent of whether they were cross-fostered by PBS- or *L. johnsonii*-supplemented females (Fig. 4 D), with no difference in eosinophils (Fig. 4 E). The PBS-PBS and PBS-LJ groups exhibited increased macrophage frequency in the airways compared with LJ-LJ or LJ-PBS animals (Fig. 4 F). The LJ-LJ group compared with PBS-PBS and PBS-LJ groups also had decreased CC11c+CD11b+ and CD11c+CD103+ DCs (Fig. 4 F). We did not find significant differences in the cross-fostering groups PBS-LJ and LJ-PBS. There were also no differences in the number of regulatory T cells in the lungs of neonatal mice (Fig. 4 G). However, the LJ-LJ and LJ-PBS groups had significantly decreased CD4+ T cells compared with the PBS-PBS and PBS-LJ groups (Fig. 4 H). The LJ-LJ group also showed decreased numbers of activated CD4+CD69+ cells, CD8+, and activated CD8+CD69+ T cells compared with the PBS-PBS and PBS-LJ groups (Fig. 4, I and J). These results further reinforce that prenatal *L. johnsonii* supplementation is associated with reduced airway inflammatory infiltration and reduced Th2 cytokine response following RSV infection of offspring.

**Maternal supplementation with *L. johnsonii* alters offspring gut microbiome***

Numerous studies have indicated that breastfeeding impacts infant microbiome development (Bergström et al., 2014; Jost et al., 2014; Roger et al., 2010). Our data indicate that the
maternal gut microbiome influences breastmilk metabolic content with consequences for offspring gut microbiome, circulating metabolome, and airway immunity. These observations provide support for an intergenerational effect of maternal microbiomes on offspring microbial and metabolic status, factors that train and strongly influence developing immunity in the offspring. Moreover, our cross-fostering experiment permits insights into the effect of pre- and postnatal maternal supplementation on promotion of airway protective adaptive and innate immunity to viral infection. In humans, memory T cell
Figure 4. Prenatal regulation of adapted immunity to RSV by maternal *L. johnsonii* supplementation. (A–C) LDLN cells were restimulated with RSV, and production of IL-4, IL-5, and IL-13 in culture supernatant was measured by Bioplex. (D–J) Total numbers of (D) ILC2s: Lin-CD45−CD90+ST2+c-Kit+CD127+GATA3+; (E) eosinophils: SSCintCD11b−SiglecF+; (F) interstitial macrophages: CD11b+CD11c−F4/80−; (G) DCs: CD11b+CD11c+MHCII+ and CD11c−MHCII−CD11b−CD103+; (H) regulatory T cells: CD3+CD4+Foxp3+; (I) CD4+ T cells: CD3+CD4+, and activated CD3+CD4+CD69+; and (J) CD8+ T cells: CD3+CD8+ and activated CD3+CD8+CD69+. Data analysis was performed using FlowJo software. Data represent the mean ± SE from four to eight mice per group from three or four experimental repeats. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001.
populations are evident as early as 11 wk of gestation (Howie et al., 1998; Spencer et al., 1986), and B cells with the capacity to respond to commensal gut microbes can be found in human fetal intestines (Chen et al., 2020). Another recent study provided evidence for the presence of low numbers of specific bacteria, including *Lactobacillus*, in the human fetal intestine by mid-gestation, which have been shown to regulate IFN-γ production by fetal memory T cells in vitro (Rackaityte et al., 2020). Thus, prenatal priming by maternal microbiomes and their products has the capacity to shape early life immune responses.

To assess how pre- and postnatal exposure to *L. johnsonii*-supplemented mothers impacted the cecal microbiota of offspring following RSV infection, i6S rRNA analysis was performed on all four offspring groups (PBS–PBS, PBS–IJ, IJ–PBS, and IJ–IJ). Offspring cecal microbiota exhibited significant between-group differences (Fig. 5 A; PERMANOVA; R² = 0.413; P = 0.001). Offspring exposed to *L. johnsonii* exhibited relatively consistent gut microbiota compositions, with those exposed both pre- and postnatally to *L. johnsonii* exhibiting the least variance in their microbiota compared with the control groups (Fig. 5 B). Offspring exposed postnatally to mothers supplemented with *L. johnsonii* also exhibited similar gut microbiota, suggesting that the effect of *L. johnsonii* supplementation on microbiota structure is mediated, at least in part, by breastmilk consumption. Mice exclusively exposed to *L. johnsonii* prenatally exhibited cecal microbiota variance similar to that of unexposed animals (Fig. 5 B). The cecal microbiota of PBS/PBS mice was highly enriched for Akkermansiaaceae, a mucin-degrading family, whereas animals exposed both pre- and postnatally to *L. johnsonii* exhibited significant enrichment of the fermentative bacterial families Lachnospiraceae and Ruminococcaceae (Fig. 5 C). Enrichment of cecal Lachnospiraceae following RSV infection was observed in both exclusive prenatal and postnatal *L. johnsonii*-exposed groups, indicating that early-life exposure to *L. johnsonii* promotes the presence of fermentative bacteria despite the perturbing effect of airway viral infection. Thus, prenatal *L. johnsonii* supplementation influences postnatal adaptive responses to airway viral infection, supporting a role for maternal prenatal microbiomes in postnatal immunity. This may provide an explanation for why maternal prenatal exposure to antimicrobials are associated with heightened risk of allergy and asthma in offspring.

**Summary**

Many studies have identified that the composition of the microbiome and/or their metabolic products can impact early life development of immunity, in humans and in murine models (Dominguez-Bello et al., 2019; Fujimura et al., 2016; Maslowski, 2019; Nash et al., 2017; Rackaityte et al., 2020). The present study demonstrates several important aspects that may help to shape developing paradigms: (1) maternal *L. johnsonii* supplementation alters offspring immune response to early-life RSV-induced pathology; (2) alteration of airway adaptive inflammatory response to RSV infection is primarily due to in utero exposure, while innate mucin responses appear to be regulated by postnatal exposure to *L. johnsonii*-supplemented mothers; (3) *L. johnsonii*-associated airway protection correlates with maternal and offspring microbiome restructuring and similarities in both microbiomes and metabolic profiles; and (4) a combination of both pre- and postnatal effects of *L. johnsonii* provides the greatest degree of offspring protection against early-life RSV infection. This latter aspect may provide important evidence for why human studies may need to consider both prenatal and postnatal interventions for future studies and may help guide future clinical studies.

**Materials and methods**

**Study design**

All experiments were approved by the Institutional Animal Care and Use Committee at the University of Michigan. All mice were maintained under standard pathogen-free conditions. BALB/c 6–7-wk-old mice were purchased from The Jackson Laboratory for breeding. Female mice were gavaged with *L. johnsonii* for 7 consecutive days, then were paired with male mice for mating. Male mice were removed after 5 d, and supplementation of female mice continued twice per week until delivery. The mice were kept in standard cages, in company with their littersmates of both sex and their mother, and we used one to two litters per group, per experiment. Offspring were nasally infected with RSV (10⁵ PFU) at 7 d of age, and collection of samples was done at 7 d after infection (Fig. 1 A). Cross-fostering experiments were performed by switching mothers from their offspring during the first 24 h of life, and infection was done intranasally as described below (Fig. 4 A). In some experiments, neonates were weaned at 3 wk of age, and were infected with RSV (4 × 10⁵ PFU) at 6 wk of age.

**Preparation of *L. johnsonii* for supplementation**

To propagate *L. johnsonii* for supplementation, we followed a previously described protocol (Fonseca et al., 2017; Fujimura et al., 2014). In brief, de Man, Rogosa and Sharpe (MRS) broth was inoculated with *L. johnsonii* from a glycerol stock before static overnight culture at 37°C. Stationary-phase cells (OD₆₀₀ = 0.89) were centrifuged at 4,000 g for 15 min at 4°C and re-suspended in a 50:50 solution of MRS broth:50% glycerol. Batches of 500 µl were snap-frozen in liquid nitrogen and stored at −80°C until used in murine studies. Viable cell count of the glycerol stock was determined by plating serial dilutions of MRS plates. For murine studies, tubes were defrosted on ice, centrifuged at 14,000 g at 4°C, and washed twice in sterile saline to remove glycerol. The cells were resuspended in 10⁶ CFU/ml of sterile saline. Each mouse received 100 µl (10⁷ CFU) of re-suspended *L. johnsonii*. The remaining suspension was plated to confirm that viable *L. johnsonii* cell counts were stable.

**RSV**

Our laboratory uses antigenic subgroup A, line 19 RSV, obtained initially from a sick infant at the University of Michigan Hospital System. This isolate has been shown in animal models to mimic human infection by eliciting airway mucus production upon inoculation with 10⁵ PFU RSV (Lukacs et al., 2006). Prior to infection, virus was concentrated by centrifugation at 100,000 rpm and re-suspended in PBS at a concentration of 2 × 10⁷ PFU/ml. To infect neonatal mice, 5 µl of RSV (10⁵ PFU) was pipetted
onto the nose of the mouse, which was held in an upright position until the total volume was inhaled.

**RNA isolation and qPCR**

Lung tissue was homogenized in TRIzol reagent. Then RNA was extracted using TRIzol reagent (Invitrogen). cDNA was synthesized using murine leukemia virus reverse transcription (Applied Biosystems) and incubated at 37°C for 1 h, followed by incubation at 95°C for 5 min to quench the reaction. Real-time qPCR was performed using custom primers to measure *Muc5ac* and *Gob5* mRNA levels as described (Miller et al., 2003). 18S rRNA gene was used as an internal control using custom primers and a VIC-conjugated probe (Applied Biosystems). Fold change was quantified using the $2^{-\Delta\Delta C_t}$ cycle threshold method. All reactions were run on 7500 Real-Time PCR System (Applied Biosystems).

**Lung histology**

The left lung was perfused with 4% (vol/vol) formaldehyde for fixation and embedded in paraffin. 5-μm lung sections were stained with PAS to detect mucus production and inflammatory infiltrates. Photomicrographs were captured using a Zeiss Axio Imager Z1 and AxioVision 4.8 software (Zeiss).

**Flow cytometry**

The lungs were removed, and single cells were isolated by enzymatic digestion with 1 mg/ml collagenase A (Roche) and 20...

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**Figure 5.** *Maternal supplementation with* L. johnsonii *alters offspring gut microbiome.* (A) Principal coordinate (PC) analysis of cecal microbiome of only cross-fostered offspring that were challenged with RSV (PERMANOVA; $R^2 = 0.413$, $P = 0.001$; Unweighted UniFrac). (B) Unweighted UniFrac distance comparisons between groups of offspring challenged with RSV. Each bar represents the mean distance between mice within a given treatment (prenatal and postnatal supplementation outcomes). (C) Differentially enriched taxa between mice receiving prenatal and postnatal PBS supplementation and mice receiving prenatal and postnatal L. johnsonii supplementation. The size of the circle represents the normalized mean of a given sequence variant in a group of offspring.
U/ml DNase I (Sigma-Aldrich) in RPMI 1640 containing 10% FCS. Tissues were further dispersed through an 18-gauge needle (10-ml syringe), RBCs were lysed, and samples were filtered through 100-µm nylon mesh twice. Cells were resuspended in PBS, and live cells were identified using the LIVE/DEAD Fixable Yellow Dead Cell Stain kit (Thermo Fisher Scientific). The cells were then washed and resuspended in PBS with 1% FCS, and Fc receptors were blocked with purified anti-CD16/32 (clone 93; Bio-Legend). Surface markers were identified using antibodies (clones) against the following antigens, all from BioLegend: anti-Gr-1 (RB6-8C5), B220 (RA3-6B2), CD3 (145-2C11), Ter119 (Ter-119), CD11b (M1/70), CD25 (PC61), CD45 (30-F11), CD127 (SB/199), S1D2 (D1H9), c-Kit (2B8), CD90 (53–2.1), CD4 (RM4-5), CD3 (17A2), CD8 (53–5.8), CD69 (H1L2F3), CD11c (N418), MHC-II (M5/I4.15.2), and CD103 (2E7). SiglecF (E50-2440) was purchased from BD Biosciences. For ILC staining, lineage markers were anti-CD3, CD11b, B220, Gr-1, and TER119. ILC2 were measured as Lin-CD45+CD90+ST2-c-Kit+CD127+GATA3+. Eosinophils were stained as SSChighCD11b+SiglecF+. DCs were stained as follows: CD11b+CD1c+MHCII+CD103+ for conventional DCs and CD11c+MHCII+CD11b+CD103+ for CD103+ DCs. Interstitial macrophages were measured as CD11b+CD1c+FCRγ80+. T cell populations were stained as follows: CD3+CD4+ or CD3+CD8+, and CD3+CD4+Foxp3+ for regulatory T cells. Data were collected in a NovoCyte flow cytometer (ACEA Bioscience, Inc.). Data analysis was performed using FlowJo software (Tree Star).

LDLN in vitro restimulation and cytokine production assay
The LDLNs were enzymatically digested using 1 mg/ml collagenase A (Roche) and 20 U/ml DNase I (Sigma-Aldrich) in RPMI 1640 with 10% FCS for 45 min at 37°C. Tissues were further dispersed through an 18-gauge needle (1-ml syringe). RBCs were lysed, and samples were filtered through 100-µm nylon mesh. Cells (5 × 10^6) from LDLN cells were plated in 96-well plates and restimulated with RSV L19 MOI 5:1 for 48 h. IFN-γ, IL-4, IL-5, IL-13, and IL-17a levels in supernatants were measured with a Bio-Plex cytokine assay (Bio-Rad Laboratories).

Metabolomics
The plasma and milk were analyzed by liquid chromatography mass spectrometry for >700 metabolites, and an estimate of the false discovery rate (q < 0.10) was calculated to take into account the multiple comparisons. Following log transformation and imputation of missing values, if any, with the minimum observed value for each compound, ANOVA contrasts were used to identify biochemicals that differed significantly between experimental groups.

Plasma was collected from the offspring at 7 d after RSV infection, or from age-matched uninfected controls. Plasma from mothers was collected at the same time. Blood was collected by cardiac puncture at euthanasia and transferred into tubes containing heparin to prevent coagulation, and plasma was separated by centrifugation. Ingested milk was removed from the stomachs of neonates at the time of sample collection, added to 200 µl of PBS, and dispersed with an 18-gauge needle to create a homogenous solution. Plasma and milk were then sent on dry ice to Metabolon (Durham, NC) to analyze the metabolic profile by mass spectrometry. The dataset comprises a total of 700 compounds from plasma and 545 compounds from ingested milk of known identity (named biochemicals).

Cecal microbiota profiling
Cecal samples were harvested immediately after the animals were euthanized, placed in RNAalater (Life Technologies), and stored for 24 h at 4°C, before storage at −80°C until processed for 16s rRNA sequencing as described previously (Fujimura et al., 2016). Briefly, frozen cecal samples were thawed on ice. DNA was extracted using a modified cetyltrimethylammonium bromide buffer-based protocol (DeAngelis et al., 2009). The V4 region of the 16S rRNA gene was amplified using primers previously described (Werner et al., 2012). PCR reactions were performed in 25 µl reactions using 0.025 U Takara Hot Start ExTaq (Takara Mirus Bio Inc.), 1× Takara buffer with MgCl2, 0.4 pmol µl−1 of F515 and R806 primers, 0.56 mg/ml of BSA (Roche Applied Science), 200 µM of deoxynucleoside triphosphates, and 10 ng of genomic DNA. Reactions were performed in triplicate under the following conditions: initial denaturation (98°C, 2 min), 30 cycles of 98°C (20 s), annealing at 50°C (30 s), extension at 72°C (45 s), and final extension at 72°C (10 min).

Demultiplexed 16S rRNA sequence reads were processed through the Divisive Amplicon Denoising Algorithm-2. Forward and reverse reads were filtered if they matched to the PhiX genome, had an expected error score >2, or were shorter than 150 bases. After denoising and merging reads, sequences were maintained if they had 253 ± 5 base pairs, the length of the V4 region. Taxonomic identification of sequence variants was assigned with the assignTaxonomy function in Divisive Amplicon Denoising Algorithm-2, and species were assigned through the addSpecies function, allowing for all possible species that matched at 100% identity. Phylogenetic trees were built using phangorn, msa, and DECIPHER packages. Sequence variants not belonging to the kingdom Bacteria were filtered out using the subset_taxa function from phyloseq. We removed sequence data arising from negative controls by removing taxa that were present in >15% of negative controls and <15% of samples; the mean counts from taxa remaining in negative controls were subtracted from samples. Any taxa present at <0.001% of the total reads were removed.

Statistical analysis
For qPCR and cytokine production, data were analyzed by Prism 6 (GraphPad Software). Data presented are mean values ± SEM. Comparison of two groups was performed with the two-sample Student t test. For metabolomics, comparisons of three or more groups were analyzed by ANOVA. Post hoc Tukey’s test was used for two group comparisons. To consider multiple testing problems for high-dimensional metabolomics data, the P values
were adjusted by controlling for the false discovery rate. Following log transformation and imputation of missing values, if any, with the minimum observed value for each compound, ANOVA contrasts were used to identify biochemicals that differed significantly between experimental groups. A summary of the numbers of biochemicals that achieved statistical significance (P ≤ 0.05) was compiled into a table.

Prior to analysis, 16S rRNA ASV tables were normalized through a variance-stabilized transformation using DESeq2. Unweighted UniFrac distance matrices were calculated with the adonis2 function from vegan. Principal coordinate analysis was conducted using the corcom function from phyloseq to visualize significant findings. Differentially abundant bacterial ASVs were identified using DESeq2 with positive counts for size factor estimation and a local fit type. P values were false discovery–corrected using the Benjamini–Hochberg method. All figures were generated using ggplot2 in R.

Online supplemental material
Fig. S1 shows that the protection from RSV pathogenesis due to exposure to L. johnsonii prenatally and during the neonatal period, as shown in Fig. 1, persists beyond weaning, and animals infected as adults continue to be protected from the Th2 response to RSV compared with mice born to PBS-treated control females. Fig. S2 highlights metabolic changes in maternal plasma, neonatal plasma, and milk. Table S1 shows genera shared between supplemented females and their offspring. Table S2 lists all significantly changed metabolites in the plasma of female mice supplemented with L. johnsonii compared with PBS-treated controls. Table S3 lists all significantly changed metabolites in the plasma of neonates born to L. johnsonii–supplemented females compared with neonates born to PBS-treated females. Table S4 lists all significantly changed metabolites in the milk harvested from the stomachs of neonates born to L. johnsonii–supplemented females compared with neonates born to PBS-treated females. Table S5 compares changes in metabolites that are common in plasma from supplemented females and offspring, and that are also found in milk.

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Disclosures: S.V. Lynch reported personal fees from Siolta Therapeutics Inc. during the conduct of the study and outside the submitted work; in addition, S.V. Lynch had a patent to Reductive prodrug cancer chemothera (Stan449-PRV) issued, a patent to combination antibiotic and antibody therapy for the treatment of Pseudomonas aeruginosa infection (WO 2010091189 A1) issued, a patent to therapeutic microbial consortium for induction of immune tolerance with royalties paid to Siolta Therapeutics Inc., a patent to systems and methods for detecting antibiotic resistance (WO 2012027302 A3) issued, a patent to nitroreductase enzymes (US 7687474 B2) issued, a patent to sinusitis diagnostics and treatments (WO 2013155370 A1) issued, a patent to methods and systems for phylogenetic analysis (US 20120264637 A1) issued, a patent to methods and compositions relating to epoxide hydrolase genes licensed to Siolta Therapeutics Inc., and a patent to novel Lactobacillus and Micrococcus species that promote tolerogenic immunity pending. No other disclosures were reported.

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Figure S1. *L. johnsonii*-dependent protection from RSV-induced pathology persists into maturity. (A) Experimental design. Female mice were supplemented with *L. johnsonii* for 7 d before mating, then twice weekly until delivery, while control mice were treated with PBS. Offspring were weaned, then infected with RSV at 6 wk of age. Samples were taken at 8 d after infection. (B) Histology sections of the lungs were stained with H&E to visualize inflammation or PAS to visualize mucus. (C) Expression of the mucus-associated gene gob5 was measured in lung tissue by qPCR. (D) Mediastinal lymph nodes were digested into a single-cell suspension and restimulated with RSV. Cytokine production was measured by Bioplex assay. (E–G) The following cell populations in the lung were measured by flow cytometry: (E) CD4+ T cells, (F) CD11c+CD11b+ DCs, and (G) CD11c+CD103+ DCs. Data represent the mean ± SE from four or five mice from two experimental repeats. *, P ≤ 0.05; **, P ≤ 0.01; ****, P ≤ 0.0001.
Table S1, Table S2, Table S3, Table S4, and Table S5 are provided online as separate files. Table S1 shows ASVs significantly enriched and shared between *L. johnsonii*–supplemented mothers and their offspring. Table S2 shows plasma metabolites significantly increased or decreased in relative concentration in *L. johnsonii*–supplemented mothers. Table S3 lists plasma metabolites significantly increased or decreased in relative concentration in offspring born to *L. johnsonii*–supplemented mothers. Table S4 shows ingested milk metabolites significantly increased or decreased in relative concentration in the stomach contents of offspring born to *L. johnsonii*–supplemented mothers. Table S5 shows metabolites found to significantly differ in *L. johnsonii*–supplemented maternal plasma and in the plasma and ingested milk of their offspring.

Figure S2. Metabolic profiles of mothers and offspring plasma and neonates ingested milk. Heatmap of differentially expressed metabolites. The row displays metabolites that achieved statistical significance (*P* ≤ 0.05), and fold change between LJ and PBS groups is presented. Metabolites significantly decreased were displayed in green, while metabolites significantly increased were displayed in red. The brightness of each color corresponded to the magnitude of the difference when compared with average value. (A) Differentially expressed metabolites in the plasma of supplemented females. (B) Differentially expressed metabolites in the plasma of offspring. (C) Differentially expressed metabolites in the milk of supplemented females. Each experiment analyzed four samples per group.