22. American Diabetes Association, *Diabetes Care* **24**, 775–778 (2001).
23. S. Marshall, O. Nadeau, K. Yamasaki, *J. Biol. Chem.* **279**, 35313–35319 (2004).

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O.L., G.W.H., and R.L.H. designed all experiments; the optogenetics experiments were also designed by Y.A. and J.E.S., and the elecotrophysiological experiments were also designed by I.H. O.L. performed all experiments and analyzed all data but the electrophysiology (I.H.) and the optogenetics (J.E.S.). The optogenetics data were analyzed by J.E.S., Y.A., and O.L. O.L., G.W.H., and R.L.H. wrote the manuscript. We thank G. Schütz for providing the αCaMKII-CreER[T2] mice, J. L. Bedont for help with in situ hybridization experiments, and R. H. White for assistance with mating and genotyping. All data necessary to understand and assess the conclusions of the manuscript are in the body of the paper and in the supplementary materials. All primary data are archived on a secure server located in the Department of Neuroscience at Johns Hopkins University (JHU). All data will be made available upon request. G.W.H. receives a share of royalty received by the university on sales of the CTD 110.6 antibody, which are managed by JHU. The research was supported by NIH (grant R01NS036715 to R.L.H. and grants R01DK6167, N01-HV-00240, and P01HL107153 to G.W.H.) and the National Institute on Drug Abuse Intramural Research Program (Y.A.).

SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/351/6279/1293/suppl/DC1
Materials and Methods
Figs. S1 to S9
References (*24*–*29*)

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MICROBIOTA

The maternal microbiota drives early postnatal innate immune development

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Postnatal colonization of the body with microbes is assumed to be the main stimulus to postnatal immune development. By transiently colonizing pregnant female mice, we show that the maternal microbiota shapes the immune system of the offspring. Gestational colonization increases intestinal group 3 innate lymphoid cells and F4/80^{+}CD11c^{+} mononuclear cells in the pups. Maternal colonization reprograms intestinal transcriptional profiles of the offspring, including increased expression of genes encoding epithelial antibacterial peptides and metabolism of microbial molecules. Some of these effects are dependent on maternal antibodies that potentially retain microbial molecules and transmit them to the offspring during pregnancy and in milk. Pups born to mothers transiently colonized in pregnancy are better able to avoid inflammatory responses to microbial molecules and penetration of intestinal microbes.

During pregnancy, the eutherian fetus inhabits a largely sterile environment in utero, protected from infections by maternal immunity. Rejection of the allogeneic fetus is avoided through maternal and fetal vascular separation, the immune privileged status of the placental trophoblast, and gestational maternal tolerance mechanisms (*1*). At birth, the situation changes dramatically as body surfaces become progressively colonized with microbes, directly exposing the immature neonatal immune system to potential pathogens (*2*, *3*). Despite continued protection from the immunoglobulins and antibacterial peptides in milk, the consequence of this transition for human health is that most of the worldwide mortality in children up to 5 years old is due to infectious disease (*4*–*6*).

Immune system development is both preprogrammed in neonatal tissues and driven later by exposure to pathogenic and nonpathogenic microbes (*3*). Germ-free mice have low immunoglobulin concentrations; lymphopenia of lymphoid structures; reduced bone marrow leukocyte pools; and aberrant innate and adaptive immune functions (*7*, *8*). It has been widely assumed that most microbiota-driven immune alterations are postnatal effects induced by the neonate's own microbiota (*2*, *9*, *10*). Here, we challenge this assumption by asking how the maternal microbiota in pregnancy alone affects the early postnatal immune system of the offspring.

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To achieve gestation-only colonization under conditions where the mice deliver their pups spontaneously at term, we used a system in which pregnant dams are transiently colonized with genetically engineered *Escherichia coli* HA107 (*11*). Because this strain does not persist in the intestine, pregnant dams become germ-free again before term and naturally deliver germ-free pups (fig. S1A). Although *E. coli* is a minor component of the adult human microbiota, it is commoner in the neonatal intestine (*12*) and a frequent cause of human neonatal sepsis (*13*).

Gestation-only colonization shapes the intestinal mucosal innate immune composition

Gestation-only colonization with *E. coli* HA107 altered the numbers of early postnatal intestinal innate leukocytes in wild-type C57BL/6 mice. At postnatal day 14, there was an increase in small intestinal innate lymphoid cell (ILC) proportions and total numbers compared with germ-free controls, particularly the NKp46^{+}RORγt^{+} ILC3 subset (Fig. 1, A and B, and fig. S1B). Small intestinal NKp46^{+}RORγt^{+} ILC3 are described in germ-free mice (*14*), but persistently increased following transient gestational colonization, reaching a maximum in 14- to 21-day-old pups: This increase persisted even after weaning (Fig. 1C and fig. S1C), consistent with increased small intestinal ILC3 content of colonized compared with germ-free mice (*15*) and the microbiota-dependent modulation of RORγt expression in this subset (*16*). Increases in the expression of the cytokine interleukin-22 (IL-22) in this population have been observed following permanent colonization or the introduction of segmented filamentous bacteria to the microbiota (*17*, *18*). Total numbers of IL-22–expressing cells increased in line with the increased NKp46^{+}RORγt^{+} ILC3 numbers as a result of gestational colonization, although individual IL-22 expression levels did not change, likely because the pups were born and raised germ-free (fig. S1, D to F).

There was also an increase in the small and large intestinal F4/80^{+}CD11c^{+} mononuclear cells (iMNCs) in day 14 (d14) pups born to gestation-only colonized dams (Fig. 1, D and E, and fig. S2, A to C), whereas the F4/80^{+}CD11c^{-} macrophages, F4/80^{lo}CD11c^{+} dendritic cells (DCs), and the CD103^{+} or CD11b^{+} DC subpopulations were not significantly affected (Fig. 1, D and E, and fig. S2, B to E). The gestational effects on increased F4/80^{+}CD11c^{+} iMNCs were also maximal between postnatal days 14 to 21, and they persisted until at least 8 weeks of age in the colon (Fig. 1F). Gestational colonization caused no significant changes in small intestinal ILC2 numbers (fig. S3, A and B) or in other early postnatal innate leukocyte populations in either systemic or intestinal tissues (table S1). These results showed that temporary colonization of a pregnant dam has long-term consequences for certain populations of innate lymphoid and mononuclear cells in the intestines of her offspring.

We next sought to verify that the effects of gestational *E. coli* on early postnatal innate leukocytes would also be seen with animals stably colonized by a different microbiota both in the mother and after birth. We compared C57BL/6 animals carrying the defined altered Schaedler flora (ASF) of eight microbes with germ-free controls. Both small intestinal NKp46^{+} ILC3 and intestinal F4/80^{+}CD11c^{+} MNC populations were increased in pups born to stably colonized ASF

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mothers compared with germ-free controls (fig. S4, A and B). Colonization of adult germ-free C57BL/6 animals with an ASF microbiota for 21 days also selectively increased intestinal NKp46$^+$ ILC3 and F4/80$^+$CD11c$^+$ iMNC populations (fig. S4, C and D, and table S2). Given that the ASF microbiota does not contain Proteobacteria, we concluded that the innate leukocyte alterations seen through gestation-only colonization with *E. coli* are also present in mice colonized with a defined microbiota, dominated by *Bacteroides distasonis* (*19*). Nevertheless, given the alterations in adaptive immunity when germ-free mice are permanently colonized with a microbiota (*8*), we next assessed the extent of adaptive immune changes after gestation-only colonization.

Maternal gestational colonization does not affect adaptive immune composition of pups

We found that gestation-only colonization did not alter relative or absolute populations of B or T cells during development in the bone marrow, spleen, or thymus (fig. S5, A and B). Intestinal and systemic CD4 or CD8 T cell numbers, T cell activation status (table S3), CD4 subpopulations (fig. S5, C to G), and intestinal microarchitecture (fig. S6) were also generally unaffected. Because all the neonatal mice in these experiments were germ-free, we concluded that the well-known microbiota-driven effects of amplification of B and T cell numbers and resulting reorganization of lymphoid structures result from postnatal colonization with an endogenous microbiota (*19*–*21*).

Maternal microbiota induces intestinal transcriptional reprogramming in offspring

Many functions of the neonatal intestine are developmentally regulated, including transport of nutrients, salts, and water; barrier function; and secretion of antibacterial peptides and mucus (*22*). Because different aspects of intestinal development determine the ability of the neonate to tolerate an incoming microbiota, we questioned whether the changes in innate leukocytes after gestational colonization were part of a much wider range of adaptations triggered by maternal exposure to intestinal microbes. We carried out RNA sequencing (RNA-Seq) analysis of whole small intestinal mucosal RNA from neonates at day 14. Unsupervised analysis showed a series of consistent transcriptional changes in the pups born to gestation-only colonized dams compared with controls (Fig. 2A). The genetic and protein interactions inferred from differentially expressed transcripts (*23*) included up-regulated gene networks for cell division and differentiation, mucus and ion channels, and the polymeric immunoglobulin receptor and mononuclear recruitment, as well as for metabolism of xenobiotics, bile acids, complex lipids, and sugars (Fig. 2B). These differentially expressed genes included significantly increased overall expression of signature genes for the different Paneth cell, goblet cell, and early/late enterocyte precursor epithelial lineages (*24*, *25*) (fig. S7, A to C). Transcripts for the C-lectin Reg family and antibacterial defensin peptides were also significantly increased in the pups of gestation-only colonized dams compared with controls (Fig. 2C and fig. S7D).

These results show that the maternal microbiota drives wide-ranging mucosal transcriptional

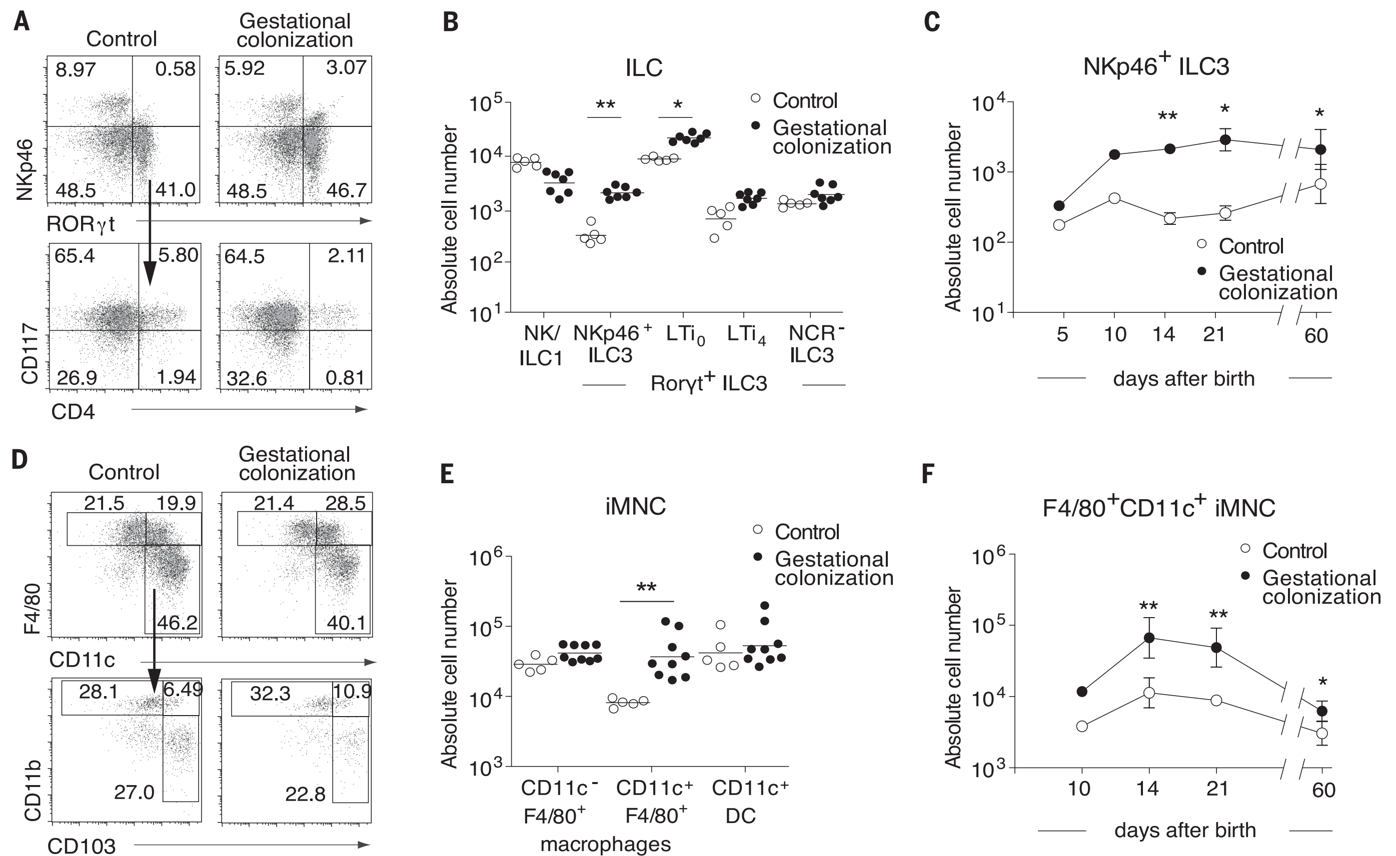


Fig. 1. Maternal microbial exposure during pregnancy shapes the frequency of intestinal innate lymphoid and mononuclear cell populations in the offspring. Germ-free C57BL/6 dams were transiently colonized with *E. coli* HA107 (gestational colonization) or kept germ-free throughout (controls). All offspring were analyzed by flow cytometry at day 14 after birth unless indicated. (**A**) Representative dot plots showing Lin$^-$ (CD19$^-$CD3$^-$) small intestinal lamina propria lymphocytes (upper row) and Lin$^-$RORγt$^+$NKp46$^-$ ILC3 (lower row). (**B**) Absolute numbers (geometric mean, sample number $n \geq 5$) of the indicated Lin$^-$ small intestinal ILC populations. (**C**) Absolute numbers of small intestinal Lin$^-$NKp46$^+$RORγt$^+$ ILC3 at indicated time points after birth. Data represent geometric mean ± SD, n = 3 to 10 per time point. (**D**) Representative dot plots showing Lin$^-$MHC-II$^+$ colon lamina propria intestinal mononuclear cells (iMNCs) (upper row) and Lin$^-$MHC-II$^+$CD11c$^+$F4/80^{lo} iMNCs (lower row). (**E**) Absolute numbers (geometric mean, $n \geq 5$) of indicated Lin$^-$MHC-II$^+$ iMNC populations in the colon. (**F**) Absolute numbers (geometric mean ± SD, n = 3 to 10 per time point) of colon Lin$^-$MHC-II$^+$CD11c$^+$F4/80$^+$ iMNCs at different time points after birth. Data are each representative of four independent experiments or show pooled data from four experiments. *$P \leq 0.05$; **$P \leq 0.01$.

signatures that are consistent with adapting early postnatal immunity and intestinal function generally to postnatal microbial colonization and the metabolic consequences of inevitable bile salt and dietary xenobiotic exposure, even in pups born germ-free. Thus, many aspects of what might have been thought of as canonical host epithelial development and innate immunity are likely shaped through maternal microbial colonization.

Gestational colonization effects depend on maternal antibodies

Live intestinal microbes, including *E. coli* HA107, generally do not penetrate the body further than the lymph nodes draining the intestinal mesentery (*26*, *27*), and we found no culturable organisms in the placenta after treatment in our gestational colonization experiments. It was therefore likely that the effects of maternal gestational microbes on early postnatal innate immunity resulted from penetration of microbial molecular products, first to maternal tissues, and subsequently to the fetus or neonate. Supporting this hypothesis, we found that serum transfer from gestation-only colonized females to unexposed pregnant dams was sufficient to shape intestinal NKp46$^+$ ILC3 populations in the neonates (Fig. 3A and fig. S8, A and B), but not when immunoglobulin G (IgG) was depleted from the serum before transfer, nor when the serum was derived from gestationally colonized $J_H^{-/-}$ antibody-deficient dams (fig. S8, A and B). Given the sufficiency of serum IgG transfer, antibody transfer from the mother to her offspring was likely important to realize some features of early postnatal immune development, because the gestation-only induction of small intestinal NKp46$^+$ ILC3 by the maternal microbiota was lost in the antibody-deficient $J_H^{-/-}$ strain (Fig. 3, B and C). We confirmed that this effect was due to the lack of maternal antibodies using a heterozygous strain combination approach (fig. S8C). Nevertheless, not all aspects of maternal microbiota-driven early postnatal immune system development are antibody dependent, because induction of F4/80$^+$CD11c$^+$ iMNCs was preserved despite the lack of neonatal and/or maternal antibodies (fig. S8, D and E).

Because NKp46$^+$ ILC3 but not CD11c$^+$F4/80$^+$ iMNC increases are mediated through maternal antibody-dependent mechanisms after transient gestational colonization, we predicted that only a subset of the many transcriptional responses attributable to epithelial and other intestinal cells would be maternal antibody-dependent. RNA-Seq analysis was carried out in d14 ileum to compare the responses of C57BL/6 wild-type and $J_H^{-/-}$ groups, each of which was compared to germ-free controls. Only a subset of up-regulated genes in all networks were maternal antibody-dependent (Fig. 3D and fig. S9, A to C). For example, although *RegIIIa* transcript numbers were elevated in the pups of antibody-deficient mothers, maximal up-regulation of *RegIIIb* and *RegIIIg* was antibody dependent (Fig. 3E and fig. S9D).

Maternal microbial molecular transfer to the offspring

Maternal microbiota effects on the pups were only seen when the mother was transiently colonized during pregnancy itself (fig. S10, A

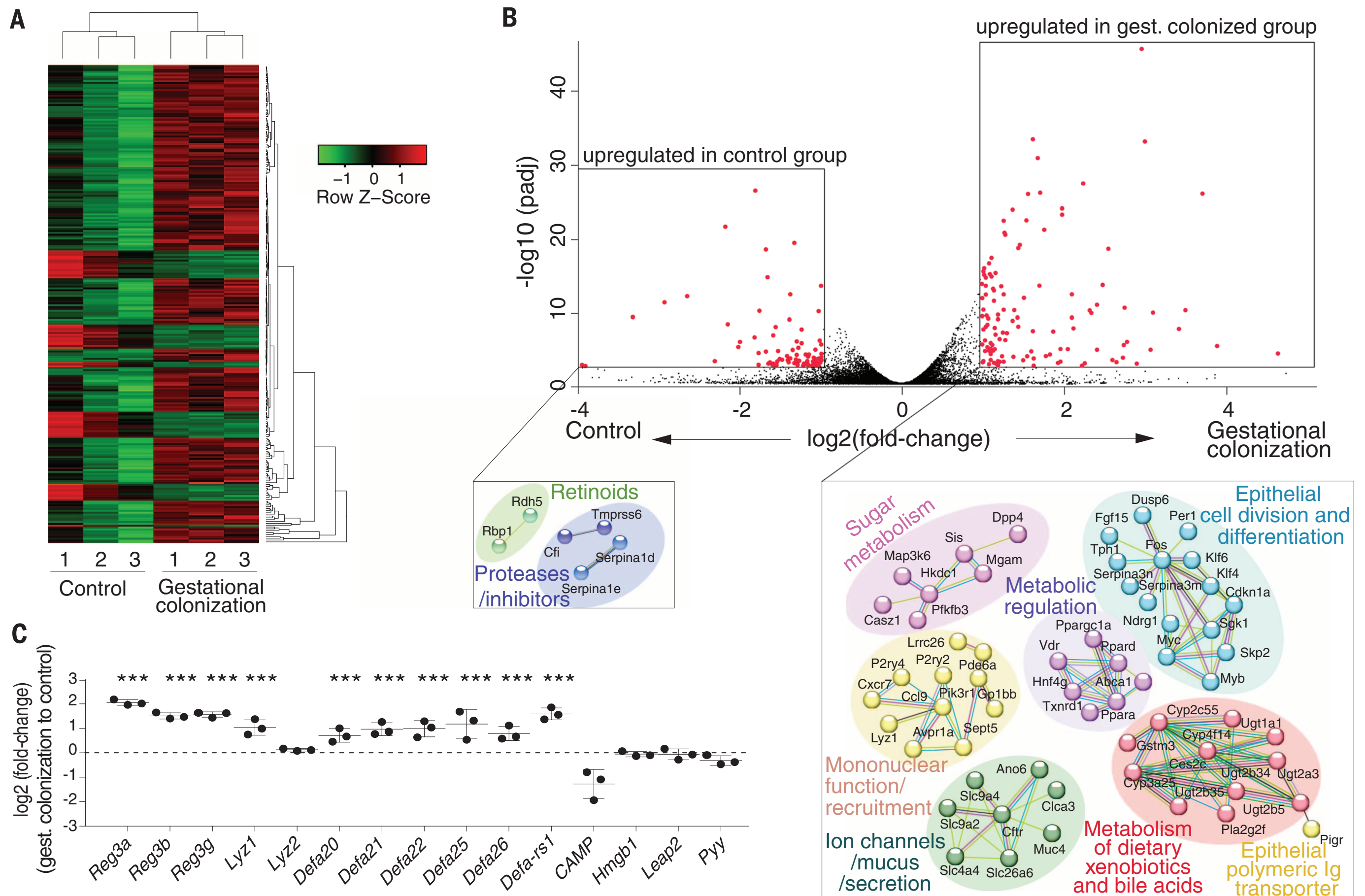


Fig. 2. Maternal microbial exposure during pregnancy causes extensive changes in the intestinal gene expression profile in neonates. Germ-free C57BL/6 dams were transiently colonized with *E. coli* HA107 during pregnancy (gestational colonization) or kept germ-free throughout (controls). Whole-tissue RNA from the small intestine was isolated from the offspring on day 14 after birth and used for RNA-Seq ($n = 3$ per group). (**A**) Heat map of genes differentially expressed (fold-change ≥ 2; adjusted P value padj < 0.001). The color scale shows the relative expression profiles. (**B**) Upper: Volcano plot showing fold-change of gene expression in offspring born to gestationally colonized mothers compared to offspring born to control mothers. Genes with significantly different expression between the two groups (fold-change ≥ 2; padj < 0.01) are highlighted in red; lower: STRING analyses for significantly altered transcripts in each case. (**C**) Fold-change of selected transcripts for antimicrobial peptides between the gestationally colonized and control pups (***padj < 0.001; see also data file S1).

and B). In mice, maternal IgG is transferred across the placenta and through intestinal uptake from the milk (*28*), so we used litter-swap experiments to distinguish between antenatal and postnatal effects of the maternal microbiota. Although there was a nonsignificant trend toward increased small intestinal NKp46$^+$ ILC3 in neonates born to an unmanipulated mother and nursed by a gestation-only colonized mother, both in utero gestation and postnatal nursing by dams that had been colonized during pregnancy were necessary for significant ILC3 induction (Fig. 4A).

These results imply that maternal microbiota-derived compounds are transferred from the mother to the offspring and that this process is increased in the presence of maternal antibodies. We next considered antibody-enhanced retention of bacterial products in the mother and antibody-mediated transfer of bacterial products. The first of these effects was demonstrated by following ^{14}C elimination from metabolically labeled *E. coli* HA107 in wild-type and antibody-deficient mice. The presence of antibodies significantly increased retention of ^{14}C-labeled molecules in the mesenteric lymph nodes, spleen, liver, and serum for at least 36 hours compared with antibody-deficient controls (Fig. 4, B and C, and fig. S11, A to D): Microbial molecular exposure of the placenta and the fetus was also enhanced at embryonic day 16 (E16) (Fig. 4, D and E). We also found significantly increased radioactivity originating from maternal microbial molecules in the milk, and from postnatal intestinal mucosa and liver of wild-type pups (Fig. 4, F to I). This shows that maternal antibodies enhance the retention and transmission of microbial molecules, although effects other than direct microbial molecular binding cannot be excluded. To verify that these compounds are really of microbial origin rather than the products of secondary metabolism in the mother, we grew HA107 on [^{13}C]glucose so that bacterial compounds became fully labeled with ^{13}C, as judged by mass spectrometry–shift data. Intestinal ^{13}C-labeled metabolite levels were equivalent whether or not the mother expressed antibodies (fig. S12); however, after intestinal administration of ^{13}C-labeled HA107 in C57BL/6 intravenously (i.v.) primed mice, serum contained bacterial metabolites comigrating with IgG that were absent from the serum of antibody-deficient mice (fig. S13 and data file S5). Even if HA107 was only delivered through the intestinal route, which does not induce high-affinity serum IgG (*11*), there was evidence of low-affinity IgG coating of *E. coli* that was absent from serum of untreated germ-free controls or from HA107-treated J_H-deficient mice (fig. S14). We therefore concluded that either sterile bacterial fragments or small molecules can potentially be bound to maternal IgG after intestinal exposure.

Given that increases in NKp46$^+$ ILC3 and components of the mucosal transcriptome were antibody dependent, whereas F4/80$^+$CD11c$^+$ iMNCs were induced by gestational colonization even in pups of antibody-deficient dams, we assumed that a number of molecular ligand–receptor systems are driving different aspects of neonatal adaptation in response to the maternal microbiota. Toll-like receptor ligand signaling was not essential for the effect (fig. S15, A and B). There was an extensive range of bacterial-derived (^{13}C-labeled) molecules passed from the mother to the offspring (Fig. 5A and fig. S16), some of which also reached neonatal tissues (fig. S16). These

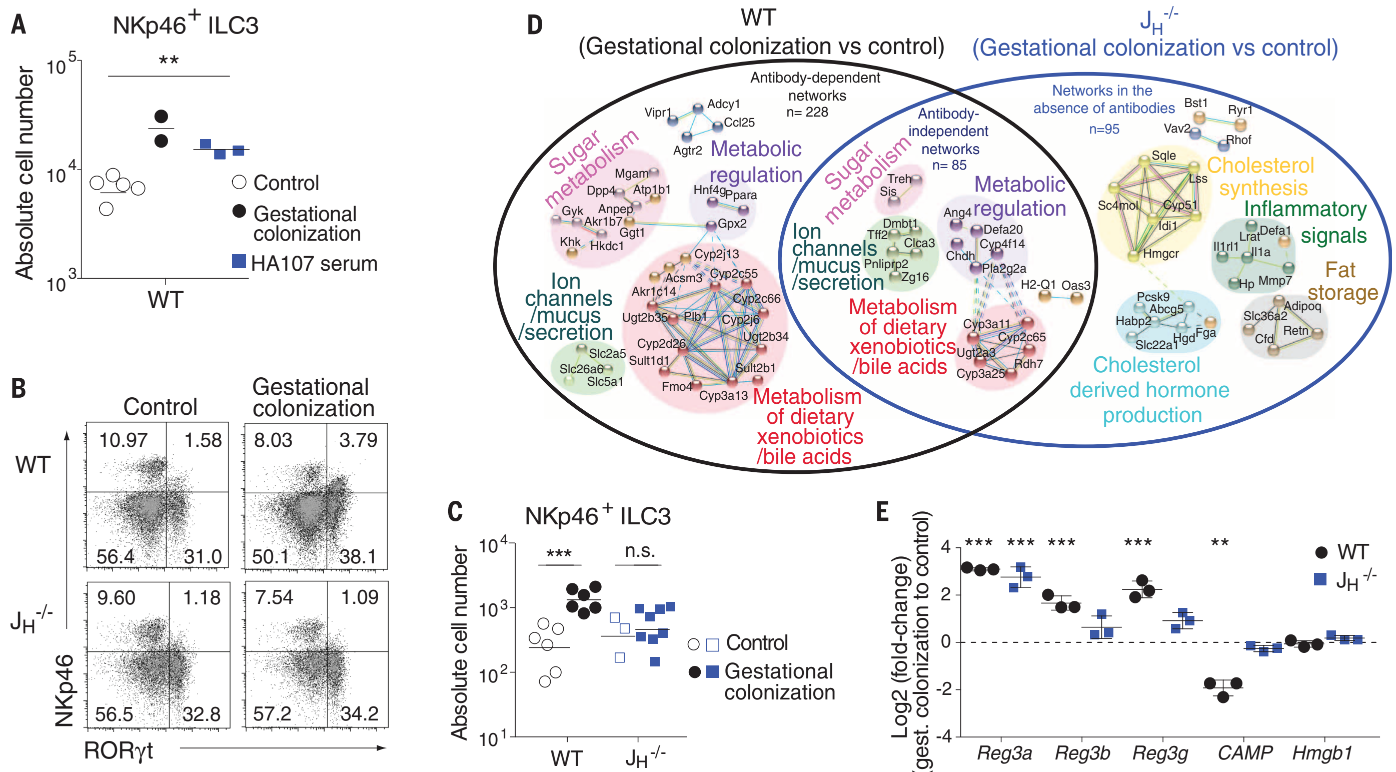


Fig. 3. Sufficiency of serum transfer and requirement for maternal antibodies for gestational colonization effects. (**A**) Pregnant germ-free C57BL/6 dams were transiently colonized (gestational colonization), left germ-free (control), or injected i.v. with serum from a germ-free donor previously gavaged with *E. coli* HA107 (HA107 serum). Total Lin$^-$RORγt$^+$NKp46$^+$ ILC3 (geometric mean, $n \geq 2$) in d14 offspring small intestine. (**B** to **E**) Germ-free C57BL/6 (WT, wild type) or $J_H^{-/-}$ dams were colonized with *E. coli* HA107 during pregnancy (gestational colonization) or kept germ-free (control). (B) Representative flow cytometry dot plots showing Lin$^-$RORγt$^+$NKp46$^+$ ILC3 in d14 small intestine. (C) Total numbers (geometric mean, $n \geq 3$) of Lin$^-$RORγt$^+$NKp46$^+$ ILC3 in d14 small intestine. (D and E) RNA-Seq of d14 distal small intestinal RNA in offspring of $J_H^{-/-}$ and wild-type dams ($n = 3$ per group). (D) STRING analysis comparing genes significantly enriched after gestational colonization versus germ-free in either WT (black circle) or $J_H^{-/-}$ (blue circle) strains. Total number of differentially regulated genes are shown. (E) Fold-change of expression of indicated genes in offspring from gestationally colonized compared to control mothers in WT or $J_H^{-/-}$ strains (***padj < 0.001, see also data files S2 and S3). Data are representative of two (A) and three (B and C) independent experiments. (A and C) $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

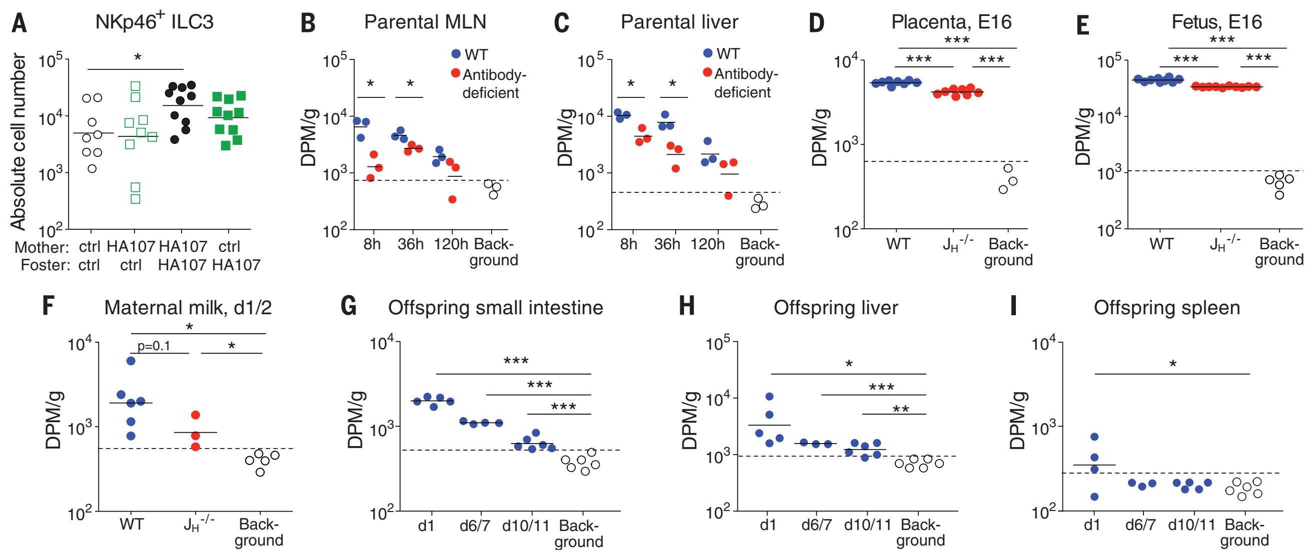
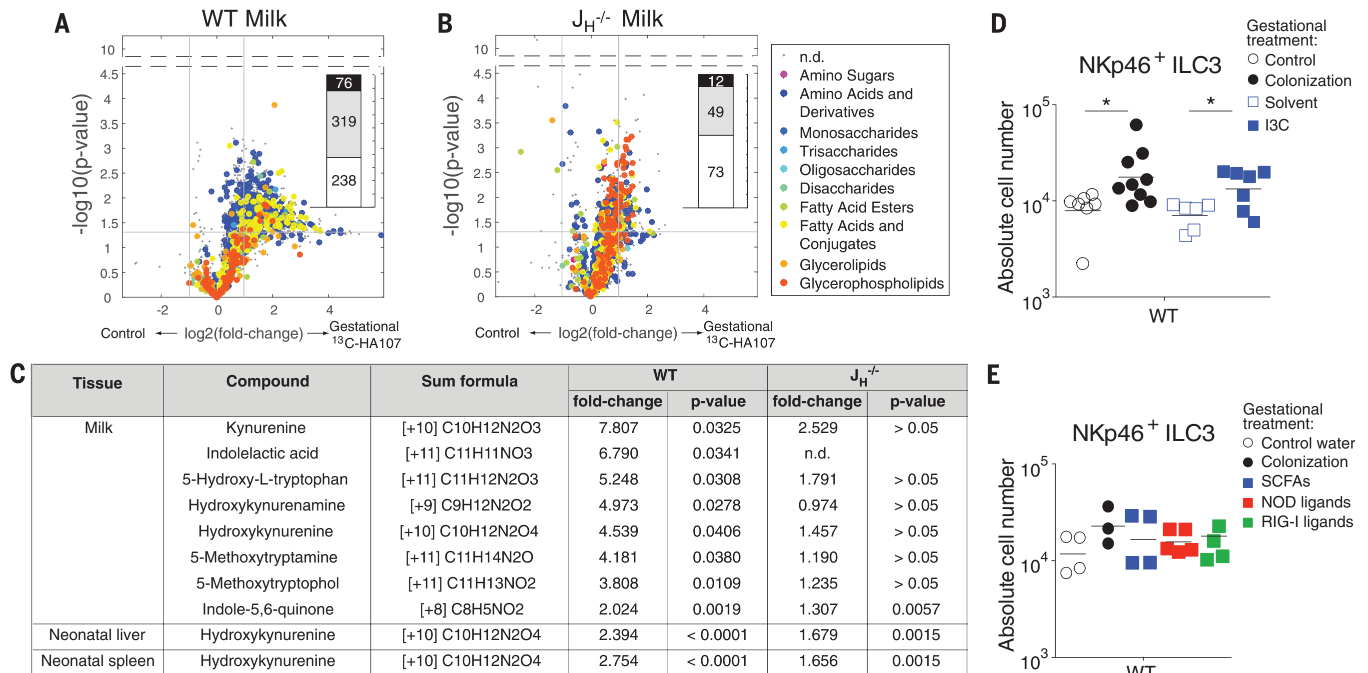


Fig. 4. Maternal microbial molecules reach the offspring during pregnancy and after birth. (**A**) Pregnant germ-free C57BL/6 mice were colonized with *E. coli* HA107 or germ-free (ctrl). Half of each litter was swapped to another group for fostering at birth. Total numbers (geometric mean, $n \geq 8$) of Lin⁻RORγt⁺NKp46⁺ ILC3 in the offspring small intestine on postnatal day 14. (**B** and **C**) Adult germ-free WT or *Rag1*$^{-/-}$ (antibody-deficient) mice received three gavages of 10^{10} colony-forming units (CFU) of unlabeled *E. coli* HA107 and one gavage of 10^{10} CFU of ^{14}C-labeled *E. coli* HA107. Radioactivity in the indicated tissues of WT and *Rag1*$^{-/-}$ adults was monitored over time. (**D** to **F**) Pregnant germ-free C57BL/6 WT or $J_H^{-/-}$ mice were gavaged with 10^{10} CFU of ^{14}C-labeled *E. coli* HA107 on E14. Placental or fetal tissues at E16 (D and E) or milk at d1/2 after birth (F) from WT or $J_H^{-/-}$ mice was analyzed for radioactivity. (**G** to **I**) Postnatal tissues from WT offspring were analyzed for persistence of transferred radioactive maternal microbial products. Geometric means are shown. Open circles show background scintillation in offspring from nongavaged mice (B to I). Results are representative of three (A) or two (D to I) independent experiments. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.



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Tissue	Compound	Sum formula	WT		$J_H^{-/-}$	
			fold-change	p-value	fold-change	p-value
Milk	Kynurenine	[+10] C10H12N2O3	7.807	0.0325	2.529	> 0.05
	Indolelactic acid	[+11] C11H11NO3	6.790	0.0341	n.d.	
	5-Hydroxy-L-tryptophan	[+11] C11H12N2O3	5.248	0.0308	1.791	> 0.05
	Hydroxykynurenamine	[+9] C9H12N2O2	4.973	0.0278	0.974	> 0.05
	Hydroxykynurenine	[+10] C10H12N2O4	4.539	0.0406	1.457	> 0.05
	5-Methoxytryptamine	[+11] C11H14N2O	4.181	0.0380	1.190	> 0.05
	5-Methoxytryptophol	[+11] C11H13NO2	3.808	0.0109	1.235	> 0.05
	Indole-5,6-quinone	[+8] C8H5NO2	2.024	0.0019	1.307	0.0057
Neonatal liver	Hydroxykynurenine	[+10] C10H12N2O4	2.394	< 0.0001	1.679	0.0015
Neonatal spleen	Hydroxykynurenine	[+10] C10H12N2O4	2.754	< 0.0001	1.656	0.0015

Fig. 5. Profiles of maternal microbial molecules shaping the neonatal immune system. (**A** to **C**) Pregnant germ-free wild-type or $J_H^{-/-}$ mice were gavaged on E10 and E12 with unlabeled *E. coli* HA107, and on E15 and E16 with ^{13}C-labeled *E. coli* HA107. Maternal milk (gestationally colonized, $n = 6$ to 8; germ-free control, $n = 2$) was analyzed by mass spectrometry. (A and B) Volcano plots of fold-change analysis between milk from treated and untreated WT (A) and $J_H^{-/-}$ (B) dams. Metabolite compound classes are color-coded. Inset histogram: percentage ^{13}C labeling for each significantly altered compound (fold-change ≥ 2; $P \leq 0.05$): unlabeled (white), ≥25% labeled (gray), 100% labeled (black). A total of 395 potentially ^{13}C-labeled compounds were significantly enriched in the milk of WT dams. (C) Fully labeled metabolites of the AhR ligand class present in d1 to d3 milk of WT and $J_H^{-/-}$ females gestationally exposed to ^{13}C-HA107. See also data file S4. (**D**) Pregnant germ-free C57BL/6 mice were gavaged with indole-3-carbinol (I3C) or solvent, or gestationally colonized. Total numbers of Lin⁻RORγt⁺NKp46⁺ ILC3 in the d14 offspring small intestine (geometric mean, $n \geq 6$). (**E**) Pregnant germ-free C57BL/6 mice were exposed to short-chain fatty acids (SCFAs), NOD1/2 ligands, or RIG-I ligand or gestationally colonized. Total numbers of Lin⁻RORγt⁺NKp46⁺ ILC3 in d14 offspring small intestine (geometric mean, $n \geq 3$). Data are representative of three (D) independent experiments. $^{*}P \leq 0.05$.

bacterial-derived metabolites present in milk or offspring tissues from gestationally colonized mice included natural microbial ligands for the aryl hydrocarbon receptor (AhR) or their precursors (Fig. 5, A to C, and table S4) (*29*). Most of these bacterial metabolites, including the fully labeled AhR ligands (Fig. 5C), were not enriched in the milk of treated $J_H^{-/-}$ mice (Fig. 5, B and C), although these data do not prove that these molecules are necessarily bound to the antibodies for transfer. Because AhR-deficient mice have a compound phenotype (*30*), and strain combination experiments reveal globally nonredundant signaling pathways, we took the approach of treating pregnant germ-free mice with authentic ligands for AhR, short-chain fatty acids, nucleotide-binding oligomerization domain (NOD) ligands and the retinoic acid–inducible gene I (RIG-I) ligand. Of these, only the AhR ligand (indole-3-carbinol, I3C) increased NKp46$^+$ ILC3 in the offspring of the treated mothers (Fig. 5, D and E). This occurred even in the absence of antibodies, although to a significantly lower extent (fig. S17, A and B). Although this shows that early postnatal NKp46$^+$ ILC3 numbers are increased in response to aryl hydrocarbons, antibodies are not essential for the effect provided that a sufficient dose of

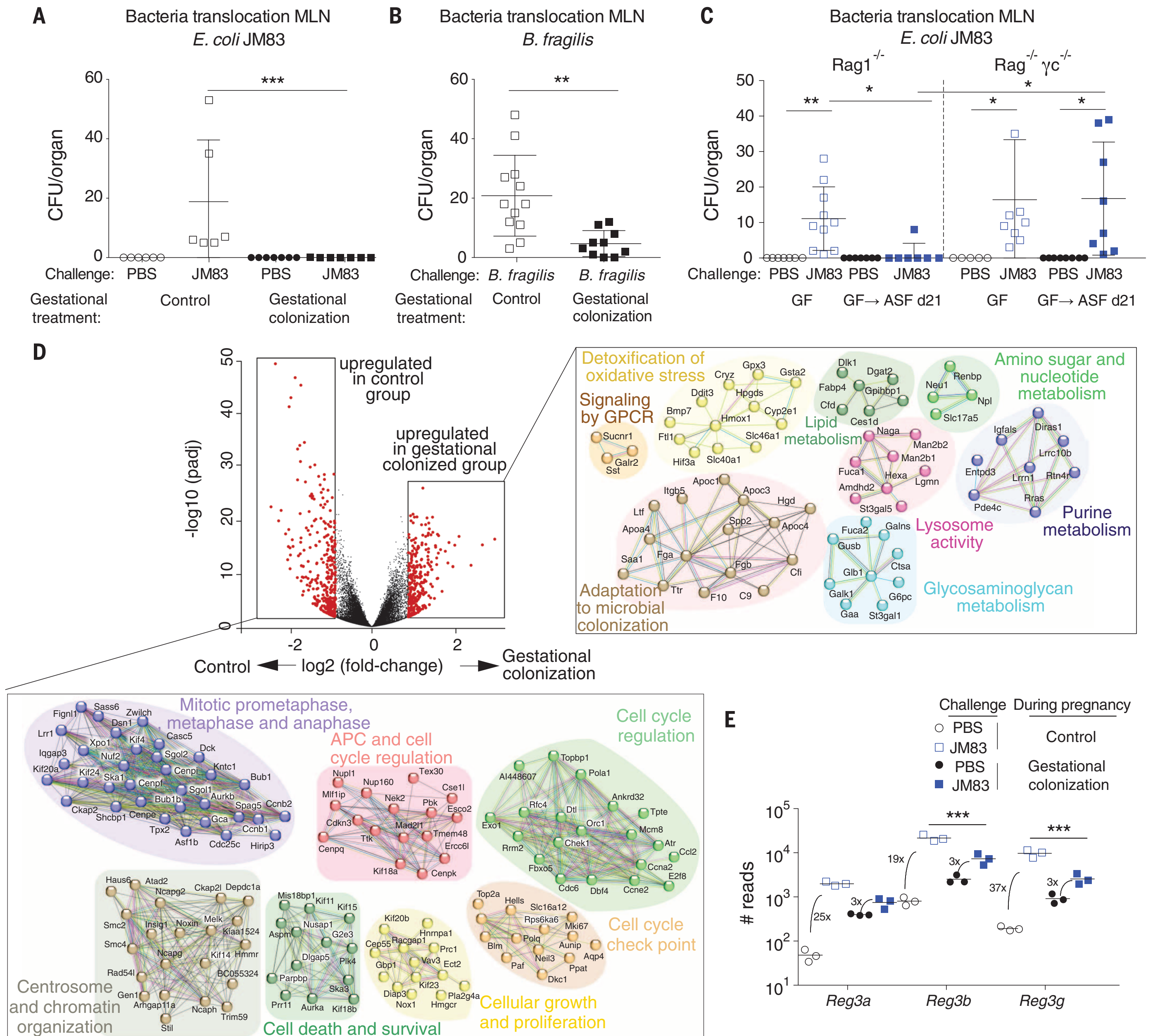

Fig. 6. Microbial exposure during pregnancy functionally protects the offspring. (**A**, **B**, **D**, and **E**) Germ-free C57BL/6 dams were gestationally colonized or kept germ-free. (A) Day 14 pups were challenged with 10^5 CFU of *E. coli* JM83 or phosphate-buffered saline (PBS). Bacterial titers in mesenteric lymph nodes (MLN) were determined at 18 hours ($\bar{x}$ ± SD, $n \geq 5$). (B) Day 14 pups were challenged with 10^{10} CFU of *B. fragilis* or PBS. Bacterial titers in MLNs were determined at 18 hours ($\bar{x}$ ± SD, $n \geq 10$). (**C**) Adult germ-free C57BL/6 *Rag1*$^{-/-}$ or *Rag2*$^{-/-}\gamma c^{-/-}$ were colonized for 21 days with ASF or kept germ-free before challenge with 10^7 CFU of *E. coli* JM83 or PBS. Bacterial titers at 18 hours in MLNs are shown ($\bar{x}$ ± SD, $n \geq 5$). (D and E) Day 14 pups were challenged with 10^5 CFU of *E. coli* JM83 or PBS (n = 3 per group) before small intestinal RNA-Seq. 18 hours later. STRING analyses for differentially expressed transcripts (fold-change ≥ 2; padj < 0.01, data file S6) compare challenged pups born to germ-free or gestationally colonized mothers. (E) Read number of indicated genes in *E. coli*– or PBS-challenged offspring (geometric mean, n = 3, ***padj < 0.001; see also data files S7 and S8). Data are representative of three (A and C) or two (B) independent experiments. (A to C) *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

AhR ligand is transmitted from the mother to her offspring. Indeed, we found that ILC3 increases induced by an endogenous ASF microbiota in adult mice were also antibody independent, presumably because endogenous colonization provides a sufficient dose of bacterial ligands (fig. S17C). We concluded that maternal antibodies assist the transfer of microbial compounds to the offspring, but are not independently required to increase ILC3 numbers. Given the diversity of maternal microbial molecular transfer, it remains probable that other microbial molecular species can also drive early postnatal adaptation.

Gestational colonization effects on innate immune precursors

The elevated number of intestinal NKp46$^+$ ILC3 and F4/80$^+$CD11c$^+$ iMNCs in the offspring born to gestation-only colonized dams may result from amplified precursor populations or increased proliferation of the mature intestinal population. Neonatal ILC3 precursors (*14*, *31*) were not increased in the liver or intestine of E17 fetuses from gestationally HA107-colonized mice (fig. S18, A to D). We did detect increased proliferative capacity of small intestinal NKp46$^+$ ILC3 isolated from 14-day-old pups born to gestation-only colonized dams (fig. S18, E and F). F4/80$^+$CD11c$^+$ iMNCs stem from CD11b$^+$CX3CR1^{int}Ly6C$^+$ monocytes (*32*, *33*), which were significantly increased in the colon lamina propria of 14-day-old pups from gestationally colonized dams (fig. S18, G and H).

Functional impact of gestational colonization on the early postnatal immune system

To test whether the integrity of the early postnatal intestine to live microbial challenge was improved by gestational colonization, we challenged pups with the replication-competent parent strain of HA107, *E. coli* JM83. Despite equal *E. coli* cecal colonization at 18 hours, only the pups of gestationally colonized mothers or dams treated with the AhR ligand I3C could avoid translocation of JM83 to the mesenteric lymph nodes (Fig. 6A and fig. S19, A and B). Because presence of HA107-specific antibodies might contribute to *E. coli*–primed protection, we confirmed these results by challenge with *Bacteroides fragilis*, where HA107-induced antibodies do not cross-react (Fig. 6B and fig. S19C).

To verify the role of ILC3 in intestinal integrity (*34*), we exploited the fact that ILC3 can be induced in adults independently of B cells and antibodies (fig. S17C and table S5). Comparison of Rag$^{-/-}$ and Rag$^{-/-}\gamma_c^{-/-}$ mice (which lack ILCs as well as B and T cells) showed that ILCs were required to mediate the microbiota-driven protection from bacterial translocation during challenge with JM83 (Fig. 6C).

Systemic immune responsiveness is also likely shaped by gestational colonization, as tumor necrosis factor–α and IL-6 proinflammatory cytokine production was reduced in the pups' splenocytes after intraperitoneal lipopolysaccharide (fig. S20). Because ILC3 are a very minor population in the spleen, the mechanism is likely to be quite distinct from the gestational effects on intestinal function.

These functional readouts of gestational colonization only show some of the potential benefits of maternal microbial molecular exposure in the pups. After challenge with replication-competent *E. coli*, small intestinal RNA-Seq analysis showed expression of antioxidant and lysosomal enzyme networks in the pups of gestation-only colonized dams, whereas control pups had wide-ranging expression signatures for cellular proliferation, cytoskeletal organization, and ribosome biosynthesis (Fig. 6D and fig. S21A). Expression of some genes for antimicrobial peptides induced by gestational colonization also increased further after intestinal bacterial challenge (Fig. 6E and fig. S21B).

Conclusion

The maternal microbiota prepares the newborn for host-microbial mutualism. This results from microbial molecular transfer because in our experimental system, live microbes are no longer present at birth; we do not detect live microbes in the placenta or the neonate; and the result can be recapitulated with sterile serum transfer. In other words, maternal antibodies not only protect the neonate through pathogen neutralization (*4*, *5*), but also have a more general effect promoting microbial molecular transfer. Short-chain fatty acids from microbes are known to shape the adult immune system (*35*, *36*). We show here that ligands for the AhR, known to drive ILC3 expansion (*37*) and limit adult bacterial translocation (*38*), can be derived from the maternal microbiota and shape the composition and function of early postnatal immunity. Nevertheless, AhR ligands are unlikely to be the only molecular mechanism involved in gestational microbial shaping.

Secretory antibodies in the milk are known to delay the maturation of the early postnatal immune system and determine long-term intestinal microbiota composition (*39*–*41*). Here we show that maternal antibodies also enhance microbial molecular levels in the fetus and the neonate. The molecular constituents of the maternal microbiota are able to ready neonatal innate immunity in time for the tsunami of microbes that successively colonize the intestine (*42*, *43*). Although these studies were focused on benign microbes, the immune morphogenesis driven by the maternal microbiota is likely also to benefit young mammals when they encounter pathogens. Postnatal microbial colonization is a pivotal early event in autonomous host-microbial mutualism. Fortunately, the maternal microbiota and maternal immunity prepare the neonate for its inevitable challenges.

REFERENCES AND NOTES

1. R. M. Samstein, S. Z. Josefowicz, A. Arvey, P. M. Treuting, A. Y. Rudensky, *Cell* **150**, 29–38 (2012).
2. M. Fulde, M. W. Hornef, *Immunol. Rev.* **260**, 21–34 (2014).
3. H. Renz, P. Brandtzaeg, M. Hornef, *Nat. Rev. Immunol.* **12**, 9–23 (2012).
4. P. Brandtzaeg, *Vaccine* **21**, 3382–3388 (2003).
5. R. M. Zinkernagel, *N. Engl. J. Med.* **345**, 1331–1335 (2001).
6. GBD 2013 Mortality and Causes of Death Collaborators, *Lancet* **385**, 117–171 (2015).
7. T. Olszak *et al.*, *Science* **336**, 489–493 (2012).
8. L. V. Hooper, D. R. Littman, A. J. Macpherson, *Science* **336**, 1268–1273 (2012).
9. A. M. Kabat, N. Srinivasan, K. J. Maloy, *Trends Immunol.* **35**, 507–517 (2014).
10. S. Rakoff-Nahoum *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **112**, 1929–1936 (2015).
11. S. Hapfelmeier *et al.*, *Science* **328**, 1705–1709 (2010).
12. F. Bäckhed *et al.*, *Cell Host Microbe* **17**, 690–703 (2015).
13. M. J. Bizzarro *et al.*, *J. Pediatr.* **166**, 1193–1199 (2015).
14. S. Sawa *et al.*, *Science* **330**, 665–669 (2010).
15. S. L. Sanos *et al.*, *Nat. Immunol.* **10**, 83–91 (2009).
16. C. Vonarbourg *et al.*, *Immunity* **33**, 736–751 (2010).
17. T. Sano *et al.*, *Cell* **163**, 381–393 (2015).
18. K. Atarashi *et al.*, *Cell* **163**, 367–380 (2015).
19. M. B. Geuking *et al.*, *Immunity* **34**, 794–806 (2011).
20. A. J. Macpherson, N. L. Harris, *Nat. Rev. Immunol.* **4**, 478–485 (2004).
21. K. Smith, K. D. McCoy, A. J. Macpherson, *Semin. Immunol.* **19**, 59–69 (2007).
22. J. Pácha, *Physiol. Rev.* **80**, 1633–1667 (2000).
23. D. Szklarczyk *et al.*, *Nucleic Acids Res.* **43**, D447–D452 (2015).
24. D. Grün *et al.*, *Nature* **525**, 251–255 (2015).
25. M. L. Robinette *et al.*, *Nat. Immunol.* **16**, 306–317 (2015).
26. A. J. Macpherson, T. Uhr, *Science* **303**, 1662–1665 (2004).
27. M. L. Balmer *et al.*, *Sci. Transl. Med.* **6**, 237ra66 (2014).
28. V. Ghetie, E. S. Ward, *Immunol. Today* **18**, 592–598 (1997).
29. C. Esser, A. Rannug, *Pharmacol. Rev.* **67**, 259–279 (2015).
30. B. Stockinger, P. Di Meglio, M. Gialitakis, J. H. Duarte, *Annu. Rev. Immunol.* **32**, 403–432 (2014).
31. M. Cherrier, S. Sawa, G. Eberl, *J. Exp. Med.* **209**, 729–740 (2012).
32. S. Tamoutounour *et al.*, *Eur. J. Immunol.* **42**, 3150–3166 (2012).
33. C. C. Bain *et al.*, *Nat. Immunol.* **15**, 929–937 (2014).
34. G. F. Sonnenberg *et al.*, *Science* **336**, 1321–1325 (2012).
35. N. Arpaia *et al.*, *Nature* **504**, 451–455 (2013).
36. Y. Furusawa *et al.*, *Nature* **504**, 446–450 (2013).
37. E. A. Kiss *et al.*, *Science* **334**, 1561–1565 (2011).
38. G. F. Sonnenberg, D. Artis, *Nat. Med.* **21**, 698–708 (2015).
39. D. R. Kramer, J. J. Cebra, *J. Immunol.* **154**, 2051–2062 (1995).
40. N. L. Harris *et al.*, *J. Immunol.* **177**, 6256–6262 (2006).
41. E. W. Rogier *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **111**, 3074–3079 (2014).
42. R. I. Mackie, A. Sghir, H. R. Gaskins, *Am. J. Clin. Nutr.* **69**, 1035S–1045S (1999).
43. Y. R. Nobel *et al.*, *Nat. Commun.* **6**, 7486 (2015).

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