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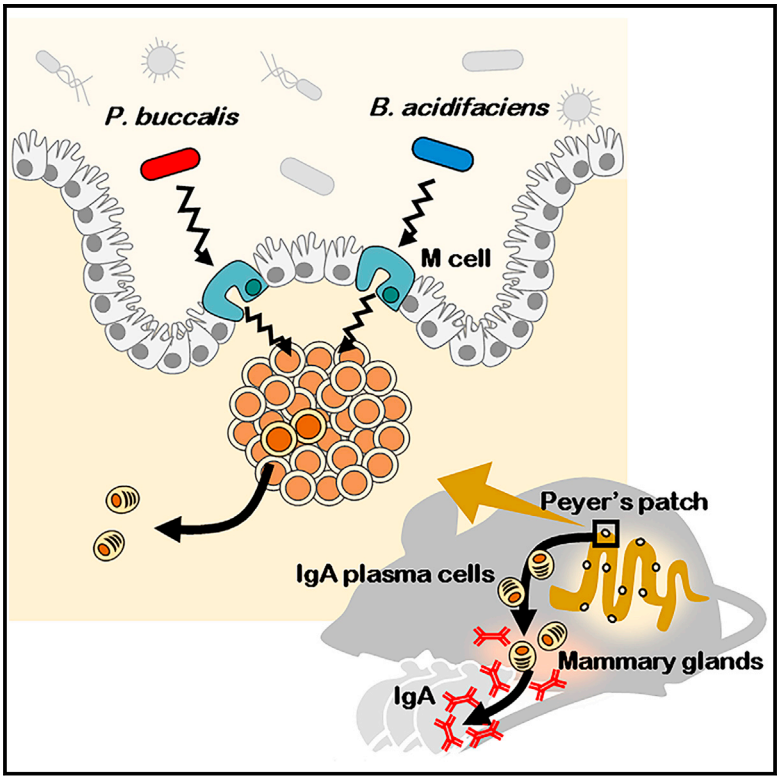
The gut microbiota induces Peyer's-patch-dependent secretion of maternal IgA into milk

Graphical abstract



Highlights

- Peyer's patches (PPs) play a key role in producing maternal IgA in milk
- Antigen sampling by M cells in PPs is important for maternal IgA production in milk
- The gut microbiota is involved in the appearance of maternal IgA in milk
- Maternal IgA production in milk is promoted by the microbiota-PP-mammary gland pathway

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In brief

Usami et al. show that Peyer's patches (PPs), a secondary lymphoid tissue in the small intestine, are involved in producing maternal IgA in milk. *Bacteroides acidifaciens* and *Prevotella buccalis*, which are intestinal residential microorganisms, play important roles in the microbiota-PP-mammary gland pathway to promote maternal IgA synthesis in milk.

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The gut microbiota induces Peyer's-patch-dependent secretion of maternal IgA into milk

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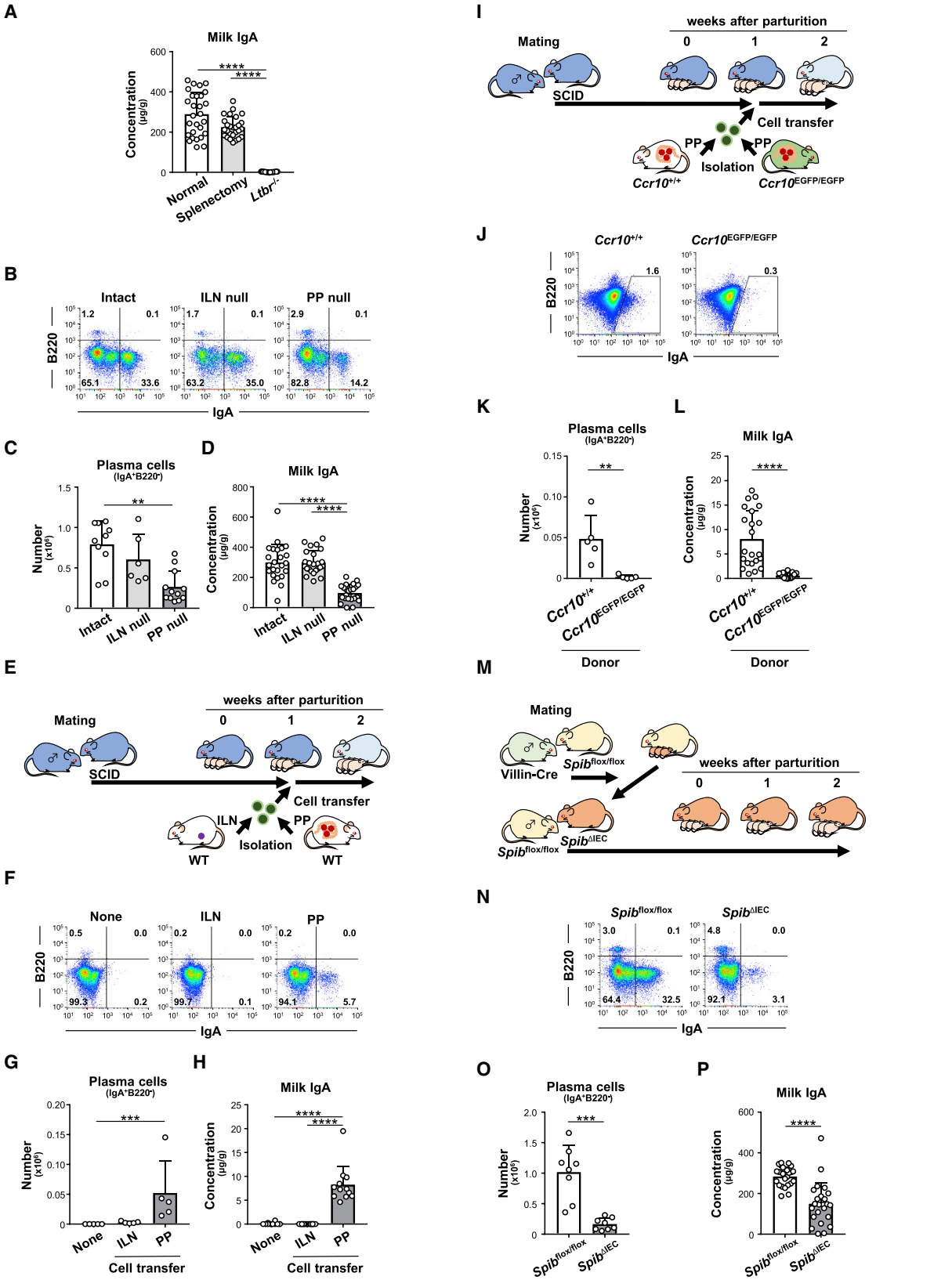
SUMMARY

The evolutionary strategy of transferring maternal antibodies via milk profoundly impacts the survival, lifelong health, and wellbeing of all neonates, including a pronounced impact on human breastfeeding success and infant development. While there has been increased recognition that interorgan connectivity influences the quality of a mother's milk, potentially to personalize it for her offspring, the underlying bases for these processes are incompletely resolved. Here, we define an essential role of Peyer's patches (PPs) for the generation of plasma cells that secrete maternal immunoglobulin A (IgA) into milk. Our metagenomic analysis reveals that the presence of certain residential microorganisms in the gastrointestinal (GI) tract, such as *Bacteroides acidifaciens* and *Prevotella buccalis*, is indispensable for the programming of maternal IgA synthesis prior to lactational transfer. Our data provide important insights into how the microbiome of the maternal GI environment, specifically through PPs, can be communicated to the next generation via milk.

INTRODUCTION

The developmental origins of health and disease (DOHaD) concept portends that adaptive responses such as an infant's future health are defined during embryogenesis and early life (Suzuki, 2018). Indeed, numerous maternal and paternal processes can potentially impact the onset of non-communicable diseases into the next generation (Barouki et al., 2012; Fleming et al., 2018). Lactation is perhaps the most pronounced and impactful strategy among these, where a mother's milk not only provides the offspring with a wide diversity of nutrients but also transfers various forms of immunity, often during a relatively short period, through the secretion of colostrum (Victora et al., 2016). Maternal antibodies, which in the milk of humans and a subset of other species are primarily of the immunoglobulin A (IgA) isotype, play a key role in protecting newborns from necrotizing enterocolitis (Gopalakrishna et al., 2019; Hurley and Theil, 2011). The effectiveness of maternal antibody transfer via breastfeeding also reflects a number of factors ranging from their production and diverse antigen specificity to their transmission (Brandtzaeg, 2010; Turin and Ochoa, 2014).

The process of maternal IgA production by the mammary glands requires humoral immune function, which originates from the distinct intestinal tract (Ramanan et al., 2020). During



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lactation, IgA plasma cells expressing the chemokine receptor CCR10 migrate into the mammary glands in response to its secretion of CCL28 (Morteau et al., 2008; Wilson and Butcher, 2004). The gut-associated lymphoid tissue (GALT), to which Peyer's patches (PPs) belong, is an important site for the differentiation of IgA-committed B cells from conventional T-cell-dependent B-2 B cells (Biram et al., 2019; Craig and Cebra, 1971; Reboldi and Cyster, 2016). Resident microbes, including *Bacteroides* spp., initiate immune responses in GALT to induce the differentiation of B cells into IgA-producing plasmablasts that become IgA plasma cells dispersed in the lamina propria of intestinal villi (Yanagibashi et al., 2009). These plasma cells secrete large amounts of dimeric or polymeric IgA to form secretory IgA (SIgA) in the lumen, which serves to eliminate pathogenic microbes by inhibiting the interaction between bacteria and host cells (Mantis and Forbes, 2010). In turn, SIgA affords symbiotic support to the resident 100 trillion microbes, spanning nearly 1,000 species (Qin et al., 2010). Furthermore, SIgA can also bind beneficial bacteria, thereby facilitating their development (Donaldson et al., 2018; Nakajima et al., 2018). Indeed, IgA-seq has consistently identified that intestinal bacteria coated by IgA (IgA^+) can be either pathogenic or beneficial (Palm et al., 2014).

Beyond the known transfer of certain viruses to the mammary glands of offspring following the ingestion of maternally derived infected milk (Finke and Acha-Orbea, 2001; Gerber et al., 2016; Golovkina et al., 1998; Langel et al., 2019), few studies have explored, and there is no direct evidence for, specific lymphoid tissues that develop in the gastrointestinal (GI) tract and function during the genesis of maternal IgA that appear in milk (Lindner et al., 2015; Roux et al., 1977). In particular, there is no information regarding how the entire intestinal bacterial domain might stimulate the immune responses in the GI tract to produce maternal milk-borne IgA.

Given the critical importance of understanding the maternal gut-mammary-infant axis and its regulation, and the potential for improving the production and specificity of IgA as a strategy for improving neonatal health and wellbeing, not only for human infants but also in fact for all mammals, we sought to define the entire maternal gut-mammary axis responsible for the transfer of IgA into milk. Our findings reveal not only that PPs are the primary origin of plasma cells within the lactating mammary glands that are responsible for producing maternal IgA found in milk but also that certain gut commensal bacteria stimulate IgA transfer to milk in this way. Specifically, we find that vancomycin-sensitive bacteria that cohabitate in the GI tract in the presence of host lymphocytes participate in maternal IgA production in milk, that most intestinal bacteria are recognized by maternal IgA in milk, and that *Bacteroides acidifaciens* and *Prevotella buccalis* are representative species of intestinal bacteria that induce PP-dependent production of IgA present in milk.

RESULTS

Identification of plasma cells that produce maternal IgA in the mammary glands

We first sought to identify the source(s) of IgA in milk from lactating mice. To eliminate any contribution of the systemic immune system, milk from lactating $Ltbr^{-/-}$ mice lacking all secondary lymphoid tissues, except the spleen, was examined alongside splenectomized and normal mice. A normal level of maternal IgA was recorded in milk from splenectomized mice, whereas no IgA was detected in milk from $Ltbr^{-/-}$ mice (Figure 1A). Therefore, the spleen, which plays an important role in the systemic immune system, is not involved in the lactational transfer of maternal IgA. We and others have previously demonstrated that the recruitment of IgA plasma cells into the mammary glands of mice is indispensable for the transfer of maternal IgA into milk (Halsey et al., 1983; Niimi et al., 2018). To facilitate further studies aimed at identifying the origin of IgA plasma cells in the lactating mammary glands, we established and optimized a strategy to characterize plasma cells by flow cytometry using multiple markers, including IgA, B220, Ly6C, I-Ad, CD11b, and CD93 (Chevrier et al., 2009; Kunisawa et al., 2013; Wrammert et al., 2002). At 2 weeks postpartum, two distinct IgA^+ populations (i.e., IgA^{high} and IgA^{low} cells) could be distinguished within the mammary glands of normal lactating mice, where the major difference between the two populations was the presence or absence of the plasma cell marker CD93 (Figure S1A). A further detailed analysis revealed that the plasma cells were IgA^{high}, $B220^-$, $Ly6C^+$, $I\text{-}Ad^+$, $CD11b^-$, and $CD93^+$ (Figure S1B), as

Figure 1. IgA plasma cells in the mammary glands originate from Peyer's patches (PPs)

(A) Whereas a significant level of milk IgA was detected in normal and splenectomized mice, levels were almost undetectable in $Ltbr^{-/-}$ mice (normal, n = 28; splenectomy, n = 27; and $Ltbr^{-/-}$, n = 17).

(B–D) The number of IgA plasma cells in the mammary glands and the level of maternal IgA in milk of PP-null mice were significantly lower than those for intact and inguinal lymph node (ILN)-null mice when analyzed 2 weeks postpartum (C: intact, n = 10; ILN null, n = 6; and PP null, n = 12; D: intact, n = 24; ILN null, n = 24; and PP null, n = 24).

(E–H) IgA plasma cells were only present in the mammary glands of ICR-*scid/scid* (SCID) mice when recipients had been xenografted with cells isolated from PPs (not ILNs) from ICR-+/+ (wild-type [WT]) mice (G: none, n = 5; ILN, n = 5; and PP, n = 5; H: none, n = 10; ILN, n = 14; and PP, n = 12).

(I–L) The number of IgA plasma cells and the level of maternal IgA in milk were elevated following the adoptive transfer of cells from the PPs of $Ccr10^{+/+}$ (not $Ccr10^{EGFP/EGFP}$) mice (K: $Ccr10^{+/+}$, n = 5; and $Ccr10^{EGFP/EGFP}$, n = 5; L: $Ccr10^{+/+}$, n = 22; and $Ccr10^{EGFP/EGFP}$, n = 23).

(M–P) Mice lacking M cells in PP had a significantly lower level of maternal IgA in their milk compared to normal mice (O: $Spib^{flox/flox}$, n = 8; and $Spib^{\Delta IEC}$, n = 8; P: $Spib^{flox/flox}$, n = 24; and $Spib^{\Delta IEC}$, n = 24).

(E, I, and M) Experimental designs for *in vivo* studies.

(B, F, J, and N) IgA plasma cells were categorized as IgA^+B220^- by flow cytometry.

(C, G, K, and O) The absolute number of IgA plasma cells in the mammary glands was determined based on the frequency of IgA plasma cells and the total number of cells isolated from the mammary glands.

(A, D, H, L, and P) The level of IgA in milk as measured by ELISA.

Data are presented as mean ± SD. $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ by multiple comparison testing (C, D, G, and H) and t test (K, L, O, and P).

confirmed by t-distributed stochastic neighbor embedding (t-SNE) analysis (Figure S1C).

PPs play a key role in maternal IgA production in the mammary glands

We next evaluated which secondary lymphoid tissue(s) could direct maternal IgA production in the mammary glands. Candidates among these are the supramammary lymph nodes (LNs) that survey the mammary glands (Sheng et al., 2011) and PPs that play a pivotal role in the mucosal immune system, especially in the GI tract, where a greater amount of SIgA is produced (Lindner et al., 2015; Moro-Sibilot et al., 2016). To this end, we generated inguinal LN (ILN)- and PP-null mice, both of which expressed normal levels of milk proteins (i.e., α, β, and k caseins) and CCL28, a chemokine that is necessary for recruiting IgA plasma cells to the mammary glands (Wilson and Butcher, 2004) (Figure S1D). Importantly, the number of IgA plasma cells in the mammary glands (but not the small and large intestines), and the level of IgA in milk, were significantly lower in PP-null mice compared to those in normal and ILN-null mice (Figures 1B–1D and S1E). We also confirmed these findings by transferring cells isolated from either PPs or ILNs of wild-type (C.B-17/ICR-+/+Jcl) mice into severe combined immunodeficiency (SCID) (C.B-17/ICR-*scid*/*scid*Jcl) mice that lack both T and B cells (Figure 1E). Transplant recipients accumulated IgA plasma cells in the mammary glands, as well as the small and large intestines (Figures 1F, 1G, and S1F). Maternal IgA was only detected in milk from lactating SCID mice that were transplanted with PP-derived cells (Figure 1H). These results strongly suggested that cells from PPs are recruited to the mammary glands to produce maternal IgA in milk.

Tropism of IgA-committed B cells to the mammary glands requires CCR10 expression in PPs

Given that CCR10 expression is critical for the migration of IgA-committed B cells to the mammary glands (Morteau et al., 2008), we next utilized a reporter strain having enhanced GFP (EGFP) knocked into the *Ccr10* gene locus to examine the sites of its expression. The number of IgA plasma cells in the mammary glands of these mice and the level of maternal IgA in their milk were consistent with the previous report (Morteau et al., 2008), where they were almost undetectable in CCR10-deficient ($Ccr10^{EGFP/EGFP}$) mice, and abundant in $Ccr10^{+/+}$ mice (Figures S2A–S2C). Importantly, appreciable numbers of both B and T cells in PPs of $Ccr10^{+/EGFP}$ mice expressed CCR10 (EGFP), whereas $CCR10^{+}$ ($EGFP^{+}$) B cells (but not T cells) were extremely rare in the ILNs and spleen (Figures S2D–S2F). To further confirm the role of PP-derived $CCR10^{+}$ B cells in the production of maternal IgA in milk, cells isolated from PPs of either $Ccr10^{+/+}$ or $Ccr10^{EGFP/EGFP}$ mice were transferred into SCID mice. The number of IgA plasma cells and the level of maternal IgA in milk only increased when $Ccr10^{+/+}$ mice were used as donors for this adoptive transfer (Figures 1I–1L). Given that cells within PP express CCR9 upon activation so as to imprint their tropism toward the lamina propria of the small intestinal villi (Mora et al., 2003), we next investigated the expression pattern of CCR9 and CCR10 in PPs. The data indicated that most cells expressing CCR10 were different from $CCR9^{+}$ cells, suggesting that CCR10 expression is regulated independently of CCR9 (Figure S2G). These results indicated that the tropism of IgA plasma cells to the mammary glands could depend on CCR10-expressing B cells in PP (but not the ILN or spleen), where various directives to cells are created individually.

Antigen sampling by M cells in PP is required for maternal IgA production in milk

We next sought to clarify the role for immune responses raised within PPs against luminal antigens, including microorganisms, during the lactational transfer of maternal IgA into milk. *Spib*, a transcription factor involved in the differentiation of antigen-sampling M cells located in the follicle-associated epithelium (FAE) (Kanaya et al., 2012; Sato et al., 2013), was depleted specifically in intestinal epithelial cells (IECs) by crossing $Spib^{flox/flox}$ and Villin-Cre transgenic mice to generate $Spib^{\Delta IEC}$ offspring (Figures S3A–S3C). Spi-B was expressed in both the FAE and germinal centers (GCs) of $Spib^{flox/flox}$ mice that normally develop M cells and undergo immune activation in PPs (Figures S3D–S3H). By contrast, Spi-B was only expressed in the GCs of $Spib^{\Delta IEC}$ mice, confirming the effective disruption of M cell differentiation in the FAE and further immune abnormalities in PPs (Figures S3D–S3H). Consequently, the number of IgA plasma cells in the mammary glands, in addition to that in the small and large intestines, as well as the level of maternal IgA in milk of $Spib^{\Delta IEC}$ mice were significantly lower than in control $Spib^{flox/flox}$ mice (Figures 1M–1P and S3I). These results suggested that the luminal antigens sampled by M cells promote immune activation within PPs prior to the tropism of stimulated cells to the mammary glands for the lactational transfer of maternal IgA into milk.

Intestinal microorganisms initiate the appearance of maternal IgA in milk

Considering that the intestinal microflora establishes homeostasis with immune cells of the GI tract, including PPs, we next explored the influence of the intestinal microbiome on the IgA profile in milk by administering various antibiotics (ampicillin, neomycin, vancomycin, and their combination) to female mice during pregnancy and lactation (Figure 2A). While antibiotic treatment did not alter the total bacterial count dramatically (Figure 2B), it did shift the microbial composition (Figures 2C, 2D, and S4A–S4G; Table S1). Among those microorganisms detected, the frequency of *Parabacteroides goldsteinii*, *B. acidifaciens*, and *P. buccalis* decreased, whereas that of *Escherichia albertii* increased, when the mice were treated with vancomycin alone or in combination (Figure 2E). More importantly, the number of IgA plasma cells in the mammary glands (but not small and large intestines) as well as the level of IgA in milk decreased significantly in mice treated with vancomycin or in combination (Figures 2F–2H and S4H). To confirm the involvement of intestinal microorganisms in the production of maternal IgA in milk, we next performed fecal microbial transplantation (FMT) using feces collected from healthy mice known to harbor *P. goldsteinii*, *B. acidifaciens*, and *P. buccalis*. Specifically, lactating mice were pretreatment with all three antibiotics and received FMT (Figures 3A). While the total number of intestinal microorganisms increased slightly after FMT (Figure 3B), changes in the microbial composition were more pronounced

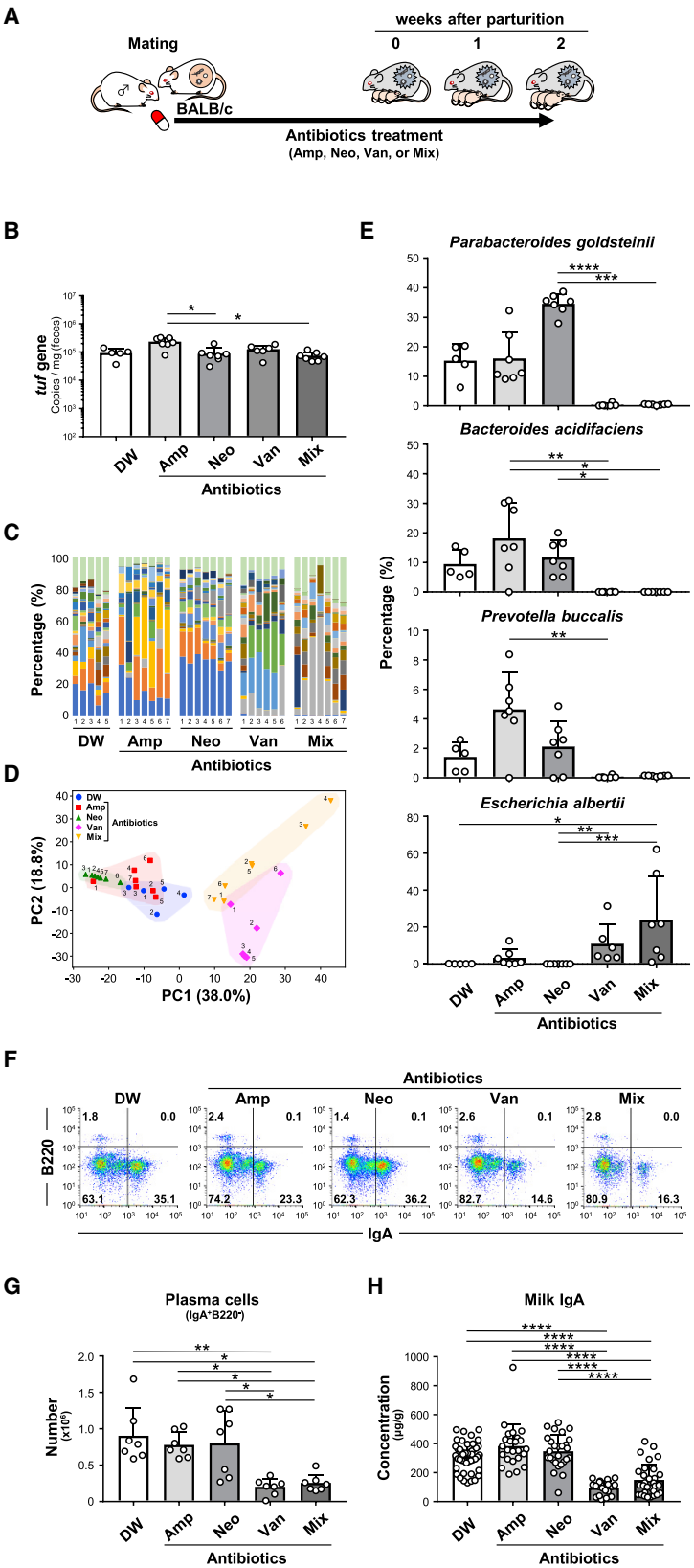

Figure 2. Intestinal microorganisms affect the presence of maternal IgA in milk

(A) Disruption of the intestinal microbiota by treatment with ampicillin (Amp), neomycin (Neo), vancomycin (Van), or all three antibiotics (Mix) administered pre- and postpartum.

(B) Following antibiotic therapy, the total number of intestinal microorganisms was only slightly altered, as determined by quantitative PCR for *tuf* gene abundance in feces (distilled water [DW], n = 5; Amp, n = 7: Neo, n = 7; Van, n = 6; and Mix, n = 7).

(C and D) Metagenomics analysis by 16S rRNA profiling revealed that the profile of the maternal microflora in the intestine varied significantly in response to Van or Mix.

(E) Identity of four representative microorganisms whose frequencies were changed significantly by treatment with Van or Mix, as identified using Illumina BaseSpace (DW, n = 5; Amp, n = 7: Neo, n = 7; Van, n = 6; and Mix, n = 7).

(F–H) The number of IgA plasma cells in the mammary glands of lactating mice and the level of maternal IgA in their milk were significantly lower in Van- and Mix-treated mice compared with those in non-, Amp-, and Neo-treated mice (G: DW, n = 7; Amp, n = 7: Neo, n = 7; Van, n = 7; and Mix, n = 7; H: DW, n = 45; Amp, n = 24: Neo, n = 27; Van, n = 20; and Mix, n = 30).

Data are presented as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 by multiple comparison testing (B, E, G, and H).

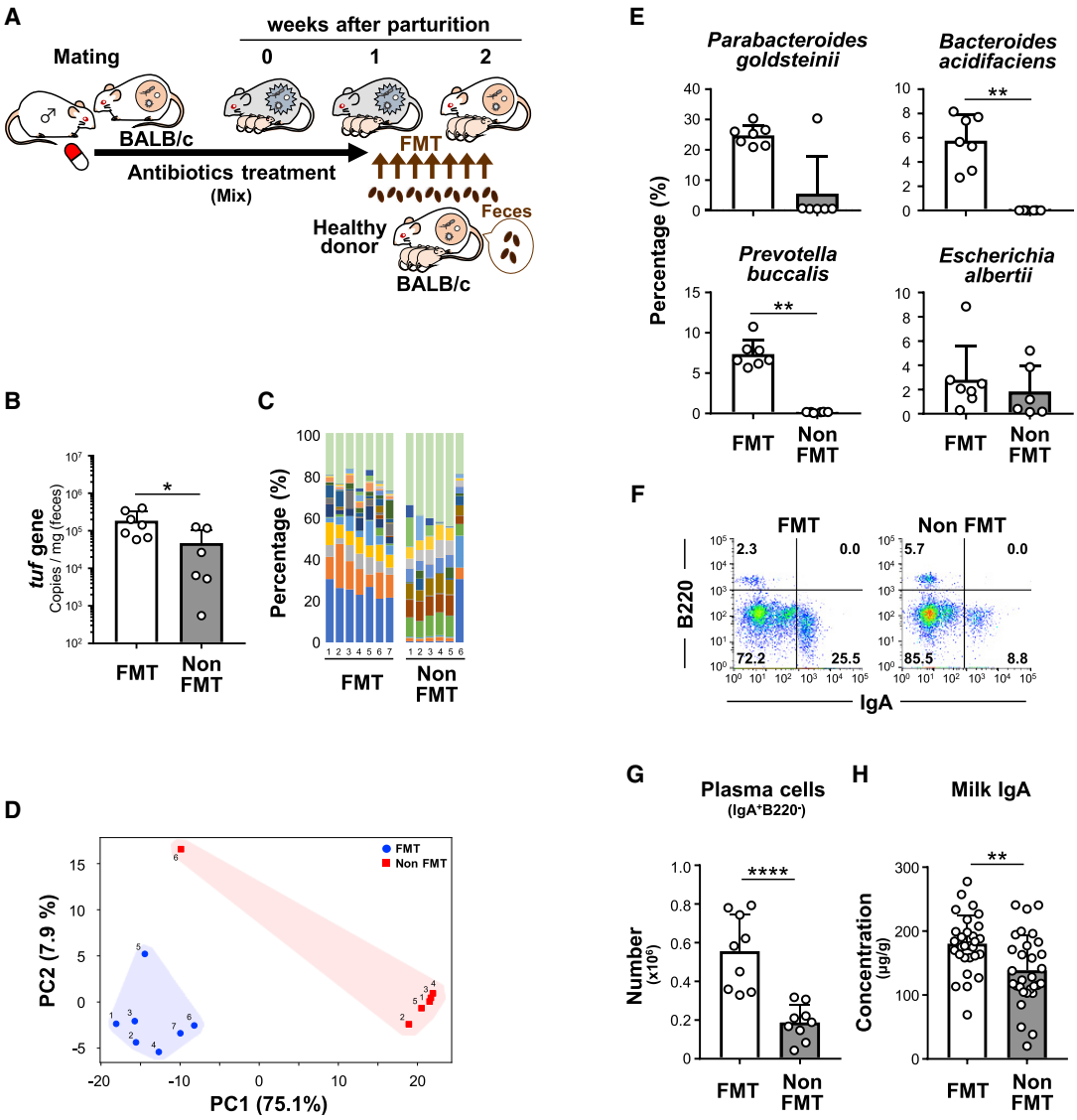

Figure 3. Fecal microbiota transplantation (FMT) of feces from untreated mice promotes the production of maternal IgA in milk by host mice having a disturbed intestinal microflora

(A) FMT with feces from untreated mice to host mice having a disrupted intestinal microflora confirmed the involvement of intestinal microorganisms during the induction of maternal IgA secretion into milk.

(B) Quantitative PCR analysis revealed that FMT significantly increased the total number of intestinal microorganisms (FMT, n = 7; and non-FMT, n = 6).

(C and D) Metagenomics analysis confirmed that the profile of the intestinal microbiota was altered significantly following FMT.

(E) Among the four representative species listed in Figure 2E, the frequency of *B. acidifaciens* and *P. buccalis* was increased by FMT (FMT, n = 7; and non-FMT, n = 6).

(F–H) The number of IgA plasma cells in the mammary glands and the level of maternal IgA in milk increased significantly following FMT (G: FMT, n = 9; and non-FMT, n = 9; H: FMT, n = 30; and non-FMT, n = 30).

Data are presented as mean ± SD. *p < 0.05, **p < 0.01, and ****p < 0.0001 by t test (B, E, G, and H).

with increased frequencies of *B. acidifaciens* and *P. buccalis* after FMT (Figures 3C–3E, S5A, and S5B; Table S2). The number of IgA plasma cells in the mammary glands (and small and large intestines) as well as the level of IgA in milk increased significantly after FMT (Figures 3F–3H and S5C). To further confirm the importance of fecal microorganisms in producing maternal IgA in milk, lactating mice received small intestinal microbial transplantation (SIMT) using small intestinal contents (Figure S5D). The number of IgA plasma cells in the mammary glands and the intestines as well as the IgA level in the milk did not increase significantly after SIMT (Figures S5E–S5H). These results indicate that the microbiome in feces rather than the small intestine may affect the PP's function of promoting the appearance of maternal IgA in milk.

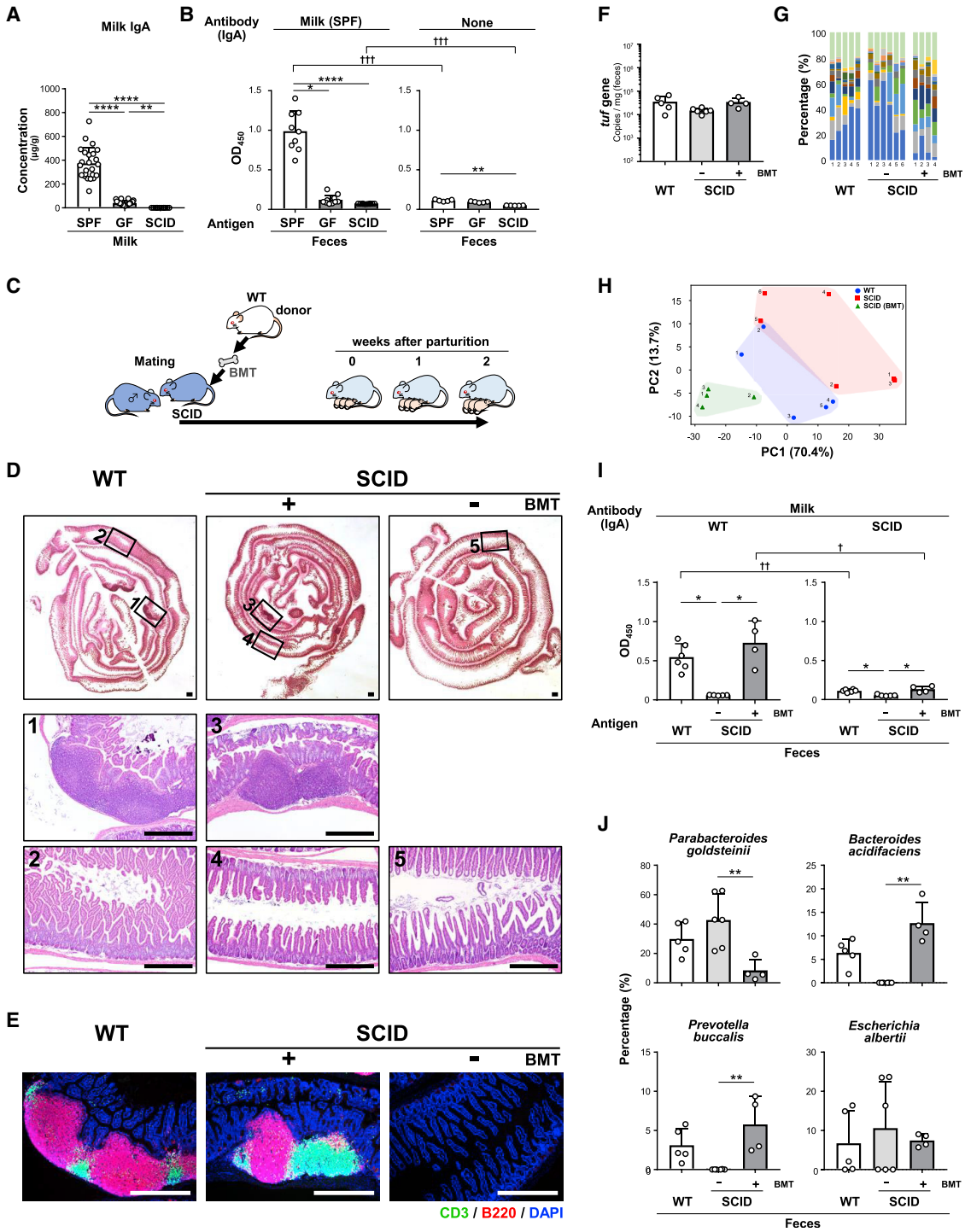

Figure 4. Host immune cells influence the developing intestinal microflora that subsequently directs maternal IgA secretion into milk

(A) The low level of IgA in milk from immunocompetent germ-free (GF) mice was almost comparable to that in immunodeficient ICR-*scid/scid* (SCID) mice (specific-pathogen-free [SPF], n = 24; GF, 25; and SCID, 12).

(B) Maternal IgA in milk from immunocompetent SPF mice recognized the intestinal microbiota in feces of immunocompetent (but not SCID) mice, both of which were maintained in an SPF (not GF) environment. Complete quenching of endogenous IgA was confirmed by an undetectable reaction of horseradish peroxidase (HRP)-conjugated anti-mouse IgA after no treatment instead of milk IgA (milk: SPF, n = 9; GF, 10; and SCID, 10; none: SPF, n = 5; GF, 5; and SCID, 5).

(C) Cells from the bone marrow of immunocompetent ICR-+/+ (WT) mice were transplanted into SCID mouse hosts.

(legend continued on next page)

The host immune system creates the intestinal microenvironment to produce maternal IgA in milk

To confirm a role for microorganisms during the production of maternal IgA found in milk, milk samples were collected from lactating mice maintained in either specific-pathogen-free (SPF) or germ-free (GF) facilities. The levels of maternal IgA in the milk of GF mice were significantly lower than those in SPF mice and similar to those in milk from SCID mice lacking all antibody isotypes (Figure 4A). When the specificity of maternal IgA in milk from SPF mice was assessed, they recognized the intestinal microorganisms present in the feces of SPF, but not GF, mice. Surprisingly, these antibodies failed to react with the intestinal microorganisms in the feces of SCID mice (Figure 4B). We subsequently inoculated SCID mice with cells isolated from the bone marrow of wild-type (WT) mice to test the hypothesis that host immune cells, especially lymphocytes, might contribute to an intestinal immune and microbial milieu responsible for directing maternal IgA production in milk (Figure 4C). Although the organogenesis of PP is initiated prenatally (Honda et al., 2001), PP-like lymphoid structures, including sufficient B and T cells, developed in the small intestine of SCID mice whose immune system was reconstituted by postnatal bone marrow transplantation (BMT) (Figures 4D, 4E, and S6A–S6D). The total number of intestinal microorganisms in SCID mice was unchanged by BMT (Figure 4F), whereas the microbial composition was changed dramatically (Figures 4G, 4H, S6E, and S6F; Table S3). Importantly, the maternal IgA in milk from WT (but not SCID) mice recognized the intestinal microorganisms present in feces from not only WT mice but also SCID mice after (but not before) BMT (Figure 4I). We next performed a comprehensive analysis of the intestinal microbiota to identify specific species that might regulate maternal IgA production in milk. Among these, *B. acidifaciens* and *P. buccalis* were identified as candidates for further analysis given their frequency increased dramatically (from undetectable levels) after BMT (Figure 4J). To further establish the importance of the intestinal microbiome for maternal IgA production into milk, we compared the efficacy of FMT using feces collected from either WT or SCID mice (Figure 5A). While there was no resultant change in the total number of intestinal microorganisms (Figure 5B), the frequency of *B. acidifaciens* and *P. buccalis* only increased after FMT using feces from WT mice (Figures 5C–5E, S6G, and S6H; Table S4). In parallel, the number of IgA plasma cells in the mammary glands (and small intestine), and the level of maternal IgA in milk, increased significantly in mice receiving FMT using feces from WT (but not SCID) mice (Figures 5F–5H and S6I). These results indicated that the host immune system, in which lymphocytes participate, could be involved in establishing an appropriate intestinal microbial environment for maternal IgA production in milk during lactation.

The specificity of maternal IgA in milk depends on the intestinal microbial environment

Given the long-standing question of how the specificity of maternal IgA in milk is established, we next performed IgA-seq analysis to identify which intestinal microorganisms were recognized by maternal IgA in milk. The microbiota from feces of lactating mice were incubated with maternal IgA from milk and subjected to flow cytometry analysis. Approximately one-quarter of the microparticles in feces were coated with IgA, and these were generally microorganisms (not debris), as recognized by the bacterial marker SYTO 9 (Figure 6A). Using magnetic cell sorting, IgA^+ and IgA^- microorganisms were separated, yielding equivalent numbers in the two populations (Figure 6A). Importantly, the intestinal microbes were distinguishable by whether they were recognized by maternal IgA in milk (Figures 6B, 6C, and S7A–S7C; Table S5). Specifically, *B. acidifaciens* and *P. buccalis* were identified as IgA^+ microorganisms (Figure 6D), highlighting that they are not only immunomodulatory in the GI tract but also antigenic for maternal immune cells in PPs so as to direct the production of maternal IgA in milk against themselves.

Oral-dosed *B. acidifaciens* and *P. buccalis* induce maternal IgA production in milk

As a final proof-of-principle for the microbiota-PP-mammary gland pathway in the appearance of maternally produced IgA in milk, lactating PP-intact and PP-null mice were orally dosed with either *B. acidifaciens* or *P. buccalis* after disturbing the intestinal microflora by pretreatment with all three antibiotics (Figure 7A). Additional lactating PP-intact mice were orally dosed with, or parenterally administered, *P. goldsteinii* that served as s negative control (Figures 7A and S7D). While there was no change in the total number of intestinal microorganisms following each bacterial dosing regardless of the species delivered (Figure 7B), the frequency of *B. acidifaciens* in the microbial population increased significantly when both PP-intact and PP null mice were dosed with this particular bacterium (Figures 7C–7E, S8A, and S8B; Table S6). No significant increase in the numbers of mature B cells or plasmablasts was recorded in PP upon the oral administration of these bacteria (Figures S8C and S8D). However, the number of IgA plasma cells in the

(D and E) PP-like structures composed of $CD3^+$ T cells and $B220^+$ B cells, similar to the PPs of WT mice, were detected in SCID mice only after bone marrow transplantation (BMT).
(F) The total number of intestinal microorganisms in SCID mice remained unchanged following BMT using WT donors (WT, n = 5; BMT−, n = 6; and BMT+, n = 4).
(G and H) The intestinal microbiome in SCID mice varied significantly after BMT, as determined by metagenomics analysis. The intestinal microbiome in SCID mice varied significantly after BMT, as determined by metagenomics analysis.
(I) Maternal IgA in milk of WT (but not SCID) mice recognized intestinal microorganisms present in feces of not only WT mice but also SCID mice after they were subjected to BMT (WT, n = 6; BMT−, n = 5; and BMT+, n = 4). Maternal IgA in milk of WT (but not SCID) mice recognized intestinal microorganisms present in feces of not only WT mice but also SCID mice after they were subjected to BMT (WT, n = 5; BMT−, n = 6; and BMT+, n = 4).
(J) Among the four representative species detailed in Figure 2E, the frequency of *B. acidifaciens* and *P. buccalis* increased in feces of SCID mice after BMT, whereas that of *P. goldsteinii* decreased (WT, n = 5; BMT−, n = 5; and BMT+, n = 4).
Data are presented as mean ± SD. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$ by multiple comparison testing (A, B, I, and J). †$p < 0.05$, ††$p < 0.01$ by t test (I). Scale bars, 500 μm.

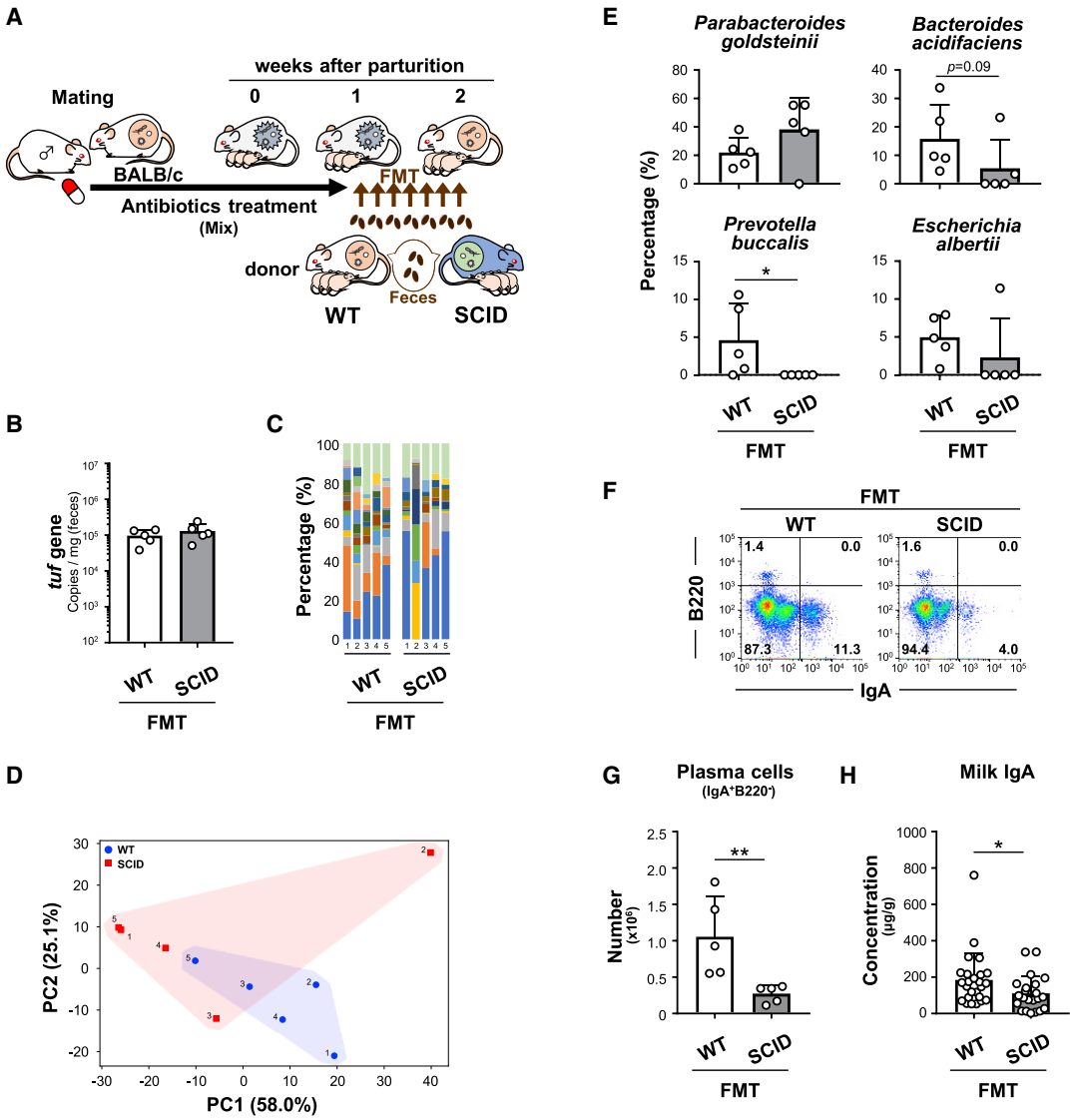

Figure 5. FMT using feces from immunodeficient mice promotes the production of maternal IgA in milk of immunocompetent mice having a disturbed intestinal microbiota

(A) Mice with disrupted intestinal microflora underwent FMT using feces collected from either ICR-+/+ (WT) or ICR-*scid*/*scid* (SCID) mice.

(B) Quantitative PCR analysis demonstrating that the total number of intestinal microorganisms was identical regardless of the source of feces used for FMT (WT, n = 5; and SCID, n = 5).

(C and D) Metagenomics analysis revealed that the intestinal microflora content varied depending on the source of feces used for FMT.

(E) Among the four representative microorganisms listed in Figure 2E, the frequency of *B. acidifaciens* and *P. buccalis* increased by FMT using feces collected from WT (but not SCID) mice (WT, n = 5; and SCID, n = 5).

(F–H) The number of IgA plasma cells in the mammary glands and the level of maternal IgA in milk increased significantly only when the feces of WT (but not SCID) mice were used for FMT (G: WT, n = 5; and SCID, n = 5, H: WT, n = 25; and SCID, n = 22).

Data are presented as mean ± SD. *p < 0.05 and **p < 0.01 by t test (E, G, and H).

mammary glands (but not intestines) and the level of maternal IgA in milk increased significantly in PP-intact mice dosed with either *B. acidifaciens* or *P. buccalis*, but not *P. goldsteinii* (Figures 7F–7H and S8E). Most importantly, maternal IgA production in milk was not induced in PP null mice even when they were orally dosed with either *B. acidifaciens* or *P. buccalis* (Figures 7F–7H). Furthermore, oral dosing of lactating PP-intact (but not PP null) mice with *B. acidifaciens* or *P. buccalis* significantly induced the secretion of bacteria-specific maternal IgA in milk (Figure 7I). It should be noted that parenteral immunization with *P. goldsteinii* induced a high level of bacteria-specific systemic IgG in serum (Figure S7E); however, it failed to produce maternal IgA specific for the bacteria in milk (Figure S7F). Taken together, these results clearly indicate that the origin of maternal IgA in milk is the intestine in which PP promote the migration of IgA plasma cells to the mammary glands in the presence of certain

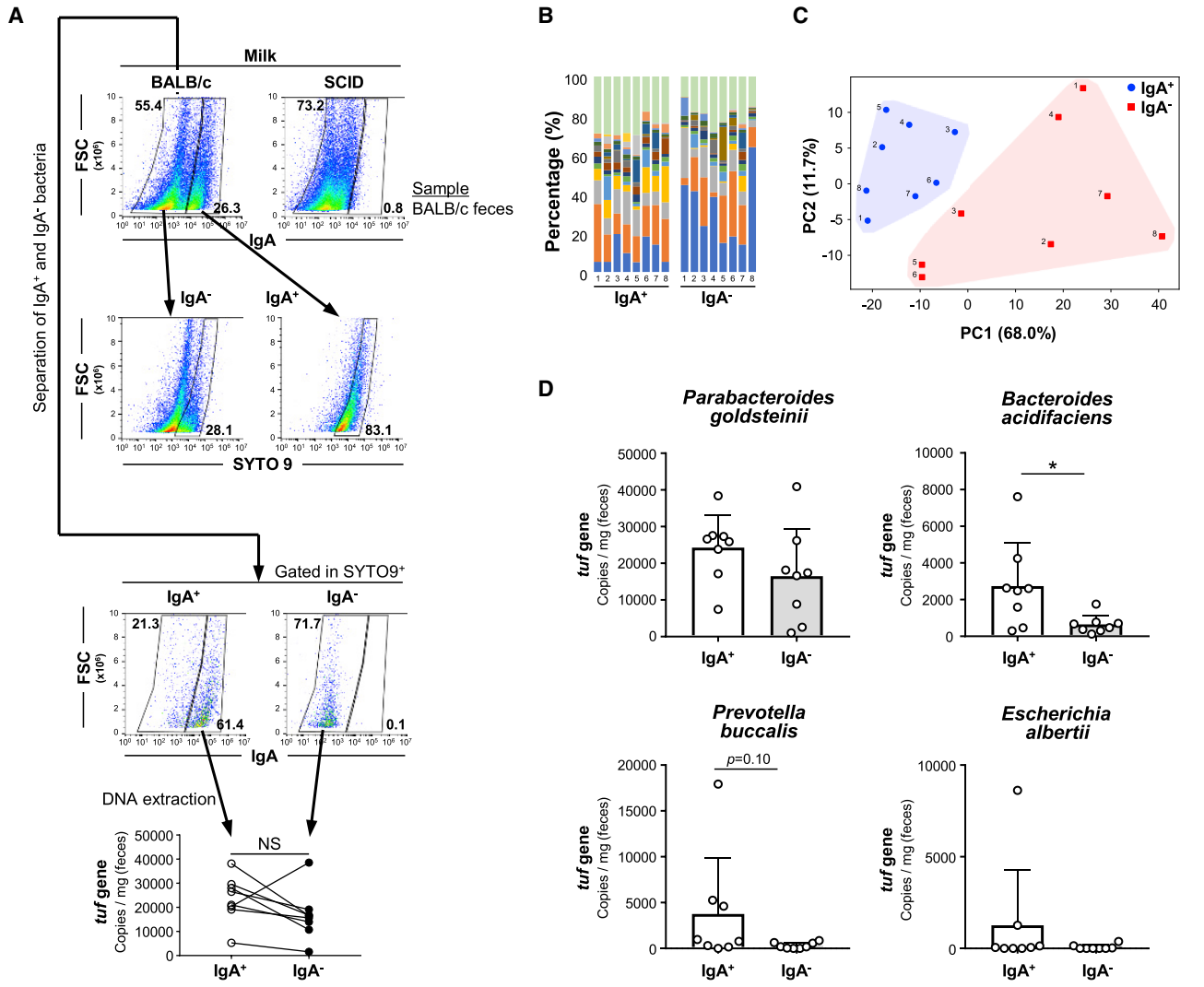

Figure 6. Maternal IgA in milk recognizes intestinal microorganisms

(A) SYTO 9$^+$ intestinal bacteria recognized by maternal IgA in milk (SYTO 9$^+$IgA$^+$) were obtained from WT (not IgA-lacking SCID) mice for species analysis alongside SYTO 9$^+$IgA$^-$ bacteria. Quantitative PCR analysis revealed that the number of SYTO 9$^+$IgA$^+$ and SYTO 9$^+$IgA$^-$ bacteria in each individual was identical. See Table S7.

(B and C) Metagenomics analysis demonstrated that the species profile for SYTO 9$^+$IgA$^+$ and SYTO 9$^+$IgA$^\times$ bacteria was completely different.

(D) Among the four representative microorganisms listed in Figure 2E, most *B. acidifaciens* were categorized as being SYTO 9$^+$IgA$^+$ (n = 8).

Data are presented as mean ± SD. *p < 0.05 by t test (D).

residential microorganisms, such as *B. acidifaciens* and *P. buccalis* (Figure S8F).

DISCUSSION

The critical role for milk in transferring nutrients and immunity to infants has undergone a renaissance in recent years, alongside the growing appreciation of the potential for interorgan transfer of immunity from a mother's intestinal microbiome (Liu et al., 2019; Milani et al., 2017; Wang et al., 2020). Through a series of genetic and transplantation strategies, we now provide the first longitudinal demonstration that PPs are the primary source of IgA-committed B cells transferred to the mammary glands during lactation. This axis has been previously implicated in viral transmission during the lymphocyte-mediated transfer of mouse mammary tumor virus (MMTV) to the mammary epithelium, leading to shedding of virus through milk (Finke and Acha-Orbea, 2001; Golovkina et al., 1998), and during the transfer of porcine epidemic diarrhea virus (PEDV)-induced immunity (Gerber et al., 2016; Langel et al., 2019). Furthermore, the IgA repertoire is similar between the small intestine and the mammary glands (Lindner et al., 2015), consistent with the demonstration that IgA plasma cells in the mammary glands originate from somewhere in the small intestine (Ramanan et al., 2020). Here, we

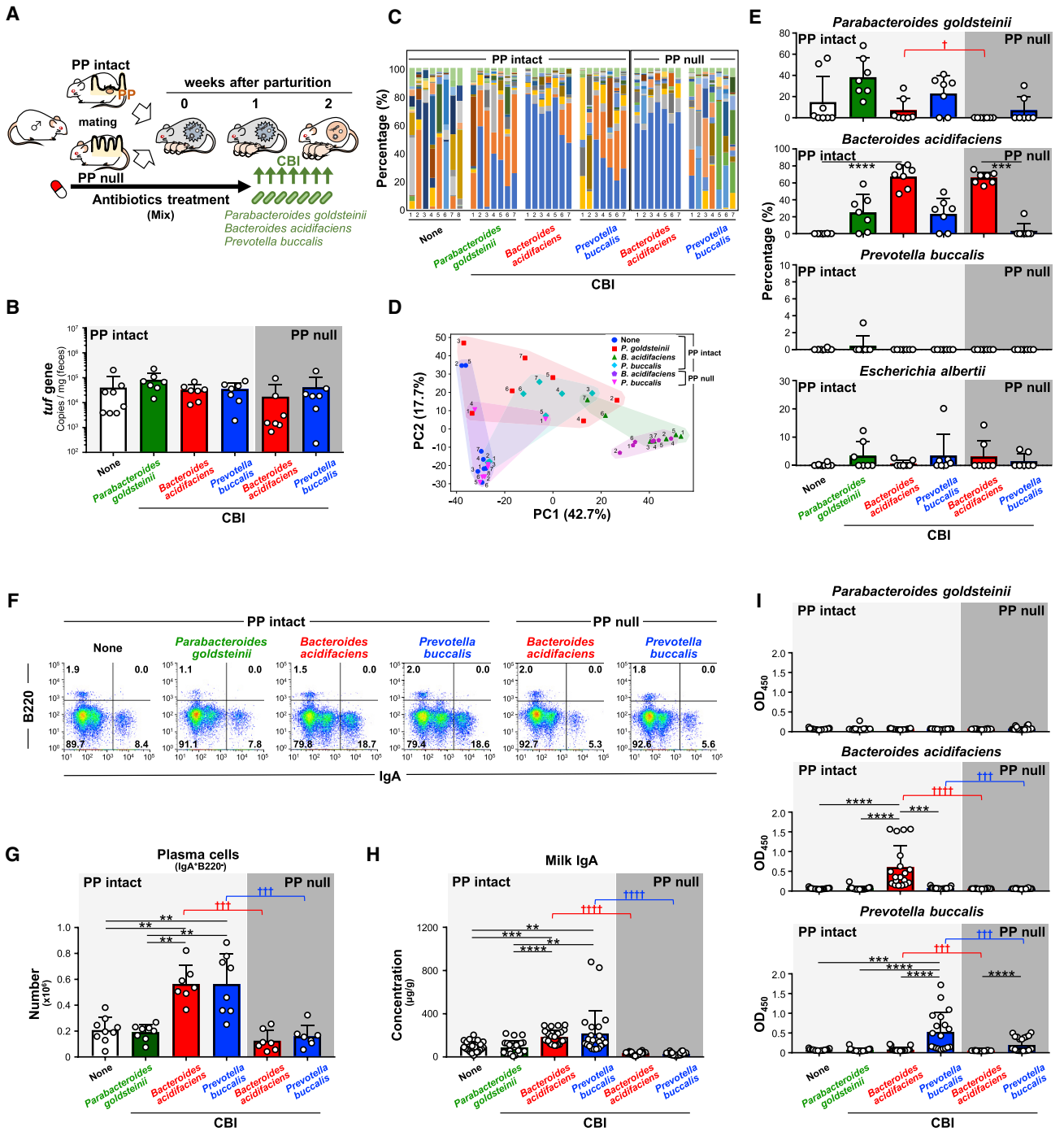

Figure 7. Certain bacterial inoculation (CBI) facilitates the production of bacteria-specific maternal IgA in milk

(A) CBI using strains of *P. goldsteinii*, *B. acidifaciens*, or *P. buccalis* was performed to confirm that the individual bacteria could rescue the production of maternal IgA in milk of PP-intact and PP-null mice having a disturbed intestinal microbiome. See Table S8.

(B) Quantitative PCR analysis revealed that the total number of intestinal microorganisms was unchanged by CBI (PP intact: none, n = 8; *P. goldsteinii*, n = 7; *B. acidifaciens*, n = 7; *P. buccalis*, n = 7; PP null: *B. acidifaciens*, n = 7; *P. buccalis*, n = 7).

(C and D) Metagenomics analysis demonstrating that the microbiome varied significantly between hosts administered different bacteria by CBI.

(E) Among the four representative bacteria listed in Figure 2E, the frequency of *B. acidifaciens* in both PP-intact and PP-null mice increased significantly after CBI (PP intact: none, n = 8; *P. goldsteinii*, n = 7; *B. acidifaciens*, n = 7; *P. buccalis*, n = 7; PP null: *B. acidifaciens*, n = 7; *P. buccalis*, n = 7).

(F–H) The number of IgA plasma cells in the mammary glands of PP-intact (but PP-null) dams undergoing CBI and the level of maternal IgA in their milk increased significantly after CBI with *B. acidifaciens* or *P. buccalis* (but not *P. goldsteinii*) (G: PP intact: none, n = 9; *P. goldsteinii*, n = 8; *B. acidifaciens*, n = 7; *P. buccalis*,

(legend continued on next page)

demonstrate that PPs in the small intestine are the primary source of maternal IgA produced in milk, activated by distinct bacteria cohabiting in the GI tract.

The present findings demonstrate that PPs are essential for the transfer of IgA into milk, whereas they are dispensable for intestinal IgA production. There have been clear demonstrations that PPs play a vital role in developing the immune environment of the GI tract (Craig and Cebra, 1971), where they preferentially induce T-cell-dependent immune activation in response to the intestinal microbiota. Our present data clearly establish that PPs are the source of migratory IgA plasma cells in the mammary glands and that these plasma cells produce maternal IgA found in milk during normal lactation. In keeping with these findings, very few IgA plasma cells were found in the mammary glands of T cell receptor β (TCRβ)/TCRδ double-deficient mice lacking T cells or in GF mice lacking residential microorganisms (Bunker et al., 2017). By contrast, in the small intestine, both PPs and mesenteric LNs (MLNs) contribute to T-cell-dependent IgA production from plasma cells that are differentiated from B-2 B cells (Bergqvist et al., 2013; Yamamoto et al., 2000). Given our data, we posit that in the absence of PPs, compensatory IgA in the intestine (but not milk) is derived from MLNs to accompany the T-cell-independent production of IgA in the isolated lymphoid follicles and by B-1 B cells (Tsuji et al., 2008).

Our present findings demonstrate that the gram-negative obligate anaerobes belonging to *Bacteroides*, namely *B. acidifaciens* and *P. buccalis*, can induce the transfer of specific maternal IgA into milk. Importantly, *B. acidifaciens* also prevents obesity and improves insulin resistance by regulating intestinal metabolism (Yang et al., 2017). Until now, few labs have studied the characteristics of *P. buccalis*, although *Prevotella* spp. are reportedly involved in glucose metabolism and the Th17-mediated proinflammatory response in the GI tract (Kovatcheva-Datchary et al., 2015; Larsen, 2017). What remains unclear is how *B. acidifaciens* and *P. buccalis* induce maternal IgA production; clearly both were antigenic given they were subsequently recognized by maternal IgA in milk. Given that both *B. acidifaciens* and *P. buccalis* are rarely detected in PPs, they could be processed immediately by antigen-presenting cells such as dendritic cells, without colonization, after being sampled through M cells. In this regard, *B. acidifaciens* can stimulate dendritic cells to induce the expression of interleukin-6 (IL-6) and IL-10 (Tsuda et al., 2007), both of which may facilitate the differentiation of B cells into IgA plasma cells resident in the mammary glands.

Here, we reveal that host immune cells, especially lymphocytes, are essential for *B. acidifaciens* and *P. buccalis* to colonize the GI tract, given they were absent from immunodeficient SCID mice lacking T and B cells, even during lactation, but formed a niche to become a member of the intestinal microbiota after allogenic BMT from immunocompetent donors. Ongoing studies are required to identify the immune factors responsible for promoting the colonization of bacteria such as these before they subsequently elicit the secretion of targeted IgA in milk. One hypothesis is that PP-derived IgA may promote a symbiotic relationship with beneficial microorganisms, not only in the maternal gut but also in offspring that ingest the maternal IgA through milk (Donaldson et al., 2018; Rogier et al., 2014). Since SCID mice develop type 3 innate lymphoid cells (ILC3s), including lymphoid tissue inducer (LTi) cells that initiate the prenatal organogenesis of PPs (Fu and Chaplin, 1999), they could form intestinal PP-like structures comprising both T and B cells, even after postnatal BMT. Subsequently, *B. acidifaciens* and *P. buccalis* could colonize the GI tract of BMT-treated SCID mice once sufficient intestinal IgA was available. In this same way, *Bacteroides fragilis* has been reported to use intestinal IgA specific for itself to colonize the GI tract of mice to become a dominant species (Donaldson et al., 2018). Furthermore, the intestinal microbiome in newborns is altered dramatically when there is no intake of maternal IgA (Rogier et al., 2014). Although there has been ongoing debate over the mechanism by which commensal microflora develop in the GI tract (Donaldson et al., 2018), our data support the notion that PP-derived IgA may be critical for maintaining beneficial microorganisms in the GI tract across generations, both in the dam and in her milk-fed offspring.

Our findings also provide important considerations and insights for using mucosal vaccination to induce local IgA production to target a range of pathogens, including those affecting neonates of all species, including human infants. There is already widespread use of maternal immunization by injection to induce high concentrations of circulating IgG that are transferred into colostrum in various livestock species (Hurley and Theil, 2011). By contrast, humans only transfer IgG to the offspring via the placenta, as occurs for vaccines such as the inactivated Tdap raised against tetanus, diphtheria, and pertussis (Centers for Disease Control and Prevention, 2011; Maertens et al., 2016; Skoff et al., 2017). The production of mostly IgA in colostrum and milk of humans presents a unique opportunity to target GI pathogens in offspring through the long-term transfer of antigen-specific IgA in milk after maternal mucosal vaccination (Van de Perre, 2003). In fact, this strategy has proven effective in pigs, where mucosal vaccination by oral delivery of live attenuated PEDV led to complete protection of nursing piglets due to locally produced maternal IgA transferred in milk (Jang et al., 2019). Furthermore, manipulating the maternal microbiome could alter the profile of IgA transferred in milk. Of relevance to this proposal is the demonstration that supplementing pregnant and lactating mothers with lactic acid bacteria (LAB) or LAB plus *Bifidobacterium* reduced the risk of their children developing allergies (Rautava et al., 2002, 2012). Since one of the obstacles for oral vaccine development is the processing of antigens in PPs leading to their effective delivery to M cells, modifying the

n = 8; PP null: *B. acidifaciens*, n = 7; *P. buccalis*, n = 7; H: PP intact: none, n = 24; *P. goldsteinii*, n = 24; *B. acidifaciens*, n = 24; *P. buccalis*, n = 24; PP null: *B. acidifaciens*, n = 24; *P. buccalis*, n = 24).

(I) The IgA in the milk of PP-intact (but not PP-null) dams undergoing CBI was reactive to the specific bacterial inoculate (PP intact: none, n = 19; *P. goldsteinii*, n = 19; *B. acidifaciens*, n = 19; *P. buccalis*, n = 19; PP null: *B. acidifaciens*, n = 24; *P. buccalis*, n = 24).

Data are presented as mean ± SD. **p < 0.01, ***p < 0.001, and ****p < 0.0001 by multiple comparison testing in PP-intact mice and by t test in PP-null mice (E, G, H, and I). †p < 0.05, †††p < 0.001, and ††††p < 0.0001 by t test between PP-intact and PP-null mice dosed with certain bacterium (E, G, H, and I).

genome of bacteria such as *B. acidifaciens* and *P. buccalis* to encode vaccine antigens might serve to enhance targeting to PPs. Alternatively, bacteria such as *B. acidifaciens* and *P. buccalis* could be used as prebiotics alongside genetically modified vaccine strains to increase the efficacy of inducing antigen-specific maternal IgA produced into milk.

In conclusion, PPs clearly play a crucial role in the health of subsequent generations by inducing maternal IgA production in milk while also cooperatively interacting with resident microorganisms in the maternal GI tract. These insights, which demonstrate a microbiota-PP-mammary gland pathway in maternal IgA production in milk, not only stand to refine approaches to enhance lactational immunity in a range of species but also provide a clearer understanding of the complex interrelationships between intestinal immunology and microbiology.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2021.109655.

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AUTHOR CONTRIBUTIONS

K.U. and T.N. designed the study. K.U., K.N., A.M., Y. Suyama, Y. Sakai, S.S., S.U., M.F., J.I., K.I., T.M., Y.K., M.T., and T.N. performed the research and analyzed the data. K.U. and T.N. wrote the paper, and all authors commented on it. K.F., H. Kiyono, R.C.H., J.S., H.Y., H. Kitazawa, K.W., and H.A. assisted in the writing and discussion.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCE TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Donkey ani-rat IgG Alexa647 (polyclonal)	Jackson ImmunoResearch Laboratories	Cat. 712-605-153
Donkey anti-rabbit IgG HRP (polyclonal)	Jackson ImmunoResearch Laboratories	Cat. 711-035-152
Donkey anti-sheep IgG Alexa488 (polyclonal)	Jackson ImmunoResearch Laboratories	Cat. 713-546-147
Goat anti-mouse IgA (polyclonal)	Bethyl Laboratries	Cat. A90-103A
Goat anti-mouse IgA HRP (polyclonal)	Bethyl Laboratries	Cat. A90-103P
Hamster anti-mouse CD3 APC (145-2C11)	BD Biosciences	Cat. 553066
Hamster IgG1 APC Isotype Control (A19-3)	BD Biosciences	Cat. 553974
Mouse anti-mouse CCR9 PE (CW-1.2)	Biolegend	Cat. 128710
Mouse anti-mouse I-Ad Alexa647 (39-10-8)	Biolegend	Cat. 115010
Mouse anti-PE MicroBeads UltraPure	Miltenyi Biotec	Cat. 130-105-639
Mouse IgG2a PE Isotype Control (MOPC-173)	Biolegend	Cat. 400212
Mouse IgG3 Alexa647 Isotype Control (MG3-35)	Biolegend	Cat. 401322
Sheep anti-mouse Spi-B (polyclonal)	R&D Systems	Cat. AF7204
Rabbit anti-CD3 (SP7)	Abcam	Cat. ab16669
Rat anti-mouse/rat CCR10 (248918)	R&D Systems	Cat. FAB2815A
Rat anti-mouse CD11b BV510 (M1/70)	BD Biosciences	Cat. 562950
Rat anti-mouse CD16/32 (2.4G2)	BD Biosciences	Cat. 553141
Rat anti-mouse CD45 BV711 (30-F11)	BD Biosciences	Cat. 563709
Rat anti-mouse CD45R (RA3-6B2)	BD Biosciences	Cat. 550286
Rat anti-mouse CD45R/B220 BV421 (RA3-6B2)	BD Biosciences	Cat. 562922
Rat anti-mouse CD93 PECy7 (AA4.1)	Biolegend	Cat. 136506
Rat anti-mouse IgA BV421 (C10-1)	BD Biosciences	Cat. 743293
Rat anti-mouse IgA FITC (C10-3)	BD Biosciences	Cat. 559354
Rat anti-mouse IgA PE (mA-6E1)	Invitrogen	Cat. 12-4204-82
Rat anti-mouse IL7Rα (A7R34)	Bio X cell	Cat. BE0065
Rat anti-mouse Ki67 PE (16A8)	Biolegend	Cat. 652404
Rat anti-mouse Ly6C PE (HK1.4)	Biolegend	Cat. 128008
Rat anti-mouse/human CD45R/B220 APC (RA3-6B2)	Biolegend	Cat. 103212
Rat IgG1 BV421 Isotype Control (R3-34)	BD Biosciences	Cat. 562868
Rat IgG1 FITC Isotype Control (R3-34)	BD Biosciences	Cat. 553924
Rat IgG1 PE Isotype Control (eBRG1)	Invitrogen	Cat. 12-4301-82
Rat IgG2a APC Isotype Control (RTK2758)	Biolegend	Cat. 400512
Rat IgG2a BV421 Isotype Control (R35-95)	BD Biosciences	Cat. 562602
Rat IgG2a PE Isotype Control (RTK2758)	Biolegend	Cat. 400508
Rat IgG2a APC Isotype Control (54447)	R&D Systems	Cat. IC006A
Rat IgG2b BV510 Isotype Control (R35-38)	BD Biosciences	Cat. 562951
Rat IgG2b PECy7 Isotype Control (RTK4530)	Biolegend	Cat. 400618
Rat IgG2c PE Isotype Control (RTK4174)	Biolegend	Cat. 400707
Bacteria Strains		
Bacteroides acidifaciens	RIKEN BioResource	JCM10556
Parabacteroides goldsteinii	RIKEN BioResource	JCM13446
Prevotella buccalis	RIKEN BioResource	JCM12246

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Commercial Assay		
Agencourt AMPure XP	Beckman Coulter	Cat. A63881
Bacteria (tuf gene) Quantitative PCR Kit	Takara-bio	Cat. RR240A
Library Quantification Kit	Takara-bio	Cat. Z8324N
Miseq Reagent kit v2 (500 cycle)	Illumina	Cat. MS-102-2003
PhiX Seqencing Control v3	Illumina	Cat. FC-110-3001
Pierce BCA protein assay kit	Thermo Fisher Scientific	Cat. 23225
PrimeScript RT reagent kit	Takara-bio	Cat. RR037A
PrimeSTAR HS DNA polymerase	Takara-bio	Cat. R010A
PrimeSTAR GXL DNA Polymerase	Takara-bio	Cat. R050B
QIAamp Fast DNA Stool Mini Kit	QIAGEN	Cat. 51604
ReliaPrep RNA tissue miniprep system	Promega	Cat. Z6111
Stool DNA Isolation Kit	Norgen	Cat. 27600
TB Green Premix Ex TaqII	Takara-bio	Cat. RR820A
Tetramethylbenzidine microwell peroxidase substrate system	SeraCare Life Sciences	Cat. 5120-0050
Experimental model		
Mouse: B6N.Cg-Tg(Vil-cre)997Gum/J mice	Jackson Laboratory	N/A
Mouse: B6-Tg(CAG-FLPe)36 mice	RIKEN	N/A
Mouse: BALB/cCrSlc mice	SLC	N/A
Mouse: C.B-17/Icr-+/+Jcr mice	CLEA	N/A
Mouse: C.B-17/Icr-*scid*/*scid*Jcr mice	CLEA	N/A
Mouse: *Ccr10*$^{EGFP/EGFP}$ mice	Dr. Bao Lu, Harvard Medical School	N/A
Mouse: C57BL/6NCrSlc mice	SLC	N/A
Mouse: germ-free BALB/c mice	Sankyo Labo Service	N/A
Mouse: *Ltbr*$^{-/-}$ mice	Dr. Mitsuru Matsumoto, The University of Tokushima	N/A
Oligonucleotides		
Primer: *Ccl28* (Forward)	Takara-bio	N/A
Primer: *Ccl28* (Reverse	Takara-bio	N/A
Primer: *Csn1s1* (Forward)	Takara-bio	N/A
Primer: *Csn1s1* (Reverse)	Takara-bio	N/A
Primer: *Csn2* (Forward)	Takara-bio	N/A
Primer: *Csn2* (Reverse)	Takara-bio	N/A
Primer: *Csn3* (Forward)	Takara-bio	N/A
Primer: *Csn3* (Reverse)	Takara-bio	N/A
Primer: floxed (Forward)	Hokkaido System Science	N/A
Primer: floxed (Reverse)	Hokkaido System Science	N/A
Primer: Neo insertion (Forward)	Hokkaido System Science	N/A
Primer: Neo insertion (Reverse)	Hokkaido System Science	N/A
Primer: proximal-floxed and Neo deletion (Forward)	Hokkaido System Science	N/A
Primer: proximal-floxed and Neo deletion (Reverse)	Hokkaido System Science	N/A
Primer: Southern blot probe (Forward)	Hokkaido System Science	N/A
Primer: Southern blot probe (Reverse)	Hokkaido System Science	N/A
Primer: V3 and V4 region of bacterial 16S rRNA (Forward)	Nihon Gene Research Laboratories	N/A
Primer: V3 and V4 region of bacterial 16S rRNA (Reverse)	Nihon Gene Research Laboratories	N/A
Primer: 2nd PCR (Forward)	Eurofins Genomics	N/A
Primer: 2nd PCR (Reverse)	Eurofins Genomics	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
Attune™ NxT Software	Thermo Fisher Scientific	Version 2.7
FlowJo	BD	Version 10
LEfSe	The Huttenhower Lab	Version 3.1
Python	Python Software Foundation	Version 3.7.8
Prism	GraphPad	Version 9
QIIME2	QIIME 2 development team	Version 2020.2
Rstudio	Bioconductor	Version 1.4.1106
Chemicals		
0.5M EDTA (pH8.0)	Nippon gene	Cat. 311-90075
Ampicillin	Wako	Cat. 014-23302
β-mercaptoethanol	Nakalai Tesque	Cat. 21438-82
Bovine serum albumin	Sigma-Aldrich	Cat. A3059
Carbonate bi Carbonate	Sigma-Aldrich	Cat. C3041
Collagenase from Clostridium histolyticum Type III	Sigma-Aldrich	Cat. C2139
Donkey serum	Jackson ImmunoResearch Laboratories	Cat. 713-546-147
Eosin Y	Wako	Cat. 058-00062
Target retrieval solution	Dako	Cat. S1699
DAPI solution	Dojindo	Cat. 340-07971
Deoxyribonuclease I from bovine pancreas	Sigma-Aldrich	Cat. D4513
GAM Broth	Nissui	Cat. 05422
Hematoxylin	Sigma-Aldrich	Cat. 104302
Mouse reference serum	Bethyl Laboratries	Cat. RS10-101
Neomycin	Wako	Cat. 146-08871
Paraformaldehyde	Nakalai Tesque	Cat. 30525-89-4
Rat serum	Sigma-Aldrich	Cat. R9759
Saponin	Nakalai Tesque	Cat. 30502-42
Sodium hydroxide solution	Sigma-Aldrich	Cat. 72068
SYTO 9 Green Fluorescent Nucleic Acid Stain	Thermo Fisher Scientific	Cat. S34854
TNB blocking reagent	PerkinElmer	Cat. FP1020
Turk's solution	Wako	Cat. 277-09491
Vancomycin	Wako	Cat. 222-01303
Via-Probe	BD Biosciences	Cat. 555815

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Tomonori Nochi (nochi@tohoku.ac.jp).

Materials availability

Transfer of materials requires a material transfer agreement (MTA) to be signed.

Data and code availability

All data reported in this paper will be shared by the lead contact upon request. This study did not generate code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

All mice were maintained in animal facilities at the Graduate School of Agricultural Science in Tohoku University or The Institute of Medical Science at The University of Tokyo. BALB/c (BALB/cCrSlc), C57BL/6 (C57BL/6NCrSlc), ICR (C.B-17/ICR-+/+Jcr), ICR with severe combined immunodeficiency (C.B-17/ICR-*scid*/*scid*Jcl), B6N.Cg-Tg(Vil-cre)997Gum/J, and germ-free (GF) BALB/c mice (female, 6 weeks old) were purchased from either Japan SLC, Inc., CLEA Japan, Inc., The Jackson Laboratory, or Sankyo Labo Service. B6-Tg(CAG-FLPe)36 and *Ltbr*$^{-/-}$ and *Ccr10*$^{EGFP/EGFP}$ mice were provided by Drs. Mitsuru Matsumoto and Bao Lu, respectively. BALB/c mice lacking Peyer's patches (PP) were generated by in utero administration of 1 mg of purified anti-mouse IL-7Rα antibody (A7R34) at embryonic day 14 as described elsewhere (Yoshida et al., 1999). Splenectomy and removal of the inguinal lymph node was performed surgically under anesthesia (0.3 mg/kg of medetomidine, 4 mg/kg of midazolam, and 5 mg/kg of butorphanol tartrate). To disturb the intestinal microflora, BALB/c mice were dosed with ampicillin (1 g/l), neomycin (1 g/l) or vancomycin (500 mg/l) alone or together via the drinking water to which mice had *ad libitum* access. Antibiotics were delivered orally from the time of mating to 14 days postpartum. In some experiments, fecal microbiota transplantation (FMT), small intestinal microbial transplantation (SIMT), and certain bacterial inoculation (CBI) were performed once daily from 8 to 14 days after parturition in PP-developing or PP-lacking BALB/c mice whose intestinal microflora was first disturbed by oral administration with three mixed antibiotics from mating to 7 days postpartum. Specifically, 3–4 pellets of fresh feces, obtained from either lactating BALB/c, C.B-17/ICR-+/+Jcr, or C.B-17/ICR-*scid*/*scid*Jcl mice, contents of small intestine obtained from BALB/c mice, or approximately 10^{10} cfu of *P. goldsteinii*, *B. acidifaciens*, or *P. buccalis,* were dissolved in 500 μL of PBS, and administered to each recipient mouse via oral gavage. Additionally, BALB/c mice were parenterally administered with 10^9 cfu of *P. goldsteinii* at 3 days and 10 days after parturition. In addition, 10^6 bone marrow-derived mononuclear cells obtained from C.B-17/ICR-+/+Jcr mice was used for bone marrow transplantation (BMT) into C.B-17/ICR-*scid*/*scid*Jcl recipient mice. All animal experiments were designed in accordance with the protocols approved by the institutional animal care and use committee of both Tohoku University and The University of Tokyo.

Generation of Spi-B conditional knockout mice

A construct was designed to flank exon 2 to 5 of the *Spib* gene with loxP sites and to insert an Frt-flanked neo-resistance cassette into the intron between exon 5 and 6. JM8A1.N3 embryonic stem cells, obtained from the KOMP Repository, were transformed using 30 μg of SalI-linearized targeting vector. Neomycin- and ganciclovir-resistant embryonic stem cell colonies were selected and screened by PCR and Southern blot using the following primer sets: for distal-floxed, primer 1: 5′-TAGTTCATTGCTGCTCCTAGTCT CAGC-3′ and primer 2: 5′-TGGGATTTGCATGCACAGACGCTGGGG-3′; for Neo insertion, primer 4: 5′-CTAAAGCGCATGCTCCA GACTGCCTTG-3′ and primer 5: 5′-GAGGCAAGAGGACTGCCACTAGCTTGAG-3′; for proximal-floxed and Neo deletion, primer 3: 5′-CTTATTTATACTCCCGCGCTCACTGGAGG-3′ and primer 5; for exon deletion, primer 1 and primer 5; for Southern blot probes, forward: ^{32}P-labeled 5′-GGTAGACAGACTTCTAGACTTGGTCAACCC-3′ and reverse: ^{32}P-labeled 5′-GTTTAGCCTTGACCACATG GACATCTGG-3′. Selected homologous recombinants were used for microinjection into the blastocysts of C57BL/6 mice to generate chimeric mice. To delete the neo-resistance cassette and to obtain floxed-*Spib* mice (*Spib*$^{flox/flox}$), heterozygous F1 mice were mated with B6-Tg(CAG-FLPe)36 mice. *Spib*$^{flox/flox}$ mice were then mated with B6N.Cg-Tg(Vil-cre)997Gum/J mice to generate M-cell-deficient mice as the result of Spi-B deletion in intestinal epithelial cells (IEC).

Bacterial strains

Parabacteroides goldsteinii (JCM13446), *Bacteroides acidifaciens* (JCM10556), and *Prevotella buccalis* (JCM12246) were obtained from The Japan Collection of Microorganisms at RIKEN BioResource Research Center. These bacteria were cultured overnight in GAM culture medium under anaerobic conditions created using Anaeropacks, and plated on GAM-Agar plates to quantify colony-forming units prior to their use for certain bacterial inoculation (CBI).

METHOD DETAILS

Cell isolation

Mononuclear cells were isolated from the mammary glands, Peyer's patches (PP), small intestine (one-third in the middle), large intestine, inguinal lymph nodes (ILN), spleen, and bone marrow. Specifically, the mammary glands and PP were digested with 0.5 mg/ml of collagenase for 60 min at 37°C. The small and large intestines were first cut into 2- to 3-cm pieces and stirred in 1 mM of EDTA for 25 min at 37°C to remove the epithelial cell layer. The tissues were then treated with 0.5 mg/ml of collagenase, 5 U/ml of DNase I, and 50 μM of β-mercaptoethanol for 75 min at 37°C to isolate cells present in the intestinal lamina propria. The ILN and spleen were processed mechanically to collect cells. All cells obtained were counted by staining with Turk's solution and subjected to flow cytometry. Cells isolated from the bone marrow of femurs were harvested with PBS using a syringe prior to adaptive transfer.

Flow cytometry

Mononuclear cells isolated from each tissue were first blocked with 5 μg/ml of anti-mouse CD16/32 (2.4G2) for 15 min at 4°C and then stained with appropriate combinations of the following antibodies: 2 μg/ml of PE anti-mouse CCR9 (CW-1.2), 0.2 μg/ml of

PE anti-mouse Ly6C (HK1.4), 2 μg/ml of PE-Cy7 anti-mouse CD93 (AA4.1), 10μl/test of APC anti-mouse CCR10 (248918), 2 μg/ml of APC anti-mouse CD3 (145-2C11), 2 μg/ml of APC anti-mouse/human CD45R/B220 (RA3-6B2), 5 μg/ml of APC anti-mouse I-Ab (AF6-120.1), 5 μg/ml of Alexa 647 anti-mouse I-Ad (39-10-8), 2 μg/ml of BV421 anti-mouse CD45R/B220 (RA3-6B2), 2 μg/ml of BV510 anti-mouse CD11b (M1/70), and 2 μg/ml of BV711 anti-mouse CD45 (30-F11) for 30 min at 4°C. Alternatively, 2 μg/ml of PE mouse IgG2a (MOPC-173), 2 μg/ml of APC hamster IgG1 (A19-3), 5 μg/ml of Alexa 647 mouse IgG3 (MG3-35), 2 μg/ml of APC rat IgG2a (RTK2758), 10 μl/test of APC rat IgG2a (54447), 2 μg/ml of BV421 rat IgG2a (R35-95), 2 μg/ml of BV510 rat IgG2a (R35-38), 2 μg/ml of PE-Cy7 rat IgG2b (RTK4530), and 2 μg/ml of PE rat IgG2c (RTK4174) were used as isotype controls. Cell Viability Solution (10ul/test) was added to the antibody cocktails to exclude dead cells from the analyses. For intracellular staining, the cells were then fixed in 4% (w/v) paraformaldehyde for 20 min at room temperature (RT), treated with 0.1% (w/v) saponin for 15 min at RT, and stained with either 5 μg/ml of FITC anti-mouse IgA (C10-3), 2 μg/ml of BV421 anti-mouse IgA (C10-1), or 2 μg/ml of PE anti-mouse Ki67 (16A8) for 30 min at RT. Alternatively, 2 μg/ml of PE rat IgG2a (PTK2758), 5 μg/ml of FITC rat IgG1 (R3-34), and 2 μg/ml of BV421 rat IgG1 (R3-34) were used as isotype controls. Flow cytometry analysis was performed using either an Attune™ NxT Acoustic Focusing Cytometer (Thermo Fisher Science) or an Accuri™ C6 flow cytometer (BD Bioscience).

ELISA

The level of IgA in feces or milk collected from the stomach contents of mouse pups was measured by ELISA. Both sample types were suspended in PBS (10 μL per mg), and the supernatants were collected by centrifugation. Next, 96-well ELISA plates were coated with 100 μg/ml of anti-mouse IgA overnight at 4°C. After blocking with 1% (w/v) BSA for 1 h at RT, two serially-diluted fecal or milk samples were incubated in the plates for 2 h at RT. After washing, the plates were treated with 100 ng/ml of HRP anti-mouse IgA for 1 h at RT, and the signals developed using a tetramethylbenzidine microwell peroxidase substrate system. Mouse reference serum that contains IgA at a known concentration was used as a standard. The reactivity of IgA in milk to fecal contents, consisting of intestinal microorganisms or certain bacterium (i.e., *P. goldsteinii*, *B. acidifaciens*, and *P. buccalis*), was also determined by ELISA. In brief, plates were coated overnight at 4°C with either fecal suspension (100 μg/ml in PBS, filtered at 100 μm), or 100 μg/ml of certain bacterium. After blocking with BSA (1%, w/v) and 1 μg/ml of anti-mouse IgA (when reactivity to fecal content was tested) for 1 h at RT, milk (undiluted or diluted 1:64) or serum samples (diluted 1:16, 32, 64, and 128) were reacted on the plates for 2 h at RT. After washing, the plates were treated with HRP anti-mouse IgA, IgG, or IgM (100 ng/ml) for 1 h at RT, and the signal developed using a tetramethylbenzidine microwell peroxidase substrate system. The titer of milk IgA to intestinal microorganisms or certain bacterium was described as the OD_{450}.

IgA-Seq

Intestinal microorganisms recognized by IgA in milk were identified by IgA-Seq analysis as described (Palm et al., 2014) with some modifications. In brief, fresh feces was suspended in PBS and filtered using cell strainers (40 μm) to remove debris. Fecal suspension containing 0.1 mg of feces was blocked with 50 μg of anti-mouse IgA, 10% (v/v) normal rat serum, and 0.5% (w/v) BSA for 30 min at 4°C. This fecal suspension was then mixed with milk that had been prediluted in PBS (10 μL per mg) at a ratio of 1:1 for 30 min at 4°C. After washing, the suspension was treated for 30 min at 4°C with 2 μg/ml of PE anti-mouse IgA (mA-6E1) and 500 nM of SYTO 9 followed by anti-PE MicroBeads UltraPure (30 μl/test) for 30 min at 4°C. IgA-coated microorganisms were collected using an AutoMACS magnetic cell sorter (Miltenyi Biotec), and genomic DNA was extracted using a Stool DNA Isolation Kit.

Metagenomics analysis

Genomic DNA obtained from IgA-Seq or directly extracted from feces using a QIAamp DNA Stool Mini Kit was subjected to metagenomics analysis as described previously (Nochi et al., 2020). Specifically, the V3 and V4 regions of the bacterial 16S ribosomal RNA gene were amplified by PCR using PrimeSTAR HS DNA polymerase and the following primers: forward primer mixed (5′-TGCTCTTCCGATCTGACNNNCCTACGGGNGGCWGCAG-3′, 5′-TGCTCTTCCGATCTGACNNNNCCTACGGGNGGCWGCAG-3′, 5′-TGCTCTTCCGATCTGACNNNNNCCTACGGGNGGCWGCAG-3′, 5′-TGCTCTTCCGATCTGACNNNNNNCCTACGGGNGGCWGCAG-3′) and reverse primers mixed (5′- CGCTCTTCCGATCTCTGNNNGACTACHVGGGTATCTAATCC-3′, 5′-CGCTCTTCCGATCTCTGNNNNGACTACHVGGGTATCTAATCC-3′, 5′-CGCTCTTCCGATCTCTGNNNNNGACTACHVGGGTATCTAATCC-3′, 5′-CGCTCTTCCGATCTCTGNNNNNNGACTACHVGGGTATCTAATCC-3′). The adaptor tag sequence is singly underlined, and the spacer sequences are double underlined. The PCR fragments obtained from the first round of PCR were purified using AMPure XP and then amplified in a second round of PCR using the following primers: forward (5′-CAAGCAGAAGACGGCATACGAGATxxxxxxxxxGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGAC-3′) and reverse (5′-AATGATACGGCGACCACCGAGATCTACACxxxxxACACTCTTTCCCTACACGACGCTCTTCCGATCTCTG-3′), including nine and five base indices, shown as "xxxxxxxxx" and "xxxxx," to distinguish each sample. All PCR products were then sequenced using the MiSeq platform (Illumina) with the MiSeq reagent kit v2 (500 cycles). Demultiplexed sequences were attained from the Illumina BaseSpace, and they were further investigated using the Quantitative Insights into Microbial Ecology 2 (QIIME 2, version 2020.2) (Bolyen et al., 2019). The sequences were filtered, denoised, and merged for quality control using the DADA2 pipeline (*q2-dada2* plugin) available in QIIME2 to cluster operational taxonomic units (OTUs) with default parameters. Later, taxonomy was assigned using a pre-trained Naive Bayes classifier and the *q2-feature-classifier* plugin against the Greengenes 13_8 99% OTUs (https://greengenes.lbl.gov/) (McDonald et al., 2012). The data were also analyzed by LEfSe to identify bacterial species from each sequencing run. A principal component analysis was also conducted using the statistical

program Python to compare intestinal microflora. Alpha diversity analysis of microbial abundance data was performed by three different approaches (i.e., Chao1, Sannon, Simpson) using the "vegan" (version 2.5-6) R package (https://cran.r-project.org/web/packages/vegan/index.html).

Quantitative RT-PCR

Total RNA was extracted from the mammary glands using a ReliaPrep RNA Tissue Miniprep System, and cDNA was synthesized by reverse transcription using the PrimeScript RT reagent kit with oligo (dT) primers and random hexamers. Quantitative PCR was performed using TB Green *Premix Ex TaqII* to quantify the expression levels of mRNA coding for *Ccl28*, *Csn1s1* (α-S1-casein), *Csn2* (β-casein), *Csn3* (κ-casein), and *Gapdh* (Glyceraldehyde-3-phosphate dehydrogenase: GAPDH). Gene expression levels were normalized to those for GAPDH. All primers were designed using the Takara perfect real-time support system. The number of copies of the bacteria-specific gene *tuf* in genomic DNA extracted from feces and IgA-coated bacteria was measured using a bacteria (*tuf* gene) quantitative PCR kit.

Histochemistry

Samples of small intestine obtained from BMT studies were fixed in 4% (w/v) paraformaldehyde and embedded in paraffin. Tissue sections (5 μm) were blocked with Tris-NaCl-Blocking buffer (TNB) for 30 min at RT and stained overnight 4°C with PE anti-mouse CD45R/B220 (RA3-6B2) and purified anti-human CD3 (SP2) that cross-reacts with murine CD3. After washing, the tissue sections were incubated with HRP anti-rabbit IgG for 1 h at RT before being subjected to the Tyramide Signal Amplification (TSA) Plus fluorescein system for 10 min at RT to amplify the CD3 signal. Sections were counterstained with 1 μg/ml of DAPI. For histopathological analysis, the tissue sections were stained with hematoxylin and eosin. The images were obtained using either BZ-9000 (Keyence) or BX63 (Olympus).

QUANTIFICATION AND STATISTICAL ANALYSIS

Values are presented as mean ± standard deviation (SD). N = number of animals (Figures 1C, 1G, 1K, 1O, 2B, 2E, 2G, 3B, 3E, 3G, 4B, 4F, 4I, 4J, 5B, 5E, 5G, 6A, 6D, 7B, 7E, 7G, S1D, S1E, S1F, S2B, S2F, S2G, S3F, S3H, S3I, S4B, S4F, S4H, S5B, S5C, S5F, S5H, S6B, S6D, S6F, S6H, S6I, S7C, S7E, S8B, S8D, and S8E) or number of milk samples (Figures 1A, 1D, 1H, 1L, 1P, 2H, 3H, 4A, 5H, 7H, 7I, S2C, S5G, and S7F). Statistical analyses were performed by t test in Figures 1K, 1L, 1O, 1P, 3B, 3E, 3G, 3H, 5B, 5E, 5G, 5H, 6A, 6D, 7B, 7E, 7G–7I, S2B, S2C, S2E, S3F, S3H, S3I, S5B, S5C, S5F–S5H, S6H, S6I, S7C, S7E, S7F, S8B, and S8E, and multiple comparison test in Figures 1C, 1D, 1G, 1H, 2B, 2E, 2G, 2H, 4A, 4B, 4F, 4I, 4J, 7B, 7E, 7G, 7H, 7I, S1D–S1F, S2F, S4B, S4F, S4H, S6B, S6D, S6F, S8B, S8D, and S8E using Prism 7 (GraphPad). Principal component analyses were performed using the statistical program Python in Figures 2D, 3D, 4H, 5D, 6C, and 7D. All statistical details can be found in the figure legends while *p* values are shown in the figures.