Lactobacillus maintains healthy gut mucosa by producing L-Ornithine

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Gut mucosal layers are crucial in maintaining the gut barrier function. Gut microbiota regulate homeostasis of gut mucosal layer via gut immune cells such as ROR\textsubscript{yt} (+) IL-22(+) ILC3 cells, which can influence the proliferation of mucosal cells and the production of mucin. However, it is unclear how gut microbiota execute this regulation. Here we show that lactobacilli promote gut mucosal formation by producing L-Ornithine from arginine. L-Ornithine increases the level of aryl hydrocarbon receptor ligand L-kyurenine produced from tryptophan metabolism in gut epithelial cells, which in turn increases ROR\textsubscript{yt} (+)IL-22(+) ILC3 cells. Human REG3A transgenic mice show an increased proportion of L-Ornithine producing lactobacilli in the gut contents, suggesting that gut epithelial REG3A favors the expansion of L-Ornithine producing lactobacilli. Our study implicates the importance of a crosstalk between arginine metabolism in Lactobacilli and tryptophan metabolism in gut epithelial cells in maintaining gut barrier.
Gut mucous layers play a crucial barrier role in both separating the host from the noxious external environment and inhibiting the entrance of gut microbiota and/or their metabolites into the bloodstream and tissues. The small intestine has one layer of unattached mucus to directly form a soluble mucous gel, which may act as a matrix to limit the contact of gut microbiota with gut cell surface. The colonic mucous layer forms a physical barrier against bacteria and their metabolites. Although gut mucus layers are vitally important for individual health, the mechanism(s) underlying the maintenance of gut mucosal homeostasis is not completely clear.

Gut mucus layer consists of high-molecular-weight glycoproteins called mucus, that are synthesized and secreted by goblet cells. Goblet cells originate by their own mitosis or by differentiation of stem cells, which may be regulated by gut immune cells through the production of cytokines, such as IL-6 or direct cell–cell contact by activated macrophages. IL-22 produced by innate lymphoid cells group 3 and other immune cells such as Th17, Th22, natural killer cells, y6 T cells, and lymphoid tissue inducer can also promote the production of gut epithelial stem cells, which potentially increase mucus production through goblet cells. These immune cell responses are dictated not only via “endogenous” host-derived but also “exogenous” signals, such as gut microbiota/metabolites. Indeed, gut microbiota may not only regulate gut innate immune but also adaptive immune cells, such as that L. reuteri has a role in IL-22 production, and segmented filamentous bacteria may induce Th17 cell differentiation. The products of bacteria may also interrupt T-cell homeostasis, we generated human REG3Atg mice by injecting IL-22-neutralizing antibodies. Reduced mucus gel in the ileum tissues and thinning of mucus layers may be derived from the decreased goblet cells (Fig. 1f). Interestingly, mucus layers in the proximal colon tissues of REG3Atg mice were also markedly thicker, as compared with their control cohoused littermates (Fig. 1g). The thickened mucus layer in the colon tissues may be derived from the expression of REG3A in colon Paneth cell-like cells and/or the secreted REG3A by intestinal Paneth cells. Higher levels of mucin 2 were detected in proximal colon tissues of human REG3A mice (Fig. 1h). Ki67 cells in the colon crypt also remarkably increased in these REG3A mice (Fig. 1i). The Cdkn1a (p21) and Cdkn2d (p19) were downregulated in the colonic epithelial cells (Fig. 1f). The REG3A mice also conferred a marked resistance to DSS-mediated colitis (Fig. 1i–n). Levels of serum LPS were lower in DSS-treated human REG3A mice (Fig. 1o). The bacterium numbers in the organs and tissues, such as the spleen of DSS-treated REG3A mice, were much less than wt control littermates (Fig. 1p). Furthermore, there had been much more goblet cells and Ki67 cells with upregulated Clec3, Retnib, and Tjf2 and downregulated Cdkn1a and Cdkn2d in the colon crypt of DSS-treated human REG3A mice (Supplementary Fig. 2a–d). Taken together, these data indicate that REG3A is involved in the maintenance of gut mucosal homeostasis through modulating gut epithelial regeneration and repair.

**REG3A-mediated formation of gut mucus layers is dependent on ILC3.** Gut immune cells may influence mucosal cell proliferation through the direct cell–cell contact or the production of cytokines, such as IL-22. We thus assessed IL-22-associated gut immune cell population and subpopulations according to the described gated strategy (Supplementary Fig. 3). IL-22(+) cells include innate lymphocyte cell 3 (ILC3), CD4(+)T17, and CD4(+)T22 cells in gut tissues. We found that the increased IL-22(+) cells in human REG3A mice mainly belonged to CD4(−) IL-22(+) cells but not CD4(+)IL17(+) (Fig. 2a), implying that these cells may be ILC3 cells. ILC3 cells are RORyt-positive cells and constitute at least two bona fide subsets NCR(+) ILC3 expressing NKP46 and LTI-like ILC3, which includes CD4(+) and CD4(−) subsets. The increased ILC3 cells in the ileum and colon of human REG3A mice were CD45(+)lin(−)RORyt(+) IL-22(+) NKP46(−) CD4(−) ILC3 cells (Fig. 2b, c), which may strongly produce IL-22. Increased CD45(+)lin(−)RORyt(+) IL-22(+) NKP46(−) CD4(−) ILC3 cells were also found in REG3a adenovirus-injected mice (Fig. 2d and Supplementary Fig. 1b, d, f). Higher levels of IL-22 in the ileum and colon tissues were detected in REG3A mice and REG3a/adenovirus- injected mice (Fig. 2e). All of these imply that CD45(+)lin(−)RORyt(+) IL-22(+)NKP46(−) CD4(−) ILC3 cells may be involved in REG3A/Reg3a-mediated formation of gut mucus layers. To further confirm the role of IL-22 in REG3A-mediated gut mucus layers, we treated REG3A mice by injecting IL-22-neutralizing antibodies. Reduced mucin gel in the ileum tissues and thinned mucus layers in the colon tissues of REG3A mice were observed after administering IL-22-neutralizing antibodies (Fig. 2f, g), indicating that REG3A-mediated mucus layers were dependent on IL-22. In addition, the proportion of CD11c(+)CD103(+)CD11b(+) dendritic cells, CD11b(+)Ly6C(+) myeloid-derived monocytes, and CD11b(+)F4/80(+) macrophages, which may be promoted by GM-CSF from RORyt(+) IL-22(+) ILC3 cells, also increased (Supplementary Fig. 4). Taken together, gut REG3A (Reg3a in mouse) promotes the formation of gut mucous layers in the small intestine and colon through RORyt(+) IL-22(+) ILC3 cells.

**Lactobacilli promote the accumulation of ILC3.** We next investigated whether the accumulation of CD45(+)lin(−)RORyt(+) IL-22(+)NKP46(−) CD4(−) ILC3 cells in human REG3A mice is dependent on the altered microbiota. We performed the
transplantation experiment of REG3A-shaped microbiota in pan-
antibiotic-treated WT mice. More RORγt(+)IL-22(+) cells increased mucus gel in the ileum, thickened mucus layers in the colon tissues, and increased goblet cells and Ki67 cells in the gut tissues were observed in REG3A tg feces-transplanted mice (Supplementary Fig. 5). We next analyzed the composition of gut microbiota and found that the proportion of lactobacilli was high in the ileum and colon of human REG3A tg mice, as compared with their control cohoused littermates (Fig. 3a–e). Although mouse Reg3 may kill some Gram-positive bacteria, Gram-positive
**Fig. 1** Gut human REG3A promotes the formation of gut mucus layers. a Fluorescence in situ hybridization of 16S rRNA and immunostaining of mucin in the ileum of human REG3A<sup>tg</sup> mice (REG3A<sup>tg</sup> and control cohoused littermate wt mice (ten slides/mouse; n = 6). b qRT-PCR of mucin 2 (MUC2) in the ileum of human REG3A<sup>tg</sup> and control cohoused littermate wt mice (n = 6). c Staining of goblet cells in the ileum of control cohoused littermate wt and human REG3A<sup>tg</sup> mice. Ten slides/mouse, n = 6; VCU, villus-crypt units. d qRT-PCR of Cica3, Retnlb, and Thi2 (n = 6). e Staining of Ki67 cells (ten slides/mouse, n = 6) and qRT-PCR of Cdkn1a and Cdkn2d (n = 6). f Crypt and transit-amplifying (TA) heights in the ileum of wt and human REG3A<sup>tg</sup> mice. Eighty wt (WT) versus 86 human REG3A<sup>tg</sup> (REG3A<sup>tg</sup>) transit-amplifying compartments; ten slides/mouse, n = 6. g Fluorescence in situ hybridization of 16S rRNA and immunostaining of mucin in the proximal colon of human REG3A<sup>tg</sup> mice (REG3A<sup>tg</sup>) and control cohoused littermate wt mice (ten slides/mouse; n = 6). h qRT-PCR of mucin 2 (MUC2) in the colon tissues (n = 6). i Staining of Ki67 cells in the colon (ten slides/mouse, n = 6) and qRT-PCR of Cdkn1a and Cdkn2d (n = 6). j, k Survival rate (j), body weight, and the disease activity index (DAI) (k) after DSS (n = 18). l Length of colon tissue. m qRT-PCR of TNFa, IL7, and IL-6 in the colon tissues after DSS (n = 6). n Hematoxylin/eosin staining and histological scores of distal colon samples after DSS. Scale bars = 40 μm. o LPS in the peripheral sera of REG3A<sup>tg</sup> and control cohoused littermate wt mice after DSS (n = 6). p Bacterium clones in the spleen after DSS (n = 6). Student’s t test, mean ± SD in b, d, and (RE), h, i (RE), and l, m, o, and p, mean ± SEM in e (ki67 cells). f, g, and i the Mann–Whitney U test in c and n, Wilcoxon’s test in j, k analysis of variance test in k, NS no significance; RE relative expression. Data are representative of three independent experiments. Also see Supplementary Figs. 1 and 2.

**lactobacilli** are not sensitive to Reg<sup>314,23</sup>. We further analyzed the composition of lactobacilli via in vitro culture and sequencing analyses, and found that increased lactobacillus in human REG3A<sup>tg</sup> mice was close to L. murinus isolates, which was named as L. NK2 (Fig. 3a, d and Supplementary Fig. 6a, b). We next employed germ-free (GF) mice to examine the effects of L. NK2 strain on RORγt (+) IL-22 (+) ILC3 cells and formation of gut mucus layers (Supplementary Fig. 6c). Infusion of L. NK2 caused increased mucus and accumulation of RORγt (+) IL-22 (+) ILC3 cells (Fig. 3f, g). Non-transplanted control GF mice housed under separated but similar conditions had less RORγt(+) cells in GF mice (Fig. 3f, g). Increased mucus and RORγt(+) accumulation in both the ileum and colon tissues were also observed in L. NK2 strain (Fig. 3c, d and Supplementary Fig. 6a, b). We next employed germ-free (GF) mice to examine the effects of L. NK2 strain on RORγt (+) IL-22 (+) ILC3 cells and formation of gut mucus layers (Supplementary Fig. 6c). Infusion of L. NK2 caused increased mucus and accumulation of RORγt (+) IL-22 (+) ILC3 cells (Fig. 3f, g). Non-transplanted control GF mice housed under separated but similar conditions had less RORγt(+) cells in GF mice (Fig. 3f, g). Increased mucus gel in the ileum, thickened mucus layers in the colon tissues, and increased goblet cells and Ki67 cells in the gut tissues were also observed in L. NK2 strain- transfected GF mice (Fig. 3h, i and Supplementary Fig. 6d–f). The increased RORγt (+) IL-22 (+) cells, thickened gut mucus, and increased Ki67 cells were also not found in germ-free REG3A<sup>tg</sup> mice (Fig. 3j–m). Thus, we demonstrate that lactobacillus L. NK2 alone promotes the accumulation of RORγt (+) IL-22(+) in gut tissues.

**Increased ILC3 is related to lactobacillus-induced L-Kyn.** We next determined how REG3A-associated lactobacillus was formed to cause the accumulation of CD45(+)(Lin(−))RORγt(+)IL-22(+) cells. Previous studies showed that AhR ligand indole-3-aldehyde (1Ald) from lactobacillus may contribute to AhR-dependent IL-22 transcription<sup>40</sup>. However, this was not the case for REG3A-associated lactobacillus (Supplementary Fig. 7). Studies have also shown that diverse host-derived signals can regulate and cause the accumulation of RORγt(+)IL-22(+) cells, such as AhR ligands derived/generated from host cells<sup>26,27</sup>, chemotaxis, and IL-23 by CXCR1(+) mononuclear phagocytes<sup>28</sup>. To investigate the factor(s) which is responsible for an increase in CD45(+)Lin(−)RORγt(+)IL-22(+) cells, we employed a microarray to compare the gene expression of gut epithelial cells and gut immune tissues (Payer’s patch node). We did not find RORγt(+) IL-22(+) cells associated with chemokines and/or IL-23 (GSE111111). Interestingly, L. NK2 colonization in GF mice induced at least a twofold change in the expression of multiple other genes in gut epithelial cells, typically indoleamine 2,3-dioxygenase 1 (IDO1) (Fig. 4a and GSE111111), which is a critical enzyme for tryptophan (Trp) metabolism to produce AhR ligands such as L-Kyn (Fig. 4b) <sup>26,27</sup>. qRT-PCR and immunoblotting also exhibited the higher expression of IDO1 in the gut epithelial tissues of lactobacillus-infused mice (Fig. 4c and Supplementary Fig. 11). Importantly, IDO1 was mainly expressed in gut epithelial cells in L. NK2-infused mice (Fig. 4d). Since Trp metabolites by IDO1 are primarily L-Kyn, more L-Kyn was detected in the gut epithelial cells of L. NK2-infused GF mice than control lactobacillus (Fig. 4e). L-Kyn in the gut epithelial cells of REG3A<sup>tg</sup> mice was higher (Fig. 4f). Trp metabolism-associated components such as IDO1 and p-Src<sup>29</sup> were also higher in REG3A<sup>tg</sup> mice (Fig. 4g–i and Supplementary Fig. 11). In vivo-administered L-Kyn caused the accumulation of RORγt(+) IL-22(+) cells in both the ileum and colon tissues (Fig. 4j, k). Thus, the AhR ligand L-Kyn produced by gut epithelial cells is responsible for lactobacillus-mediated RORγt(+) IL-22(+) cells.

**Lactobacillus-derived L-Orn is a critical factor for ILC3.** We next sought to address how lactobacillus regulates the expression of IDO1 in gut epithelial cells. In addition to cytokine-mediated activation, IDO1 signaling can also be triggered by metabolites such as L-Orn<sup>39</sup>; thus, we hypothesized that REG3A-associated lactobacillus might produce some metabolites to regulate the expression and activity of IDO1. Indeed, there was increased L-Orn in the gut contents of GF mice with L. NK2 colonization (Supplementary Fig. 8a). Increased L-Orn was further confirmed by ELISA in the ileum but also in the colon contents (Supplementary Fig. 8b). Higher levels of L-Orn were also detected in the contents of the ileum and colon of REG3A<sup>tg</sup> mice (Supplementary Fig. 8c). L-Orn may upregulate IDO1 in macrophages and dendritic cells<sup>30</sup>. When gut ileum and colon epithelial cells were exposed to different concentrations of L-Orn in vitro, L-Orn also upregulated the expression of IDO1 in these tissues (Supplementary Fig. 8d, e). Importantly, L-Orn-infused mice had a high level of IDO1 in their gut epithelial cells; whereas L-Orn inhibitor DFMO, which may inhibit L-Orn to putrescine<sup>29</sup>, suppressed the expression of IDO1 (Supplementary Fig. 8f, g and Fig. 5a). Notably, spermidine, a metabolite of L-Orn also induced the expression of IDO-1 (Supplementary Fig. 8h), implying that L-Orn-mediated IDO-1 expression may be through its metabolites. L-Kyn increased in the epithelial cells in L-Orn-infused mice, but DFMO caused reduced L-Kyn in REG3A<sup>tg</sup> mice (Fig. 5b). Thus, lactobacillus-derived L-Orn promotes the production of the AhR ligand L-Kyn in gut epithelial cells. Increased RORγt(+) IL-22(+) ILC3 cell populations and higher levels of IL-22 were also detected in the ileum and colon of L-Orn-infused mice (Fig. 5c, d). Conversely, L-Orn inhibitor (DFMO) decreased accumulation of RORγt(+) IL-22(+) cells in the gut tissues (Fig. 5c, d). Unsimilar to wt mice, IDO-1 KO mice did not exhibit the same responses to L-Orn (Fig. 5e). Administration of L-Orn also promoted mucin secretion, goblet cell production, and cell proliferation in wt mice. Conversely, L-Orn inhibitor-infused REG3A<sup>tg</sup> mice had reduced mucin secretion, goblet cell production, and cell proliferation (Fig. 5f–h). Thus, lactobacillus-derived
Fig. 2 REG3A-mediated gut mucus layers depend on RORγt (+) IL-22 (+) ILC3 cells. a Flow cytometry of CD4(+)IL22(+), CD4(-)IL-22(+), CD4(+)Th17 (+), and Th22(+)IL17(-) cells in the ileum lamina propria (LP) of human REG3A^9^ (REG3A) and control cohoused littermate wt mice (n=6). b, c, Flow cytometry of RORγt(-)IL22(+) cells and their subsets in the ileum (b) and colon (c) LP of wt and human REG3A^9^ mice (n=6). d Flow cytometry of RORγt(-)IL22(+) cells in the ileum LP of mice with (Reg3^–/–^/ad) or without (NC/ad) Reg3^–/–^ adenovirus injection (n=3). e QRT-PCR of IL-22 in the ileum and colon of human REG3A^9^ and control littermate wt mice (upper) and in the ileum (left) and colon (right) of mice with (Reg3^–/–^/ad) or without (NC/ad) Reg3^–/–^ adenovirus injection (lower). f, g Staining of ileum mucus (f) and colon tissues (g) in human REG3A^9^ or control littermate wt mice using IL-22-neutralizing antibody or control isotype antibody (Iso) (ten slides/mouse in g); scale bars = 40 µm. Student’s t test, mean ± SD in a–e, mean ± SEM in g. n = 6; NS no significance; RERelative expression; data are representative of three independent experiments. Also see Supplementary Figs. 3, 4.
L-Orn is a critical factor for lactobacillus-mediated RORγt(+)IL-22(+) cells and gut mucus formation.

**L-OCT deficiency impede the effect of lactobacillus.** L-Orn may be derived from Arg metabolism through the ADI pathway in lactobacillus (Supplementary Fig. 9a)\(^1\). Indeed, L. NK2 produced L-Orn through Arg metabolism, but L. NK2 and L. reuteri more effectively used Arg to produce L-Orn than L. NK1 (Supplementary Fig. 9b). The ADI pathway comprises three reactions catalyzed by Arg deminase (ADI; EC3.5.3.6), ornithine...
Discussion

We found that *lactobacillus* may promote the homeostasis of gut mucus layer through producing L-Orn. L-Orn stimulates Trp metabolism to produce AhR ligands in gut epithelial cells, which induce accumulation of ROtyt (+) IL-22 (+) ILC3 cells in gut tissues. We demonstrate that the proportion of L-Orn-producing *lactobacillus* in the gut contents may be regulated by gut epithelial *REG3A*. Thus, there exists a gut epithelial *REG3A-lactobacillus*-derived L-Orn—L-Kyn in gut epithelial cells—ROtyt (+) IL-22 (+) ILC3 immune cell axis to maintain gut mucosal homeostasis. Our data improve understanding of the mechanism of gut mucosal homeostasis. Since the gut mucosal homeostasis plays a critical role in human diseases such as colitis and metabolism-associated diseases, our findings also offer insight for prevention and treatment of these diseases.

We demonstrate that *lactobacillus*-derived L-Orn may upregulate IDO1 in gut epithelial cells to produce AhR ligand L-Kyn. AhR ligands that drive the differentiation of ILC3 immune cells may be entirely derived from the endogenous ligands, such as the Trp metabolites L-Kyn. Several cell types, including specific subsets of dendritic cells (DCs), macrophages, and immature monocytes, express increased levels of IDO1, which may promote the AhR ligand production in response to inflammatory cues, such as interferon γ (IFNγ) or signal transducer and activator of transcription 3 (STAT3)-activity stimuli, and CpG oligodeoxynucleotides (ODNs). However, our data exhibit that gut epithelial cells also produce AhR ligand L-Kyn by L-Orn through upregulating IDO1. Previous studies show that IDO1-positive staining may be primarily detected within the intestinal space of the villus or mucosal layer. IDO1 expression at mucosal sites may be modulated during immune activation. Recent studies also reveal that L-Orn may upregulate IDO1 in the macrophages and DCs.

*Lactobacillus* may produce L-Orn through arginine (Arg) metabolism. Others also reported that *lactobacillus* could produce L-Orn. Interestingly, Arg has been found to preserve intestinal barrier integrity in animals after intestinal obstruction and dextran sodium sulfate (DSS) colitis. It has also been established that Arg may improve the migration of epithelial cells, increase villus height and crypt depth, and decrease cell apoptosis in methotrexate (MTX)-induced mucositis and DSS colitis. Long-term increased polyamine (L-Orn metabolites) intake elevate blood spermine levels and inhibited aging-associated pathologies in mice and humans. Since L-Orn from *lactobacillus* through Arg metabolism may promote gut mucus homeostasis, our results expand understanding of the mechanism by which Arg contributes to this homeostasis. Others also found that some lactobacilli, such as *L. reuteri*, have an effect on IL-22 production by producing indole-3-aldehyde. Thus, multiple lactobacillus strains or multiple effects of one lactobacillus strain may exist in the gut.
**Fig. 4** REG3A-associated *lactobacillus* promotes production of L-Kyn in gut epithelial cells. **a** Microarray of the ileum epithelial cells in REG3A-associated *lactobacillus*-colonized GF mice (GF/LNK2) and control uncolonized GF mice (n = 6). **b** Metabolism map of tryptophan in mouse gut epithelial cells. **c** QRT-PCR and immunoblotting of IDO1 in the ileum tissues of L. NK2 or L. NK1-colonized GF mice and control uncolonized GF mice. SPF, wt mice raised in SPF environment. **d** Immunostaining of IDO1 in the ileum tissues of REG3A-associated *lactobacillus*-colonized GF mice and control GF mice (representative image, n = 6). **e** L-Kyn ELISA of the ileum (left) and colon (right) epithelial cells of REG3A-associated *lactobacillus*-colonized GF mice and control GF mice (n = 6). **f** L-Kyn ELISA of the ileum (left) and colon (right) epithelial cells of human REG3A12 mice and their control littermates (n = 6); **g** QRT-PCR (g) and immunoblotting (h) of IDO1 in the ileum or colon epithelial cells of human REG3A12 mice and their control littermates. Immunostaining of p-Src in the ileum epithelial cells of human REG3A12 mice and their control littermates; **j** Flow cytometry of CD45+Il-22+RORyt+ and their subsets in the ileum (j) and colon (k) LP of mice with or without L-Kyn infusion. Scale bars = 40 μm; Student’s t test was used in **f, g, j, and k**. ANOVA plus post-Bonferroni analysis in **c, d, and e**. NS no significance; RE relative expression.
Fig. 5 *Lactobacillus*-derived L-Orn promotes the production of AhR ligand L-Kyn in gut epithelial cells. **a** Immunostaining of IDO1 in the ileum after L-Orn administration in wt mice or L-Orn inhibitor DFMO administration in human REG3A<sup>tg</sup> mice. **b** HPLC/MASS of L-Kyn in the ileum epithelial cells after administrating L-Orn or L-Orn inhibitor DFMO (n = 6). **c, d** Flow cytometry of RORγt (+) IL-22(+) cells in the ileum (c) and colon (d) of mice after administering L-Orn or L-Orn inhibitor DFMO. **e** Flow cytometry of RORγt (+) IL-22(+) cells in the ileum and colon of IDO-1 KO with or without L-Orn. **f** Immunostaining and qRT-PCR of mucin in the ileum of mice after administering L-Orn or L-Orn inhibitor DFMO (ten slides/mouse, n = 6). **g** Staining of Ki67 cells in the ileum of mice after administering L-Orn or L-Orn inhibitor DFMO (ten slides/mouse, n = 6). **h** Immunostaining of mucin in the colon of mice after administering L-Orn or L-Orn inhibitor DFMO (ten slides/mouse, n = 6). WT, wild-type mice; WT/L-Orn, L-Orn-fed mice; REG3A, human REG3A<sup>tg</sup> mice; REG3A/DFMO, L-Orn inhibitor DFMO-fed mice. IDO-1 KO/L-Orn, L-Orn-fed IDO-1 KO mice. Scale bars = 40 μm; Student’s t test, mean ± SD in e; ANOVA plus post-Bonferroni analysis in a, b, c, d, f, g, and h; NS no significance; RE, relative expression. Data are representative of at least three independent experiments. Also see Supplementary Fig. 7.
lactobacilli. There also exist increased Gram-positive lactobacilli in other REGT mice.14,23

**Methods**

**Mice.** Four- to six-week-old male or female C57BL/6 mice were obtained from Nanjing Animal Center. IDO-1−/− mice were from Nanjing Animal Center. All experimental litters were bred and maintained under specific pathogen-free conditions in the Animal Center of Nankai University. Experiments were carried out using age- and gender-matched mice. All procedures were conducted according to the Institutional Animal Care and Use Committee of the Model Animal Research Center. Animal experiments were approved by the Institute's Animal Ethics Committee of Nankai University. All experimental variables such as husbandry, parental genotypes, and environmental influences were carefully controlled.
C57BL/6 germ-free (GF) mice were generated by Shanghai SLAC Laboratory Animal Co. Ltd. All experiments in GF mice were performed in Shanghai SLAC Laboratory Animal Co. Ltd. Human REG3a transgenic mice (human REG3α mRNA) were generated by Nanjing Animal Center. HD5 promoter, which may specifically promote the REG3α expression in gut Paneth cells, was conjugated into Pseudomonas-pHD5 promoter-β-gal-sulfadiazine. The fragments of REG3α-β-gal and polyA were cloned into HD5 promoter-Pseudomonas. This construction was demonstrated using primers (M13F: GCCAGGGTATTTCGACGCA and HD5-REG3α-R: TTATGAGTATGAGTGTGTTG) and sequencing using the primers (HD5-K:CGAGACTTGTGGATACGCT and CDS-FP: GGCAACATATGCGCAATTC). The HD5-REG3α mRNA, and the downstream chromosomal DNA fragments (primers (REG3α-F3: GAGCCCAAATGGGAAGTGTG and REG3α-R3: GTCTCCAGGTAGGAGACAC, which produced a 323-bp band in Fig and no band in wt mice), were used to identify these mice, which were identified as human REG3α-positive mice (human REG3α mRNA) and human REG3α-negative mice (wt). Human REG3α mRNA or wt siblings were from a cross between wt mice and human REG3α mRNA (heterozygous mice). The mice were from different mothers.

For preparation of mouse Reg3α adenovirus-injected mice, mouse Reg3α adenoviruses (Reg3α Ad) were first prepared by ABM, Canada and expanded by JIKAI, China, and then ip injected into mice according to the indicated time (1 × 10⁷ viral particles/mouse). Control empty adenoviruses (NC/Ad, 1 × 10⁷ control viral particles) were from ABM, Canada.

Mouse models. For DSS-induced colitis, dextran sodium sulfate (DSS)-induced colitis was performed according to the previous method. Briefly, mice received 2.5% (wt/vol) DSS (40,000 kDa, ICN Biochemicals) or indicated doses in their drinking water for 7 days, and then switched to regular drinking water. The amount of DSS water drank per animal was recorded and no differences in intake between strains were observed. For survival studies, mice were followed for 14 days post start of DSS treatment. Mice were weighed every other day for the determination of body weight change. This was calculated as % weight change = (final weight at day X – weight at day 0)/weight at day 0 × 100. Diarrhea was scored daily as follows: 0, normal; 2, slight bleeding; 4, gross bleeding. Weight loss was scored as follows: 0, normal; 2, loose stools; 4, watery diarrhea. Blood in stool was scored as follows: 0, none; 1, 10%; 2, 10–20%; 3, 10–15%; 4, >15%. Disease activity index was the average of these scores (combined score of stool consistency, bleeding, and weight loss)33. Mice were killed at the indicated days for histological study. Representative colon tissues were embedded in paraffin for histology.

For microbiota transplantation, germ-free mice were orally administered 200 µl of lactobacillus (1 × 10⁹ bacteria, once/week). In wt mice, mice were first treated with ampicillin (A, 1 g/L, Sigma), vancomycin (V, 0.5 g/L), neomycin sulfate (N, 1 g/L), and metronidazole (M, 1 g/L) via the drinking water for 2 weeks. Before using to assay arginine catabolism, cells were first cultured (37 °C for 24 h) on MRS agar. Anaerobic conditions were generated by using 16S rRNA sequence analyses. The OTU memberships of the sequences were used to construct a sample distance matrix from Horn dissimilarity was used to compute a sample distance matrix from the OTU count matrices, respectively. For each clustering, a hierarchical clustering using Ward’s minimum variance method was used for the absolute numbers of gut lactobacillus, 16s rRNAs were extracted, and then amplified using strain-specific primers. The concentration of each product was detected and then exchanged into copy numbers. Standard curves were prepared from serial dilution of Lactobacillus gasseri 16S rRNAs. Primers used were listed in Supplementary Table 1.

Lactobacillus isolation and culture. Lactobacillus isolation and culture were performed according to a serial method. In Lactobacillus gasseri 16S rRNAs were collected and diluted in 2 ml of BPS solution, and cultured on Roga Sl. A selective medium (Sigma-Aldrich) for lactobacillus enumeration, and then colonies were identified and purified using 16S rRNA sequence analyses. Lactobacilli were cultured in deMan, Rogosa, Sharpe (MRS; 3 M Health Care, St. Paul, MN) media and also grown on MRS agar containing 10% sucrose. Anaerobic conditions were generated with the sachets of AnaeroPack-Anaero (Mitsubishi Gas Chemical, Japan) in an airtight jar. After 24 h of cultivation in liquid medium, lactobacilli could reach 1 × 10⁹ CFU/ml.

For L-Orn production by lactobacillus in vitro, lactobacilli were propagated routinely for 24 h at 37 °C in MRS broth medium. Before using to assay arginine catabolism, cells were first cultured (37 °C for 24 h) on MRS agar. Monodonal lactobacilli was then newly propagated in MRS broth with or without 6 mM Ado, which was then used to induce arginine catabolism. The supernatants were collected at the indicated time and L-ornithine was analyzed using ELISA.

Construction of OCT-deficient lactobacillus. Ornithine carbamoyltransferase (OCT) nucleotide sequences of L. reuteri DSM 20016 (NCBI GI 148303277, Lreu_0044) and JCM 1112 (NCBI GI 183223999, LAR_0041) were used for identifying a homologous gene in the genome of L. reuteri ATCC PTA 4659. The locus of the gene encoding the OCT and flanking nucleotide sequences in L. reuteri ATCC PTA 4659 was analyzed with the BLAST program against the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The gene encoding for OCT in L. reuteri ATCC PTA 4659 was truncated according to the following method. To construct a ΔOCT (ornithine carbamoyltransferase) deletion mutant inserted with chloramphenicol acetyltransferase (Cm) (seen in Supplementary Table 1), the upstream chromosomal DNA fragment (896 bp) and the downstream chromosomal DNA fragment (949 bp) were amplified by 36e-F/36e-R primers. Then the four PCR products were gel purified, ligated by the one-step cloning kit, and transformed into E. coli DH5α. The recombinant plasmid pMG36e fragment was amplified by 36e-F/36e-R primers. Then the four PCR products were gel purified, ligated by the one-step cloning kit, and transformed into E. coli DH5α. The recombinant plasmid pMG36e-left-cat-right was electropropared into L. reuteri with selection on MRS medium of 3 µg/mL ethyrosinum (Em) and 3 µg/mL Cm. The recombinant L. reuteri- containing plasmid pMG36e-left-cat-right was propagated in MRS medium with 5 µg/mL Em at 37 °C for 30 generations without Em at 37 °C for 20 generations with Em. The cultures were serially diluted from 10 to 10⁷ in sterilized PBS and plated onto MRS medium without any antibiotics. After cultivating for 48 h, the clones were spotted on the MRS medium with Em and Cm. After incubation at 37 °C for 48 h, the clones which were growing only on the MRS medium with Cm, were thought to be desired mutants. The sequences around the OCT gene were amplified and sequenced to confirm the mutants. PCR primers used in this study were listed in Supplementary Table 1.
Microarray. Expression of coding mRNA was analyzed by Beijing Capitalbio Technology Co., Ltd, according to our previously reported method. Total RNA was extracted using Life Technologies. Contaminating DNA was removed using RNeasy spin columns (Qagen). The quality of isolated RNA samples was evaluated with an Agilent Bioanalyzer 2100 (Agilent Technologies) and the purified RNA was quantified using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher). The Agilent Gene Expression oligo microarrays and miRNA microarrays were analyzed using Agilent Gene Expression oligo microarrays Version 6.5, May 2010 and Agilent miRNA microarrays Version 2.3. The R software (v.2.13.0) platform was applied to analyze the microarray data, and the LIMMA (linear regression model) package was used to statistically analyze differentially expressed genes. Genes having a fold change >2 or <−2 and an adjusted p < 0.05 were considered as differentially expressed.

Flow-cytometry analyses. Single-cell suspensions of Peyer’s patches (PP) and spleen of mice were prepared by mashing in a cell strainer (70 mm), stained, and analyzed by flow cytometry according to previous method. In brief, colon or small intestine were isolated and harvested in HBSS with 2 mM EDTA. The epithelial cells were removed by incubating the tissue in HBSS with 2 mM EDTA for 30 min at 37 °C with shaking. The LP cells were isolated by incubating the tissues in digestion buffer (DMEM, 5% fetal bovine serum, and 1 mg/ml Collagenase IV and DNase I) for 40 min. The digested tissues were then filtered through a 40-μm filter. Cells were resuspended in 10 ml of the 40% of a 40:80 Percoll gradient and overlaid on 5 ml of the 80% fraction in a 15-ml Falcon tube. LP cells were collected at the interphase of the Percoll gradient, washed, and resuspended in a medium, and then stained and analyzed by flow cytometry. Dead cells were eliminated through 7-AAD staining.

For intracellular staining, the cells were cultured and stimulated for 6 h with 50 ng/ml phorbol 12-myristate 13-acetate and 1 μg/ml ionomycin (Sigma) in the presence of GolgiStop. After incubation for 6 h, cells were washed in PBS, and then fixed in Cytofix/Cytoperm, permeabilized with Perm/Wash buffer, and stained with FITC-, PE-, APC- APC/cy7-, PerCP/Cy5.5-, or PE/cy7-conjugated antibodies. Meanwhile, dead cells were eliminated through 7-AAD staining.

For intracellular IL-22 staining, cells were stimulated directly ex vivo by incubating them in 6 h with 20 ng/ml IL-23 in the presence of GolgiStop for the final 3 h of culture. Cells were fixed and permeabilized by using perm buffer set, as described by the manufacturers, and stained with IL-22 and RORγt antibodies.

Histological and immunostaining. For histology and immunostaining, slides were treated with 0.1% Triton X-100, blocked with 3% H2O2. Then slides were incubated with 5 μg/ml rabbit anti-chicken IgG in PBS (1:100) for 1 h at room temperature in blocking buffer. The protein–Ab complexes were checked using peroxidase-conjugated secondary Abs (Boehringer Mannheim) and ECL (Amersham Biosciences). The primary and secondary antibodies were listed in Supplementary Table 1.

RT-PCR and qRT-PCR. RT-PCR and qRT-PCR were performed according to our previous methods. Briefly, total RNA was extracted from cells by using TRIzol reagent (Life Technologies, Carlsbad, CA) and was transcribed to cDNA using HifiScript cDNA Synthesis Kit (CWBIO, Beijing, China) diluted to a concentration of 0.01 μg/ml in hybridization buffer (20 mM Tris-HCl, pH 7.4, 0.9 M NaCl, 0.1% SDS, and 20% formamide). After washing for 10 min in wash buffer (20 mM Tris-HCl, pH 7.4, 0.9 M NaCl) and 10 min in PBS, block solution (5% PBS in PBS) was added for 30 min at 30 °C. Mucin 2 primary antibody was diluted to 1:200 in block solution and applied overnight at 4 °C. After washing in PBS, block solution containing anti-rabbit secondary antibody diluted to 1:200 was applied to the section for 2 h. Nuclei were stained using Hoechst33342. Observations were performed with a Zeiss LSM 700 confocal microscope with software Zen 2011 version 7.1. This software was used to determine the distance between bacteria and the epithelial cell monolayer, as well as the mucin thickness.

HPLC/MASS analyses of gut contents. For HPLC/MASS analyses of the gut contents, 50 mg of sample were applied to the extraction procedure, and extracted with 800 μl of methanol. In total, 10 μl of internal standard (2-9 μl/mg, DL-o-chlorophenylalanine) was then added. All samples were grinded to fine powder using a grinding mill at 65 Hz for 90 s. The samples after grinding were vortexed for 30 s, and centrifuged at 12,000 rpm and 4 °C for 15 min. In total, 200 μl of supernatant was transferred to a vial for HPLC–MS analysis.

Ex vivo ileum and colon stimulation. For ex vivo ileum and colon stimulation, the fragmented fresh ileum and colon from untreated mice were immediately added in 2 ml of RPMI-1640 medium containing 10% heat-inactivated FBS (Gibco, Invitrogen), 100 U penicillin, 100 μg/ml streptomycin, and 10 ml HEPES (Gibco, Invitrogen), and then L-ornithine was added into culture at the indicated concentration and time. For IOD1 analysis, the ileum or colon epithelial cells were separated from ileum or colon tissues using 0.1% EDTA, and expression of IOD1 was analyzed using qRT-PCR and immunoblotting.

ELISA. For ELISA of L-kynurenine and L-ornithine, the preparation of tissue homogenates was performed as previously described. L-kynurenine and L-ornithine concentration in tissue homogenates or cell culture supernatants was measured using the L-kynurenine or L-Ornithine ELISA kit (ImmuSmol).

Western blotting. Cell lysates were denatured and subjected to SDS-PAGE, and then were transferred to PVDF membranes according to our previous methods. Briefly, hybridizations with primary Abs were performed for 1 h at room temperature in blocking buffer. The protein–Ab complexes were checked using peroxidase-conjugated secondary Abs (Boehringer Mannheim) and ECL (Amersham Biosciences). The primary and secondary antibodies were listed in Supplementary Table 1.

Statistical analyses. Student’s t test, one-way analysis of variance (ANOVA), ANOVA plus post-Bonferroni analysis, Mann–Whitney U test, and Wilcoxon’s test were used to determine significance. A 95% confidence interval was considered significant and was defined as p < 0.05.

Reagents. The source of the reagents and primer sequences was listed in Supplementary Table 1.

Data availability. Raw 16S rRNA gene sequence data for the feces microbiota were deposited in the NCBI Short Read Archive under BioProject Accession Number PRJNA326574. Microarray data Accession number GSE111111. The source data underlying plots presented in figures are shown in Supplementary Data 1. The data used for the L-kynurenine HPLC–MS/ HPLC–MS/MS analyses and HPLC–MASS analyses of the gut contents are presented in Supplementary Data 2. The full blots are shown in Supplementary Fig. 11.

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