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IL-1β in eosinophil-mediated small intestinal homeostasis and IgA production

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Abstract

Eosinophils are multifunctional leukocytes that reside in the gastrointestinal (GI) lamina propria, where their basal function remains largely unexplored. In this study, by examining mice with a selective deficiency of systemic eosinophils (by lineage ablation) or GI eosinophils (eotaxin-1/2 double–deficient or CC chemokine receptor 3–deficient), we show that eosinophils support immunoglobulin A (IgA) class switching, maintain intestinal mucus secretions, affect intestinal microbial composition, and promote the development of Peyer's patches. Eosinophil-deficient mice showed reduced expression of mediators of secretory IgA production, including intestinal interleukin 1β (IL-1β), inducible nitric oxide synthase, lymphotoxin (LT) α, and LT-β, and reduced levels of retinoic acid-related orphan receptor gamma t–positive (ROR-γt$^+$) innate lymphoid cells (ILCs) while maintaining normal levels of APRIL (a proliferation-inducing ligand), BAFF (B cell–activating factor of the tumor necrosis factor family), and TGF-β

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Disclosures

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(transforming growth factor β). GI eosinophils expressed a relatively high level of IL-1β, and IL-1β–deficient mice manifested the altered gene expression profiles observed in eosinophil-deficient mice and decreased levels of IgA^+ cells and ROR-γt^+ ILCs. On the basis of these collective data, we propose that eosinophils are required for homeostatic intestinal immune responses including IgA production and that their affect is mediated via IL-1β in the small intestine.

Introduction

Eosinophils have been considered to be end-stage effector cells that have an important role in parasitic infections and allergic inflammations.[1] However, numerous lines of evidence indicate that eosinophils are multifunctional leukocytes involved not only in modulation of innate and adaptive immunity but also in various biological processes.[2,3]

Eosinophils develop in the bone marrow and migrate to the lamina propria (LP) of the gastrointestinal (GI) tract under homeostatic conditions.[4] The intestinal immune system is a unique environment that invokes strong protective immunity against pathogens while maintaining tolerance to dietary proteins or commensal bacteria.[5] A prominent feature of the intestinal immune system is the neutralization of harmful pathogens by production of immunoglobulin (Ig) A, the most abundant human antibody isotype,[6] which is normally deposited as secretory IgA (SIgA) in the intestinal lumen. IgA can be induced by T cell–dependent or T cell–independent pathways, which mainly occur in the organized lymphoid tissue of Peyer's patches (PP) and in the LP of the small intestine, respectively.[7] T cell–dependent IgA production depends on cluster of differentiation (CD) 40 signals of $CD4^+$ T cells activated by dendritic cells (DCs) under the influence of cytokines, in particular transforming growth factor β (TGF-β, *Tgfb1*).[7,8] T cell–independent IgA class switching of B cells is induced by various cytokines in the LP, mainly by APRIL (a proliferation-inducing ligand, *Tnfsf13*) and BAFF (B cell–activating factor of the tumor necrosis factor family, *Tnfsf13b*).[7,9] Local production of nitric oxide via inducible nitric oxide synthase (iNOS, *Nos2*), which regulates the expression of activation-induced deaminase and IgA germinal transcript, also has a role in IgA class switching in the LP of small intestine.[10,11] Recently, lymphotoxin (LT) α (*Lta*) and LT-β (*Ltb*) produced by retinoic acid-related orphan receptor gamma t–positive (ROR-γt^+) innate lymphoid cells (ILCs) have been shown to regulate IgA production in the small intestine.[12]

Murine eosinophils in the bone marrow support the survival of plasma cells by secreting APRIL and interleukin (IL) 6.[13] Although the involvement of intestinal eosinophils in IgA class switching has not yet been directly examined, the impaired IgA production in CD47-deficient mice suggests a potential role of intestinal eosinophils in IgA synthesis,[14] as small intestinal eosinophils highly express an inhibitory receptor signal regulatory protein (SIRP) α, a cognate receptor for CD47, and SIRP-α/CD47 signaling contributes to the prolonged survival of murine intestinal eosinophils by inhibiting their degranulation.[15] In the healthy state, eosinophils are barely present in the PP,[16] where T cell–dependent IgA class switching takes place. Therefore, it seems plausible that eosinophils contribute to T cell–independent

IgA class switching by modulating the immune environment of the small intestinal LP, where abundant numbers of eosinophils co-reside with plasma cells.[4,9]

In the present study, we investigated the role of eosinophils in the production of SIgA in the GI tract. Using four different strains of mice with absent or reduced GI eosinophils caused by independent gene deletions (i.e. two eosinophil lineage–ablated [PHIL and ΔdblGATA], eotaxin-1/eotaxin-2 double knockout [*Ccl11*/*Ccl24* DKO], and CC chemokine receptor [CCR] 3 knockout [*Ccr3* KO] mice), we demonstrate that IgA^+ cells are significantly decreased in the absence of eosinophils. In addition, eosinophil-deficient mice have reduced mucus production and PP size and alterations in commensal intestinal microbiota and oral tolerance induction. Although the expression of intestinal *Tnfsf13*, *Tnfsf13b*, and *Tgfb1* were not affected by the deficiency of eosinophils, there was a decrease in intestinal *il1b* (gene for IL-1β), *Lta*, *Ltb*, and *Nos2* expression, as well as ROR-γt^+ ILCs. In line with reduced *Il1b* in eosinophil-deficient mice and with small intestinal eosinophils being a major source of *Il1b*, IL-1β–deficient (*Il1b* KO) mice demonstrated decreased intestinal IgA. Collectively, our findings demonstrate that GI eosinophils regulate intestinal adaptive immune responses, mainly SIgA production, and we propose that this regulation involves an IL-1β–dependent mechanism involving eosinophil-dependent changes in commensal microbiota.

Results

IgA^+ plasma cells are significantly decreased in the small intestine of eosinophil-deficient mice

We first demonstrated that eosinophil-deficient mice, as modeled by ΔdblGATA, *Ccr3* KO, *Ccl11/Ccl24* DKO, and PHIL, had a marked decrease in small intestinal eosinophils defined by $CD11b^{high}CD11c^{int}$ markers (Fig. 1A).[17] A significant reduction of IgA in the serum and intestinal lavage was observed in ΔdblGATA, *Ccr3* KO, and *Ccl11/Ccl24* DKO mice (Fig. 1B). To substantiate that the decrease of IgA is due to the absence of eosinophils, we examined IgA levels in PHIL mice, designed to deplete eosinophils by lineage-specific expression of the cytocidal diphtheria toxin A.[18] Significantly decreased serum and intestinal IgA levels were also observed in PHIL mice (Fig. 1B). Reduced IgA in eosinophil-deficient mice was reflected by decreases in the frequency and number of IgA^+B220^- cells in the LP of the small intestine and of IgA^+B220^+ cells (post–class switch recombination [CSR] IgA^+ B cells)[10] in the PP of these mice (Fig. 1C). The increase of IgM^+B220^+ cells in the small intestine and the decreased germinal center IgA^+ cells expressing peanut agglutinin in the PP of ΔdblGATA mice also indicated impaired IgA class switching (Fig. S1A and B). However, the presence of eosinophils in the culture of total LP cells had no supportive effect on the survival of IgA^+ cells (Fig. S1C). In addition, it is unlikely that the B cells of ΔdblGATA are defective in IgA class switching as the expression of activation-induced deaminase and IgA germinal transcript were not impaired in B cells of ΔdblGATA under IgA CSR-inducing conditions (Fig. S1D & E). In the mesenteric lymph nodes (MLN), no differences were observed in the frequency and number of post-CSR IgA^+ cells (Fig. 1C and data not shown). In the PP, CD40-expressing B cells undergo IgA CSR in response to CD40 ligand (CD40L) signal from T cells.[7] However, the frequencies of CD40-expressing B cells and CD40L-expressing T cells were not affected in the PP of ΔdblGATA, *Ccr3* KO,

and *Ccl11/Ccl24* DKO mice (data not shown). In addition, integrin α4β7 and CCR9, molecules associated with homing of post-CSR IgA$^+$ cells to the small intestine, were comparably detected in IgA$^+$ plasma cells isolated from the small intestine of WT and eosinophil-deficient mice (data not shown).

PP and small intestinal immune cell populations in eosinophil-deficient mice and impaired PP development

Analysis of the small intestinal LP cells by flow cytometry demonstrated a significant decrease in the total cell number in ΔdblGATA, *Ccr3* KO, *Ccl11/Ccl24* DKO, and PHIL mice (Fig. 2A); but no decrease in the frequency of CD4$^+$, CD8$^+$, and CD19$^+$ cells, of DCs and plasmacytoid DCs (pDCs) in any of the eosinophil-deficient murine strains was observed except for the significant decrease of DC frequency in *Ccr3* KO mice (Fig. S2). Similarly, there was no significant reduction in mononuclear cell populations in these mice (Fig. 2A).

The weight and total cell number of PP were significantly reduced in eosinophil-deficient mice, implying a role of eosinophils in the normal development of PP (Fig. 2B & C). As total cell numbers of PP was reduced, the absolute numbers of CD4$^+$, CD8$^+$, and CD19$^+$ cells and of DCs and pDCs were decreased in eosinophil-deficient mice (data not shown), although the frequencies of CD8 cells in the PP were modestly increased in ΔdblGATA and *Ccr3* KO mice (Fig. S2). ΔdblGATA, *Ccr3* KO, *Ccl11/Ccl24* DKO, and PHIL mice did not show a significant difference compared to WT mice in their weight, cellularity, and immune cell populations in MLN (data not shown).

Adoptively transferred small intestinal LP cells restore IgA synthesis in ΔdblGATA mice

To prove that the IgA deficiency observed in ΔdblGATA mice was an acquired defect secondary to the altered intestinal immunity rather than a defect directly caused by the genetic engineering or other occult differences between WT and ΔdblGATA mice, we assessed whether IgA deficiency was reversed by adoptive transfer of small intestinal LP cells. Ideally, we would have transferred purified LP eosinophils, but getting sufficient number of LP eosinophils from the small intestine for transfer was not technically feasible. Accordingly, we isolated small intestinal LP cells from wild-type (WT) mice and transferred into recipient ΔdblGATA mice. Analysis of small intestine of reconstituted ΔdblGATA showed significantly increased side-scatter high (SSC^{hi}) and CD11b^{high}CD11c^{int} intestinal eosinophils although the degree of increase was modest (Fig. S3A). After 14 days of transfer, recipient mice showed increased numbers of small intestinal IgA$^+$B220$^-$ cells (Fig. 3A) and intestinal IgA secretion (Fig. 3B). We also observed significant restoration of weight and total cell number of PP in recipient mice (Fig. S3B), though IgA$^+$B220$^+$ number in PP of the recipient mice were not restored to the level of WT mice (Fig. 3A). The failure of adoptively transferred LP cells isolated from the small intestine of ΔdblGATA to restore IgA levels (Fig. S4A) suggests that the increased IgA in ΔdblGATA following adoptive transfer of WT LP cells was eosinophil-dependent. However, it is hard to suggest that LP eosinophils directly promote IgA class switching of small intestinal B cells as the presence of SSC^{high}CD45$^+$MHC II$^-$CD11b^{high}CD11c^{int} small intestinal LP eosinophils did not

induce a significant increase of IgA in cultures of B cells activated either with APRIL and BAFF or cecal bacterial antigen (CBA) (Fig. 3C and Fig. S3C).

Reduced mucus secretion and less efficient induction of oral tolerance in ΔdblGATA mice

A significant decrease of IgA synthesis in eosinophil-deficient mice was accompanied by reduced mucus secretion in the small intestine of ΔdblGATA mice as demonstrated by wheat germ agglutinin stains (Fig. 4A) and by periodic acid-Schiff stains (Fig. S5). Accordingly, the number of mucus expressing goblet cells present in the epithelial layer of the villi was significantly decreased in the small intestine of ΔdblGATA mice (Fig. S5B). The mucus layer of the intestine provides a physical barrier for the underlying intestinal epithelium against adhesion and invasion by microorganisms, bacterial toxins, and antigens.[19] Recently, it has been reported that mucus enhances intestinal homeostasis, as well as oral tolerance, by delivering tolerogenic signals to DCs in the intestine.[20] Therefore, ΔdblGATA and WT mice were tolerized orally to ovalbumin (OVA) to examine whether the development of oral tolerance was altered in eosinophil-deficient mice. Mice were fed 1% OVA in drinking water for five days. After intraperitoneal sensitization and boost, OVA-specific serum IgG1, IgG2a, and IgE were compared between tolerized (Tol group) and systemically immunized mice without OVA (IP group). As shown in Fig. 4B, there was a significant difference between the WT and eosinophil-deficient Tol groups of mice; the OVA-specific IgG1, IgG2a, and IgE levels were significantly higher in the tolerized ΔdblGATA mice compared with the WT mice ($P = 0.0151$, $P = 0.0416$, $P = 0.0375$, respectively, Fig. 4B). Also, as for OVA-specific IgE, the IP group of the ΔdblGATA mice showed a significant increase ($P = 0.0180$) relative to the same group of WT mice, though no significant differences were observed in the OVA-specific IgG1 ($P = 0.4177$) or IgG2a ($P = 0.4867$) (Fig. 4B). Taken together, our observations demonstrate that although oral tolerance successfully developed in ΔdblGATA mice, the degree was inefficient compared to WT mice, consistent with the decrease in intestinal mucus content, which has been suggested to modulate oral tolerance.[20]

Expression of Lta and Ltb are decreased in the small intestine of ΔdblGATA mice

APRIL, BAFF, and TGF-β in the GI tract synergistically support T cell–independent IgA class switching, and TGF-β is also required for T cell–dependent IgA class switching in organized lymphoid tissue.[7,21,22] Therefore, we analyzed the expression level of these cytokines in the small intestine and PP of WT and ΔdblGATA mice. As shown in Fig. 5A, levels of *Tnfsf13*, *Tnfsf13b*, and *Tgfb1* mRNA in the small intestine and PP of ΔdblGATA mice were not different compared to WT mice as suggested by insignificant effect of small intestinal LP eosinophils on the IgA class switching observed in B cell cultures (Fig. 3C). Consistent with these results, gene expressions for *Tnfsf13*, *Tnfsf13b*, and *Tgfb1* were not considerable in isolated eosinophils from the small intestine (Fig. 5B), thus suggesting another mechanism for the decrease of IgA in eosinophil-deficient mice. Additionally, expression of matrix metalloproteinase 9 (*Mmp9*) and thrombospondin 1 (*Thbs1*) mRNAs, responsible for the activation of latent form of TGF-β[23,24], were not decreased in the small intestine of ΔdblGATA mice, although their expressions were considerable in the small intestinal eosinophils (Fig. S6A).

Recently, it has been reported that LT controls IgA production in the small intestine[12] and also induces the development of gut-associated lymphoid tissues including PP.[25] As we observed decreased IgA synthesis and impaired PP development in eosinophil-deficient mice, we examined *Lta* and *Ltb* expression in ΔdblGATA mice. As shown in Fig. 5C, *Ltb* mRNA and *Lta* mRNA, to a lesser extent, were significantly decreased in the small intestine but not in the PP of ΔdblGATA mice. Consistent with this, RNA microarray data from sorted small intestinal eosinophils indicate a considerable level of *Ltb* mRNA and barely expressed *Lta* (Fig. 5D). As the difference of *Lta* between WT and ΔdblGATA mice was reduced when the expression of *Lta* was normalized to leukocyte-specific protein tyrosine phosphatase, receptor type, C (*Ptprc*) (Fig. S6B), the significant decrease of *Lta* in the small intestine of ΔdblGATA mice may be reflective of the decrease of total LP leukocytes in these mice (Fig. 2A). However, considering the low level of *Lta* mRNA in the small intestinal eosinophils, the major population of cells missing in the small intestine of ΔdblGATA (Fig. 1A), we suggest that the distribution of Ltα in other leukocyte subsets could be considerable as Lta is a soluble protein while Ltb is a membrane-bound molecule associated with leukocytes.[26]

Small intestinal eosinophils express Il1b

Small intestinal eosinophils were found to robustly express a high level of *Il1b* (Fig. 6A) as determined by the raw signal of RNA microarray of FACS-sorted cells and in comparison to levels of *Il23a*, *Il25*, *Il33*, *Il2*, and *Il7*, genes for cytokines involved in intestinal immunity including stimulation of ILCs.[27,28] In line with the predominant presence of eosinophils in the small intestine, *Il1b* expression was decreased only in the small intestine but not in the PP of ΔdblGATA mice, thus suggesting eosinophils as a major source for IL-1β production in the small intestine (Fig. 6B). Eosinophils isolated from the small intestine also expressed low levels of Caspase 1 mRNA (*Casp1*, raw expression value of 302.6 ± 17.0, with a significance threshold of 400), known to activate precursor form of IL-1β,[29] and the production of IL-1β by small intestinal eosinophils was confirmed by detection of secreted IL-1β protein from cultured small intestinal LP eosinophils (Fig. 6C). Accordingly, small intestinal segments of WT showed significantly higher IL-1β than those of ΔdblGATA mice and the expression of IL-β in the WT small intestine was decreased from the proximal to the distal segment compatible with intestinal distribution of eosinophils as reported (Fig. 6D).[30]

IL-1β regulates SIgA production

Notably, IL-1β can stimulate proliferation of ROR-γt$^+$ ILCs[31,32] and also enhance iNOS expression,[33,34] an enzyme that has been shown to regulate IgA class switching.[10,11] Therefore, we investigated whether frequency of IgA$^+$ cells is affected in *Il1b* KO mice. As shown in Fig. 7A, *Il1b* KO mice evidenced a significant decrease of small intestinal IgA$^+$ cells and reduced intestinal IgA levels, although gene expression for *Tnfsf13*, *Tnfsf13b*, and *Tgfb1* in the small intestine was comparable to that of WT mice (Fig. S7A). *Il1b* KO mice also showed decreased ROR-γt$^+$ ILCs, and in line with this finding, the expression of *Lta* and *Ltb* mRNA was significantly decreased in the small intestine of *Il1b* KO mice with decrease of PP weight (Fig. 7B and Fig. S7B). *Il1b* KO mice also showed decreased *Nos2* expression in the small intestine (Fig. 7C). Collectively, these findings demonstrate a novel contribution of IL-1β for the regulation of intestinal immune system and suggest that

decreased IL-1β stimuli in the small intestine are responsible for defective IgA production in ΔdblGATA mice. Consistent with findings in *Il1b* KO mice, ΔdblGATA showed decreased numbers of ROR-γt$^+$ ILCs in the LP of the small intestine and was not restored by adoptive transfer of LP cells isolated from the small intestine of *Il1b* KO mice (Fig. 7D & Fig.S4C). As a control, expression of c-Kit and IL-7Rα, which are surface phenotype markers for ILCs, were comparable between ΔdblGATA and WT mice (Fig. S7C). In addition, we observed a significant decrease of *Nos2* expression in the small intestine but not in the PP of ΔdblGATA mice (Fig. 7E).

Changes in the microbiota of ΔdblGATA mice

As both SIgA and mucus have been shown to affect luminal microflora composition,[35,36] we analyzed microflora in eosinophil-deficient mice. Deep sequencing analysis of commensal microflora in WT and ΔdblGATA revealed a marked increase of Firmicutes-containing segmented filamentous bacteria (SFB) and a decrease in Bacteroidetes in ΔdblGATA mice (Fig. 8A) which could be reversed by adoptive transfer of WT LP cells isolated from the small intestine (Fig. S8A). Real-time polymerase chain reaction of selected intestinal commensals verified increased SFB and reduced Bacteroidetes in ΔdblGATA mice not only being separated but also housed with WT mice more than 3 weeks (Fig. 8B, Fig. S8B). Intestinal commensal bacteria affect the development and maturation of organized lymphoid tissue.[36] As we observed impaired PP development in ΔdblGATA, we investigated whether aberrant intestinal microbiota in ΔdblGATA mice was connected with defective PP in these mice. IL-7 (*Il7*) is one of the central regulators of PP development,[27] and commensal microflora promote steady-state IL-7 production in the intestine epithelium.[37] As shown in Fig. 8C, *Il7* expression was decreased in the small intestine of ΔdblGATA mice. Taken together, these findings demonstrate a crucial role of eosinophils for the homeostatic interactions of the intestinal immune system with the gut microbiota, although further research is needed to determine whether the involved mechanisms are direct and/or indirect.

Discussion

Eosinophils in the steady state are much more abundant in the LP of the intestines than in other tissues;[1,38] however, their physiologic function has still remained largely unknown. In the present study, we demonstrate that eosinophils are required for mucosal production of IgA and development of PP, thus suggesting their important role in the maintenance of intestinal homeostasis. Recently, reduced IgA production has been reported in ΔdblGATA and PHIL mice, strains with systemic eosinophil deficiency.[39] By additionally examining *Ccr3* KO and *Ccl11/Ccl24* DKO mice, which have selective eosinophil deficiency specific to the small intestinal,[40] we have substantiated these findings by demonstrating that eosinophils present in the small intestine are responsible for IgA production and PP development. As decrease of IgA in ΔdblGATA mice is partially restored by adoptively transferred small intestinal LP cells of WT mice but not by LP cells of ΔdblGATA (Fig.S4A), the acquired immune defect associated with eosinophil deficiency likely results in IgA reduction in ΔdblGATA mice. B cell-depleted LP cells of WT mice also had effects on the increase of IgA in ΔdblGATA mice, thus we suggest B cells of the recipient mice

(but not donor IgA$^+$ B cells) account for the restoration of IgA following adoptive transfer (Fig. S4A).

Our data presented herein suggest eosinophils regulate IgA class switching in the small intestine indirectly by altering the intestinal microenvironment to be more favorable for IgA production. The expression levels of *Tnfsf13*, *Tnfsf13b*, and *Tgfb1*, genes for cytokines directly induce IgA class switching were found to be comparable in the small intestine and PP of ΔdblGATA mice with those of WT mice. This finding contrasts with the previous observations by Chu et al.,[39] which suggest that eosinophils are direct regulators of IgA production in the GI tract through their expression of IL-6, APRIL, and TGF-β via toll-like receptor (TLR)–mediated signaling. However, the CD11b$^+$SiglecF$^+$ cell subset, which was used by Chu et al. for eosinophils, may include CD11b$^+$ small intestinal DCs (Fig. S9A), which are reported to produce IL-6 and directly induce IgA class switching in a TLR5-dependent way.[41] Additionally, though Chu et al. reported expression of TLR2, 4, 5, 7, 9 in small intestinal eosinophils, the CD11b$^+$ DC is the major subset expressing TLR5 in the isolated small intesinal leukocytes,[41] and we observed insignificant expression of these TLRs in sorted small intestinal eosinophils (raw expression values for TLR2, TLR4, TLR7, and TLR9 are 175.8 ± 4.8, 225.8 ± 18.6, 33.7 ± 0.7, and 119.3 ± 6.7, respectively, with a significance threshold of 400). In this study, CD11b$^+$ DC were not included in eosinophils isolated from the small intestine (Fig.S3C and Fig. S9) and isolated eosinophils expressed insignificant levels of *Tnfsf13*, *Tnfsf13b*, and *Tgfb1*. Indeed, intestinal *Il1b*, *Lta*, *Ltb*, *Nos2* expression and ROR-γt$^+$ ILCs numbers were decreased in ΔdblGATA mice, and showed a significant or almost significant increase after adoptive transfer of LP cells of WT mice (Fig. S4B & C). The decrease of IgA and ROR-γt$^+$ ILCs and the altered gene expression profiles observed in ΔdblGATA were also seen in *Il1b* KO mice, implicating IL-1β expression by eosinophils in the control of intestinal homeostasis and supported by insignificant restoration of IgA, ROR-γt$^+$ ILCs, and gene expressions in ΔdblGATA mice following adoptive transfer of small intestinal LP cells of *Il1b* KO mice (Fig. S4). IgA is also known to regulate intestinal microbiota via shielding, immune exclusion, and modulating bacterial gene expression and maintaining healthy bacterial composition in the intestine.[35] Therefore, mice having IgA defects show aberrant changes in microbial composition as represented by outgrowth of SFB and decrease of bacteroidetes,[42,43] which are present in ΔdblGATA mice. Notably, Chu et al. observed changes in SFB and bacteroidetes that were in the opposite direction of what we observed. The reasons for this difference are unclear, but both studies are consistent with a dominant role for eosinophils in regulating intestinal commensal microbiota. We propose that altered intestinal microbiota in eosinophil-deficient mice have a role in the impaired development of PP in these mice since *Il7*, which is critical for PP development and regulated by commensal microflora [27,37], is significantly decreased in ΔdblGATA mice.

The intestinal immune system protects against microbial pathogens and maintains a homeostatic interaction with commensal bacteria. In this environment, intestinal IgA neutralizes pathogenic toxins and microbes in a non-inflammatory manner, thereby promoting both immune protection and intestinal homeostasis.[44] Intestinal IgA is synthesized through T cell–dependent, and T cell–independent pathways.[6,45] T cell–

dependent IgA class switching is initiated in the organized tissue, mainly in the PP, by CD40L signaling of activated T cells and cytokines, especially TGF- β.[46,47] Though the size and cellularity of PP in eosinophil-deficient mice are decreased, $CD40L^+$ T cell and $CD40^+$ B cell frequencies in the PP of ΔdblGATA are normal and have genetic expression of *Tgfb1* comparable to that of WT mice. These findings are consistent with the limited number of eosinophils in PP under the steady state[16] and imply an indirect role of eosinophils in the impaired PP development in eosinophil-deficient mice.

APRIL, BAFF, and TGF-β in the small intestine facilitate IgA class switching in the LP without help from activated T cells.[6,7] However, the gene expression levels of *Tnfsf13*, *Tnfsf13b*, and *Tgfb1* were not affected in ΔdblGATA mice in accordance with the insignificant expression of these cytokines in isolated small intestinal eosinophils. We propose that eosinophils have a role in the maintenance of intestinal IgA through their expression of IL-1β. LT produced by ROR-γt^+ ILCs induces T cell–independent IgA production in the LP of the small intestine even in the absence of PP and MLN.[12,48] Considering that IL-1β promotes proliferation and LT expression of ROR-γt^+ ILCs,[31,32] it is reasonable to suggest that the selective decrease of IL-1β in the small intestine of ΔdblGATA mice is responsible for the reduction of ROR-γt^+ ILCs and LT expression. Our findings are also supported by the decrease of *Nos2*, an essential mediator for LT-dependent IgA production,[12,49] in the small intestine of *Il1b* KO and ΔdblGATA mice. As *Nos2* is barely expressed in small intestinal eosinophils (raw expression value 83.2 ± 4.0, threshold for significance being 400), it seems likely that small intestinal eosinophils are not the direct source of *Nos2*. IL-1β production by eosinophils is increased by SIgA,[50] accordingly *Il1b* mRNA expression was significantly higher in eosinophils isolated from the small intestinal than eosinophils isolated from the lung (data not shown). In addition, secreted IgA enhances survival of eosinophils,[50] and we observed considerable IgA signals in the cytoplasm of small intestinal eosinophils (Fig. S10), suggesting a positive feedback loop between IL-1β–expressing eosinophils and IgA for the immune homeostasis in the gut.

The decrease of IgA in eosinophil-deficient mice was associated with an imbalanced homeostatic state in the GI tract, including decreased IgA, mucus, and less efficient induction of oral tolerance, with the antibody titers of the tolerized ΔdblGATA mice being significantly increased relative to those of the tolerized WT mice. However, eosinophil-deficient mice still successfully developed oral tolerance. Indeed, in contrast to the findings of Chu et al.,[39] we observed no significance differences between the ΔdblGATA and WT mice in the frequencies of $CD103^+$ DCs and regulatory T cells (Treg), cells that are responsible for the development of oral tolerance[51,52] (Fig. S11). Considering the critical role of TGF-β in the development of Treg,[53] normal frequencies of Treg in ΔdblGATA mice corresponds with their level of *Tgfb1* being comparable to that of WT mice.

In summary, we show that eosinophils are required for mucosal production of IgA and normal development of PP in the gut. Furthermore, we also demonstrate less efficient induction of oral tolerance and aberrantly changed intestinal microflora in the absence of eosinophils. Though the levels of *Tnfsf13*, *Tnfsf13b*, and *Tgfb1* are maintained in eosinophil-deficient mice, the decreases in ROR-γt^+ ILC number and in expression of *Lta/Ltb* and *Nos2* indicate the eosinophils' role in the modulation of LT signaling–mediated IgA class

switching. Additionally, the decrease IL-1β in the small intestine of eosinophil-deficient mice and the reduced production of IgA in *Il1b* KO mice suggest that IL-1β is a key eosinophil-derived modulator of IgA class switching. Collectively, these findings demonstrate the crucial role of eosinophils in the homeostatic interactions with the intestinal immune system, and we propose on the basis of our findings that the involved mechanisms are unlikely to be largely dependent upon eosinophil-derived APRIL and BAFF.

Methods

Mice

Ccl11/Ccl24 DKO and *Ccr3* KO mice were generated as previously described.[40] ΔdblGATA were provided by Dr. Stuart H. Orkin (Howard Hughes Medical Institute), PHIL mice were provided by James J Lee (Mayo Clinic), *Il1b* KO mice were generated by Dr. David Chaplin and provided by Dr. Yui Hsi Wang. Littermate controls were used as WT controls for PHIL mice. In all experiments, we used 6– to 10–week-old, age- and sex-matched mice, which were housed in specific pathogen–free conditions at Cincinnati Children's Hospital Medical Center or Gachon University under an Institutional Animal Care and Use Committee–approved protocol. All mice were housed at ambient temperature and a 12-hour light cycle.

Isolation of leukocytes from small intestinal LP, PP, and MLN

Segments of the small intestine were incubated with FACS buffer (phosphate buffered saline [PBS] containing 10% FCS, 20 mM HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin, 1 mM sodium pyruvate, 10 mM EDTA, and 10 μg/mL polymyxin B) for 30 minutes at 37°C to remove epithelial cells and were washed extensively with PBS. Small intestinal segments, PP, and MLN were digested with 2.4 mg/mL collagenase A (Roche) and 10 μg/mL DNase I (Roche) in RPMI 1640/10% FCS with continuous stirring at 37°C for 30 minutes. Before enzyme digestion, weight of total isolated PP was measured using an analytical balance. EDTA was added (10 mM final), and the cell suspension was incubated for an additional 5 minutes at 37°C. After washing, the cells were subjected to density-gradient centrifugation in 40%/75% Percoll. The cells harvested from the interface were washed and used as leukocytes in assays. For adoptive transfer assay, 1×10^7 isolated small intestinal LP cells were transferred into ΔdblGATA mice by intravenous injection.

Flow cytometry

To characterize the surface phenotype, the cells were isolated and resuspended in FACS buffer. After Fc receptor blocking with anti-mouse CD16/CD32 (2.4G2, BD Biosciences) for 15 minutes at 4°C, the cells were stained with antibodies against various markers. Anti-mouse B220 (RM3-6B2), CD11c (HL3), and ROR- γt (Q31-378) were from BD Biosciences. Antibodies against CD11b (M1/70), CD45 (30-F11), CD3 (145-2C11), and Gr-1 (RB6-8C5) were from eBioscience. For IgA stain, the cells were fixed and permeabilized using the Cytofix/Cytoperm Kit (BD Biosciences) and subsequently were stained intracellularly with anti-IgA (C10-3, BD Biosciences). Transcription Factor Buffer Set (BD Bioscience) was used for ROR- γt staining. Each sample was acquired with a

FACSCalibur (BD Biosciences), and the data were processed with FlowJo software (Tree Star).

Cell culture

CBA was prepared as previously described.[39] To induce IgA class switching, 1×10^5 of $SSC^{low}CD45^+MHC\ II^+IgM^+CD19^+$ B cells isolated from the small intestine of WT mice were cultured with 100 μg/mL CBA or APRIL (100 ng/mL) plus BAFF (50 ng/mL). An equal number of small intestinal eosinophils sorted as $SSC^{high}CD45^+MHC\ II^-CD11b^{high}CD11c^{int}$ were added to some of the B cell cultures to determine whether eosinophils directly support IgA class switching. To examine IL-1β secretion, sorted small intestinal eosinophils were cultured for 24 hours in the presence of IL-5 (10 ng/mL). In some experiments, phorbol myristate acetate (20 ng/mL) and ionomycin (1 μM) or eotaxin 1 (25 ng/mL) was added for the stimulation of eosinophils.

ELISA

For determination of total IgA, 96-well plates were coated with anti-mouse IgA (C10-3, BD Pharmingen) and blocked with PBS/BSA. Diluted serum or intestinal washes were incubated, and biotinylated anti-mouse IgA (C10-2, BD Pharmingen) was added. After reacting with streptavidin-HRP, the plates were developed with TMB substrates (BD Pharmingen), and the absorbance was read at 450 nm. A mouse IL-1β ELISA kit (R&D Systems) was used according to the manufacturer's instructions to measure the levels of IL-1β in cell culture supernatants and small intestinal segments of WT and ΔdblGATA mice.

Immunohistochemistry and immunofluorescence

PP-containing small intestines were fixed in 10% formalin, routinely processed, and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E). For immunofluorescence staining, fixed, frozen small intestinal sections were rehydrated in PBS for 30 minutes and permeabilized in 0.2% Triton X-100/PBS for 10 minutes. The slides were blocked with 1% BSA/PBS for 1 hour at room temperature and incubated with Alexa Fluor 488 wheat germ agglutinin (WGA, Molecular Probes) and Alexa Fluor 568 Phalloidin (Molecular Probes) for 30 minutes. Sections were washed 3 times with PBS after each antibody incubation. After a 10-minute stain with DAPI, slides were mounted using Prolong Gold antifade (Molecular Probes). Sections were visualized using the BX51 microscope, DP72 camera, and DP2-BSW imaging software (Olympus America Inc.). WGA-positive luminal signals were quantified using Imaris software (Bitplane).

Induction of oral tolerance

Oral tolerance to OVA was induced as previously reported.[54] Mice were provided 1% OVA in drinking water *ad libitum* for 5 days, while a control group was given normal drinking water. Two days after the final oral treatment, immunization with OVA was performed via intraperitoneal injection of 100 μg of OVA adsorbed to 1 mg of aluminum hydroxide gel (Sigma-Aldrich).[55] Mice were boosted with 20 μg of OVA in PBS 14 days after immunization, and blood samples were collected 7 days after antigen boost. OVA-specific IgG1, IgG2a, and IgE were analyzed as readout of oral tolerance induction.[54,55]

Real-time PCR

RNA from the small intestine and PP was extracted using QIAzol lysis reagent (Qiagen) and subsequently column-purified with an RNeasy Mini Kit (Qiagen). RNA (500 ng) was treated with DNase I (New England Biolabs), and cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad). Real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad). PCR was performed using a CFX96 Real-Time System (Bio-Rad). Sequences of primers are shown in Table S1.

Genome-wide microarray analysis on sorted eosinophils

Microarray analysis on sorted intestinal eosinophils was performed as previously described.[56] Small intestinal eosinophils were sorted as $DAPI^{-}CCR3^{+}SiglecF^{+}CD45^{+}CD4^{-}CD8a^{-}CD19^{-}B220^{-}SSC^{high}$ cells from 10 animals using FACS Aria (BD). Total RNA from sorted eosinophils was extracted by standard TRIzol RNA isolation (Invitrogen) and subsequently column-purified with an RNeasy Mini Kit (Qiagen). mRNA integrity was validated by the Agilent 2100 bio-analyzer (Agilent Technologies). Eosinophil mRNA was amplified and labeled with the WT-Ovation Pico RNA Amplification System (NuGen) and subjected to the GeneChip Mouse Gene ST 1.0 Array chip (Affymetrix). Microarray expression analysis was performed at Cincinnati Children's Hospital Medical Center's Chip Core facility, and expression data were analyzed by the software of Genespring GX 11 (Agilent Technologies). The Affymetrix raw expression values were filtered with the significance threshold of 400 as previously reported[56,57] and validated by the levels of eosinophil-specific major basic protein gene (also known as proteoglycan 2 [*Prg2*]) and eosinophil non-expressed glucagon (*Gcg*) (Fig. S12).

Microbiota analysis

Genomic DNA was isolated from fresh or frozen ileum contents using the QIAamp fast DNA stool kit (Qiagen) according to manufacturer's instructions. Levels of the 16S rRNA gene of each bacterium were quantified by real-time PCR analysis as described above. The quantity of the 16S rRNA gene from each bacterial group was normalized to the quantity of the Eubacteria 16S rRNA gene. Sequences of primers are shown in Table S1. For deep sequencing, 16S rRNA was amplified using a combination of the universal bacterial/archael primers 515F and 806R.[58] The 16S rRNA sequence analysis was performed via MiSeq Reporter v2.3.32 (Illumina).

Statistical analysis

The data are presented as the mean ± SEM. All of the experiments were performed in triplicate. When necessary, a two-group comparison was performed using a student's t-test. A P value < 0.05 was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Rothenberg ME, Hogan SP. The eosinophil. Annu Rev Immunol. 2006; 24:147–174. [PubMed: 16551246]
2. Jung Y, Rothenberg ME. Roles and regulation of gastrointestinal eosinophils in immunity and disease. J Immunol. 2014; 193:999–1005. [PubMed: 25049430]
3. Jacobsen EA, Helmers RA, Lee JJ, Lee NA. The expanding role(s) of eosinophils in health and disease. Blood. 2012; 120:3882–3890. [PubMed: 22936660]
4. Mishra A, Hogan SP, Lee JJ, Foster PS, Rothenberg ME. Fundamental signals that regulate eosinophil homing to the gastrointestinal tract. J Clin Invest. 1999; 103:1719–1727. [PubMed: 10377178]
5. Mowat AM. Anatomical basis of tolerance and immunity to intestinal antigens. Nat Rev Immunol. 2003; 3:331–341. [PubMed: 12669023]
6. Fagarasan S, Kawamoto S, Kanagawa O, Suzuki K. Adaptive immune regulation in the gut: T cell-dependent and T cell-independent IgA synthesis. Annu Rev Immunol. 2010; 2(8):243–273. [PubMed: 20192805]
7. Cerutti A. The regulation of IgA class switching. Nat Rev Immunol. 2008; 8:421–434. [PubMed: 18483500]
8. Lamm ME, Phillips-Quagliata JM. Origin and homing of intestinal IgA antibody-secreting cells. J Exp Med. 2002; 195:F5–8. [PubMed: 11805155]
9. Suzuki K, Fagarasan S. How host-bacterial interactions lead to IgA synthesis in the gut. Trends Immunol. 2008; 29:523–531. [PubMed: 18838301]
10. Tezuka H, et al. Regulation of IgA production by naturally occurring TNF/iNOS-producing dendritic cells. Nature. 2007; 448:929–933. [PubMed: 17713535]
11. Lee MR, Seo GY, Kim YM, Kim PH. iNOS potentiates mouse Ig isotype switching through AID expression. Biochem Biophys Res Commun. 2011; 410:602–607. [PubMed: 21684254]
12. Kruglov AA, et al. Nonredundant function of soluble LTalpha3 produced by innate lymphoid cells in intestinal homeostasis. Science. 2013; 342:1243–1246. [PubMed: 24311691]
13. Chu VT, et al. Eosinophils are required for the maintenance of plasma cells in the bone marrow. Nat Immunol. 2011; 12:151–159. [PubMed: 21217761]
14. Westlund J, Livingston M, Fahlen-Yrlid L, Oldenborg PA, Yrlid U. CD47-deficient mice have decreased production of intestinal IgA following oral immunization but a maintained capacity to induce oral tolerance. Immunology. 2012; 135:236–244. [PubMed: 22070457]
15. Verjan Garcia N, et al. SIRPalpha/CD172a regulates eosinophil homeostasis. J Immunol. 2011; 187:2268–2277. [PubMed: 21775684]
16. Mishra A, Hogan SP, Brandt EB, Rothenberg ME. Peyer's patch eosinophils: identification, characterization, and regulation by mucosal allergen exposure, interleukin-5, and eotaxin. Blood. 2000; 96:1538–1544. [PubMed: 10942403]
17. Carlens J, et al. Common gamma-chain-dependent signals confer selective survival of eosinophils in the murine small intestine. J Immunol. 2009; 183:5600–5607. [PubMed: 19843944]
18. Lee JJ, et al. Defining a link with asthma in mice congenitally deficient in eosinophils. Science. 2004; 305:1773–1776. [PubMed: 15375267]
19. Corazziari ES. Intestinal mucus barrier in normal and inflamed colon. J Pediatr Gastroenterol Nutr. 2009; 48 (Suppl 2):S54–55. [PubMed: 19300126]

20. Shan M, et al. Mucus enhances gut homeostasis and oral tolerance by delivering immuno regulatory signals. Science. 2013; 342:447–453. [PubMed: 24072822]
21. Litinskiy MB, et al. DCs induce CD40-independent immunoglobulin class switching through BLyS and APRIL. Nat Immunol. 2002; 3:822–829. [PubMed: 12154359]
22. Castigli E, et al. TACI and BAFF-R mediate isotype switching in B cells. J Exp Med. 2005; 201:35–39. [PubMed: 15630136]
23. Crawford SE, et al. Thrombospondin-1 is a major activator of TGF-beta1 in vivo. Cell. 1998; 93:1159–1170. [PubMed: 9657149]
24. Yu Q, Stamenkovic I. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. Genes Dev. 2000; 14:163–176. [PubMed: 10652271]
25. Alimzhanov MB, et al. Abnormal development of secondary lymphoid tissues in lymph otoxin beta-deficient mice. Proc Natl Acad Sci U S A. 1997; 94:9302–9307. [PubMed: 9256477]
26. Ware CF. Network communications: lymphotoxins, LIGHT, and TNF. Annu Rev Immunol. 2005; 23:787–819. [PubMed: 15771586]
27. Eberl G, Lochner M. The development of intestinal lymphoid tissues at the interface of self and microbiota. Mucosal Immunol. 2009; 2:478–485. [PubMed: 19741595]
28. Spits H, Cupedo T. Innate lymphoid cells: emerging insights in development, lineage relationships, and function. Annu Rev Immunol. 2012; 30:647–675. [PubMed: 22224763]
29. Franchi L, Munoz-Planillo R, Nunez G. Sensing and reacting to microbes through the inflammasomes. Nat Immunol. 2012; 13:325–332. [PubMed: 22430785]
30. Chu DK, et al. Indigenous enteric eosinophils control DCs to initiate a primary Th2 immune response in vivo. J Exp Med. 2014; 211:1657–1672. [PubMed: 25071163]
31. Cella M, Otero K, Colonna M. Expansion of human NK-22 cells with IL-7, IL-2, and IL-1beta reveals intrinsic functional plasticity. Proc Natl Acad Sci U S A. 2010; 107:10961–10966. [PubMed: 20534450]
32. Hughes T, et al. Interleukin-1beta selectively expands and sustains interleukin-22+ immature human natural killer cells in secondary lymphoid tissue. Immunity. 2010; 32:803–814. [PubMed: 20620944]
33. Carpenter L, Cordery D, Biden TJ. Protein kinase Cdelta activation by interleukin-1 beta stabilizes inducible nitric-oxide synthase mRNA in pancreatic beta-cells. J Biol Chem. 2001; 276:5368–5374. [PubMed: 11087760]
34. Adams V, et al. Induction of iNOS expression in skeletal muscle by IL-1beta and NFkappaB activation: an in vitro and in vivo study. Cardiovasc Res. 2002; 54:95–104. [PubMed: 12062366]
35. Sutherland DB, Fagarasan S. IgA synthesis: a form of functional immune adaptation extending beyond gut. Curr Opin Immunol. 2012; 24:261–268. [PubMed: 22503962]
36. Maynard CL, Elson CO, Hatton RD, Weaver CT. Reciprocal interactions of the intestinal microbiota and immune system. Nature. 2012; 489:231–241. [PubMed: 22972296]
37. Shalapour S, et al. Commensal microflora and interferon-gamma promote steady-state interleukin-7 production in vivo. Eur J Immunol. 2010; 40:2391–2400. [PubMed: 20690180]
38. Rothenberg ME. Eosinophilic gastrointestinal disorders (EGID). J Allergy Clin Immunol. 2004; 113:11–28. quiz 29. [PubMed: 14713902]
39. Chu VT, et al. Eosinophils promote generation and maintenance of immunoglobulin-A-expressing plasma cells and contribute to gut immune homeostasis. Immunity. 2014; 40:582–593. [PubMed: 24745334]
40. Pope SM, Zimmermann N, Stringer KF, Karow ML, Rothenberg ME. The eotaxin chemokines and CCR3 are fundamental regulators of allergen-induced pulmonary eosinophilia. J Immunol. 2005; 175:5341–5350. [PubMed: 16210640]
41. Uematsu S, et al. Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5. Nat Immunol. 2008; 9:769–776. [PubMed: 18516037]
42. Suzuki K, et al. Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut. Proc Natl Acad Sci U S A. 2004; 101:1981–1986. [PubMed: 14766966]

43. Geuking MB, McCoy KD, Macpherson AJ. The function of secretory IgA in the context of the intestinal continuum of adaptive immune responses in host-microbial mutua lism. Semin Immunol. 2012; 24:36–42. [PubMed: 22138187]
44. Macpherson AJ, McCoy KD, Johansen FE, Brandtzaeg P. The immune geography of IgA induction and function. Mucosal Immunol. 2008; 1:11–22. [PubMed: 19079156]
45. Cerutti A, Rescigno M. The biology of intestinal immunoglobulin A responses. Immunity. 2008; 28:740–750. [PubMed: 18549797]
46. Bergqvist P, Gardby E, Stensson A, Bemark M, Lycke NY. Gut IgA class switch recombination in the absence of CD40 does not occur in the lamina propria and is independent of germinal centers. J Immunol. 2006; 177:7772–7783. [PubMed: 17114448]
47. Cazac BB, Roes J. TGF-beta receptor controls B cell responsiveness and induction of IgA in vivo. Immunity. 2000; 13:443–451. [PubMed: 11070163]
48. Kang HS, et al. Signaling via LTbetaR on the lamina propria stromal cells of the gut is required for IgA production. Nat Immunol. 2002; 3:576–582. [PubMed: 12006975]
49. Fritz JH, et al. Acquisition of a multifunctional IgA+ plasma cell phenotype in the gut. Nature. 2012; 481:199–203. [PubMed: 22158124]
50. Bartemes KR, Cooper KM, Drain KL, Kita H. Secretory IgA induces antigen-independent eosinophil survival and cytokine production without inducing effector functions. J Allergy Clin Immunol. 2005; 116:827–835. [PubMed: 16210057]
51. Coombes JL, et al. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. J Exp Med. 2007; 204:1757–1764. [PubMed: 17620361]
52. Worbs T, et al. Oral tolerance originates in the intestinal immune system and relies on antigen carriage by dendritic cells. J Exp Med. 2006; 203:519–527. [PubMed: 16533884]
53. Rubtsov YP, Rudensky AY. TGFbeta signalling in control of T-cell-mediated self-reactivity. Nat Rev Immunol. 2007; 7:443–453. [PubMed: 17525753]
54. Mucida D, et al. Oral tolerance in the absence of naturally occurring Tregs. J Clin Inves t. 2005; 115:1923–1933.
55. Tunis MC, Dawicki W, Carson KR, Wang J, Marshall JS. Mast cells and IgE activation do not alter the development of oral tolerance in a murine model. J Allergy Clin Immunol. 2012; 130:705–715. e701. [PubMed: 22607990]
56. Wen T, et al. The pan-B cell marker CD22 is expressed on gastrointestinal eosinophils and negatively regulates tissue eosinophilia. J Immunol. 2012; 188:1075–1082. [PubMed: 22190185]
57. Wen T, et al. Carbonic anhydrase IV is expressed on IL-5-activated murine eosinophils. J Immunol. 2014; 192:5481–5489. [PubMed: 24808371]
58. Bates ST, et al. Examining the global distribution of dominant archaeal populations in soil. ISME J. 2011; 5:908–917. [PubMed: 21085198]

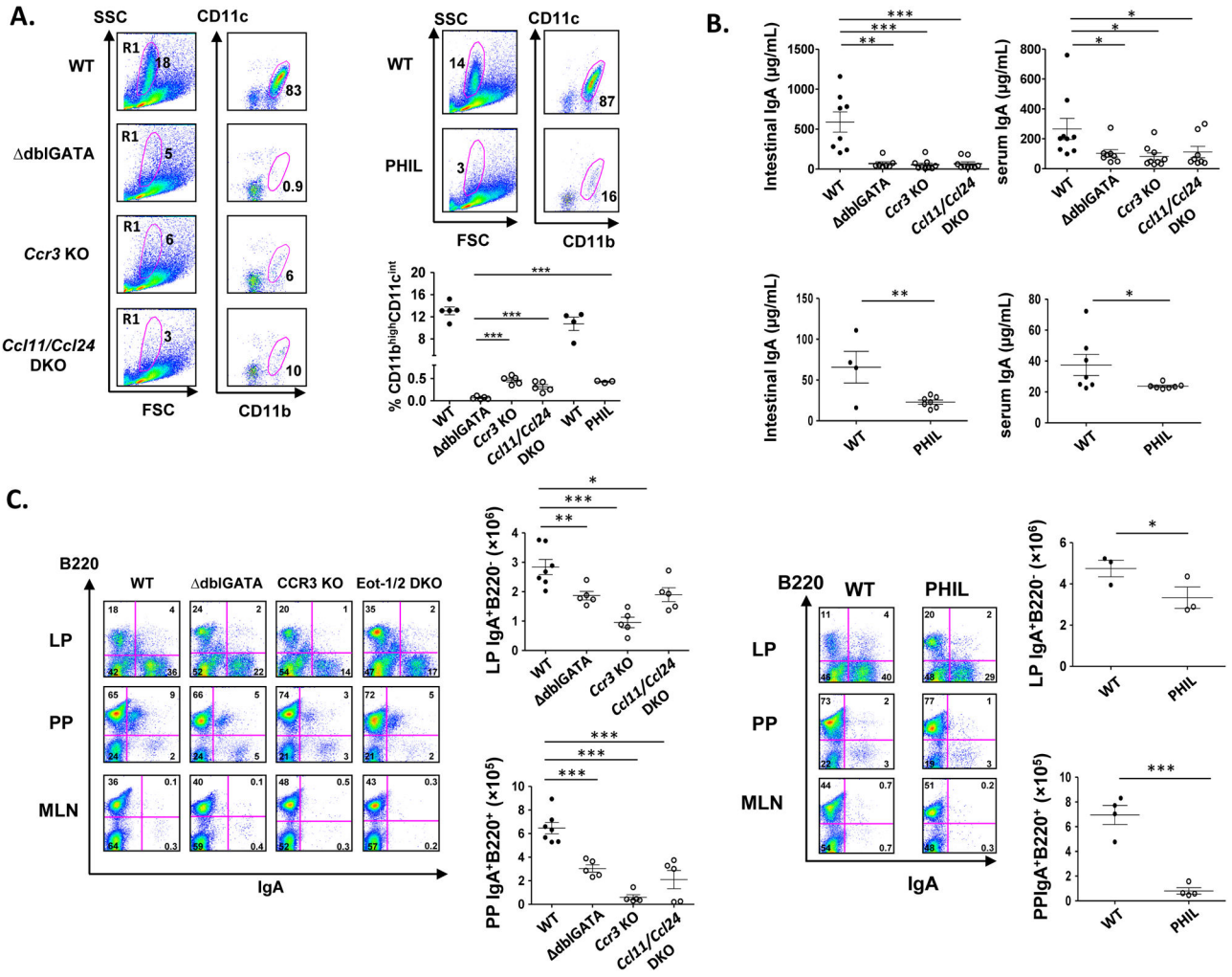

Figure 1. Reduced immunoglobulin A (IgA) levels in serum and intestinal lavage of eosinophil-deficient mice and their decreased IgA$^+$ cell numbers in the small intestine and Peyer's patches (PP)

(A) The small intestinal lamina propria (LP) cells of the wild-type (WT; black circles), ΔdblGATA, CC chemokine receptor 3–deficient (*Ccr*3 knockout [KO]), eotaxin-1/2 double–deficient (*Ccl11*/*Ccl24* double knock out [DKO]), and PHIL mice (eosinophil deficient; white circles). The frequencies of CD11b^{high}CD11c^{int} small intestinal eosinophils were analyzed with R1-gated cells representing the medium-to-high SSC subset. (B) IgA levels in intestinal lavage and serum of WT and eosinophil-deficient mice (ΔdblGATA, *Ccr3* KO, *Ccl11*/*Ccl24* DKO, and PHIL). (C) Frequencies and/or numbers of IgA$^+$ cells in the small intestinal LP, PP, and mesenteric lymph nodes (MLN) of WT, ΔdblGATA, *Ccr3* KO, *Ccl11*/*Ccl24* DKO, and PHIL mice. All data are representative of two or more independent experiments. Data are mean ± SEM values. *P < 0.05, **P < 0.01, ***P < 0.001 (Student's *t*-test).

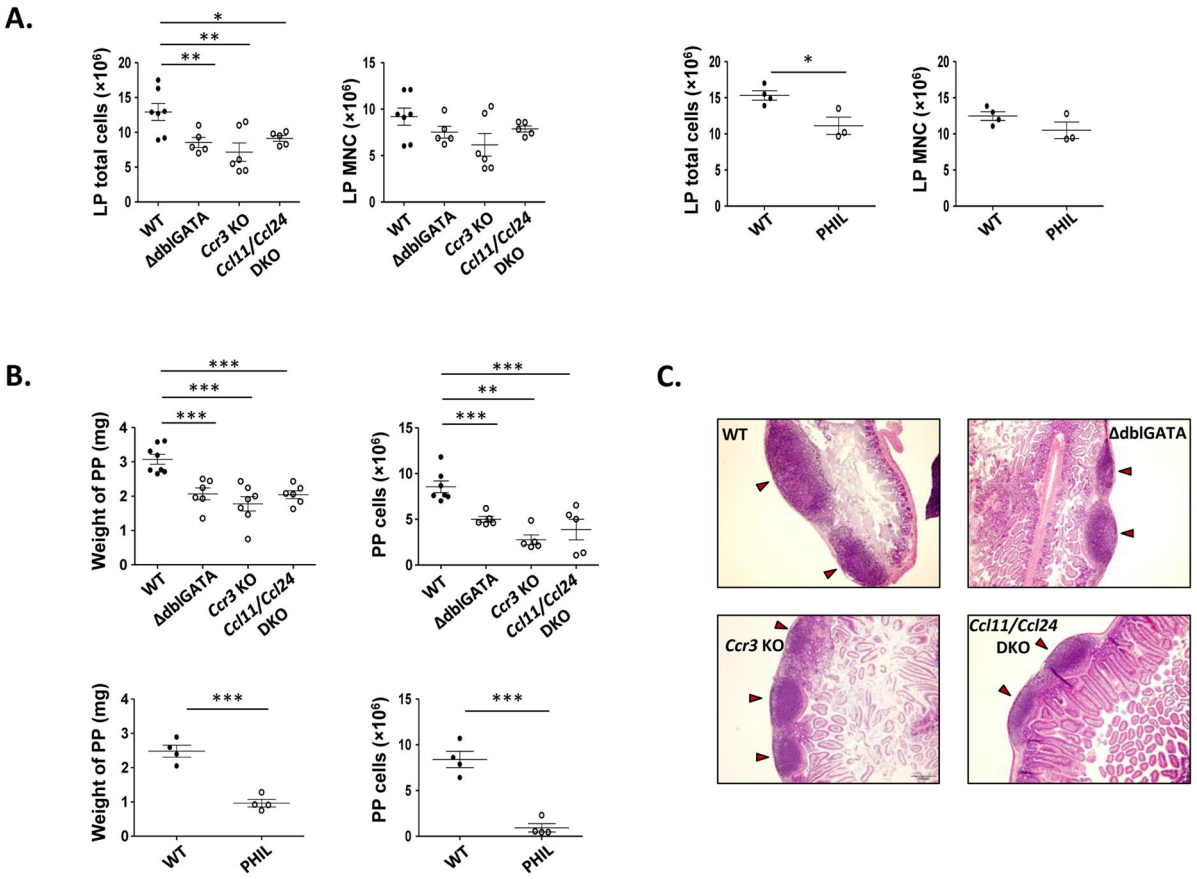

Figure 2. Small intestine and Peyer's patches (PP) immune cell populations in eosinophil-deficient mice and impaired PP development

(A) Total lamina propria (LP) cells and mononuclear cell (MNC) numbers in the small intestine of wild-type (WT; black circles), ΔdblGATA, CC chemokine receptor 3–deficient (*Ccr*3 knockout [KO]), eotaxin-1/2 double–deficient (*Ccl11*/*Ccl24* double knock out [DKO]), and PHIL mice (eosinophil deficient; white circles). (B) Weight and cell numbers of PP isolated from WT and eosinophil-deficient mice. (C) Hematoxylin and eosin (H&E) staining of PP in WT and eosinophil-deficient mice. Arrowheads indicate PP in the small intestine. Original magnification × 10. All data are representative of two or more independent experiments with n ≥ 3 mice. Data are mean ± standard error of the mean (SEM) values. **P* < 0.05, ***P* < 0.01 (Student's *t*-test).

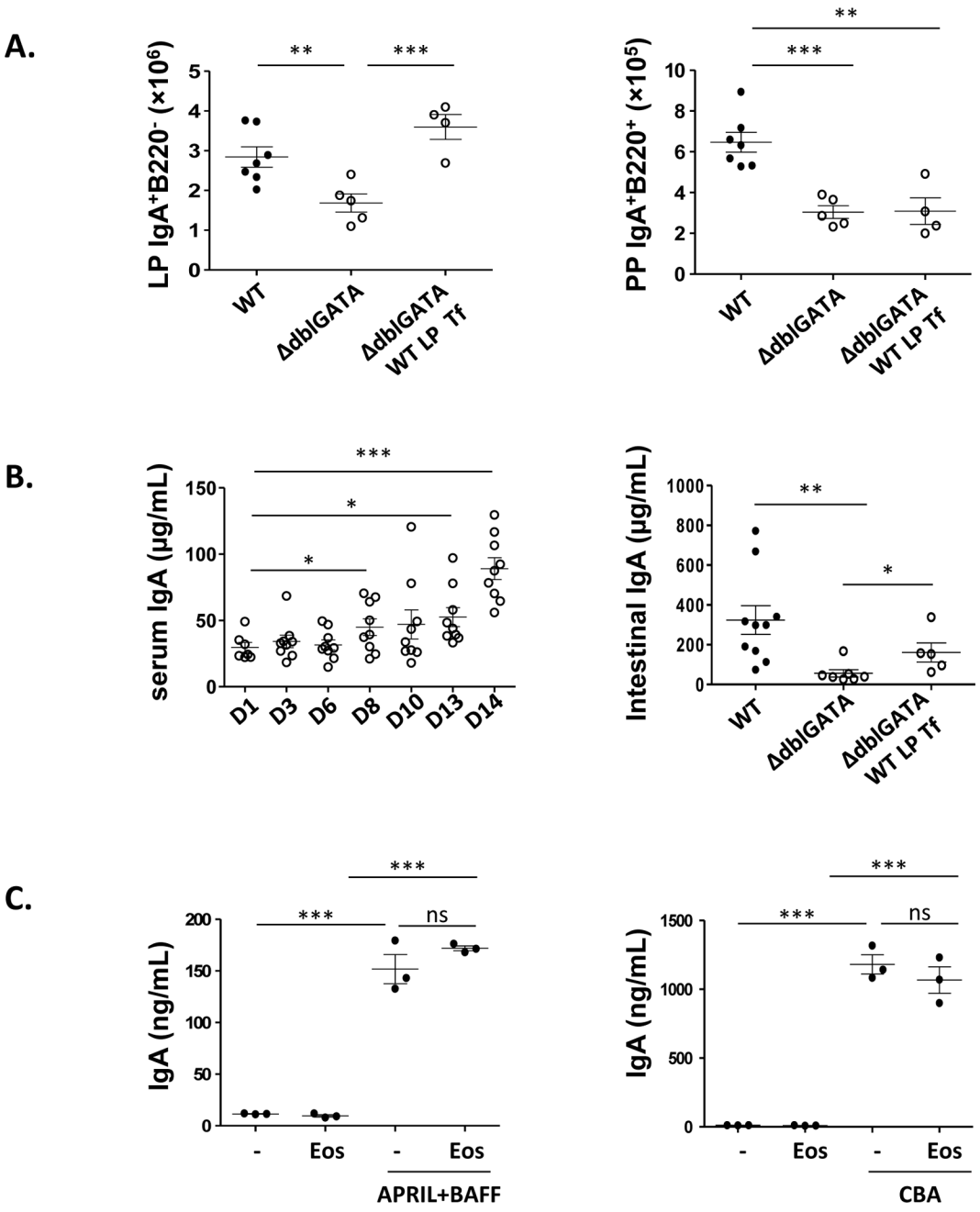

Figure 3. Effect of adoptively transferred small intestinal lamina propria (LP) cells on immunoglobulin A (IgA) synthesis in ΔdblGATA mice

(A) Numbers of IgA^+ cells in the LP of small intestine and Peyer's patches (PP) of wild-type mice (WT; black circles), ΔdblGATA (eosinophil-deficient; white circles), and ΔdblGATA mice after adoptive transfer of small intestinal LP cells isolated from WT mice (ΔdblGATA WT LP Adoptive Tf; white circles). Data are mean ± standard error of the mean (SEM) values. $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's *t*-test). (B) IgA levels in serum of ΔdblGATA mice were analyzed over the indicated time period after adoptive transfer of WT LP cells. IgA levels in intestinal lavage of WT, ΔdblGATA mice, and ΔdblGATA mice 14 days after

adoptive transfer of WT LP cells. Data are mean ± standard error of the mean (SEM) values. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (Student's *t*-test). (C) Naïve B cells isolated from the small intestine of WT mice were cultured with the indicated stimuli in the presence or absence of small intestinal eosinophils for 7 days. IgA production by stimulated B cells was determined by ELISA. All data are representative of two or more independent experiments. Data are mean ± standard error of the mean (SEM) values. ****P* < 0.001 (Student's *t*-test).

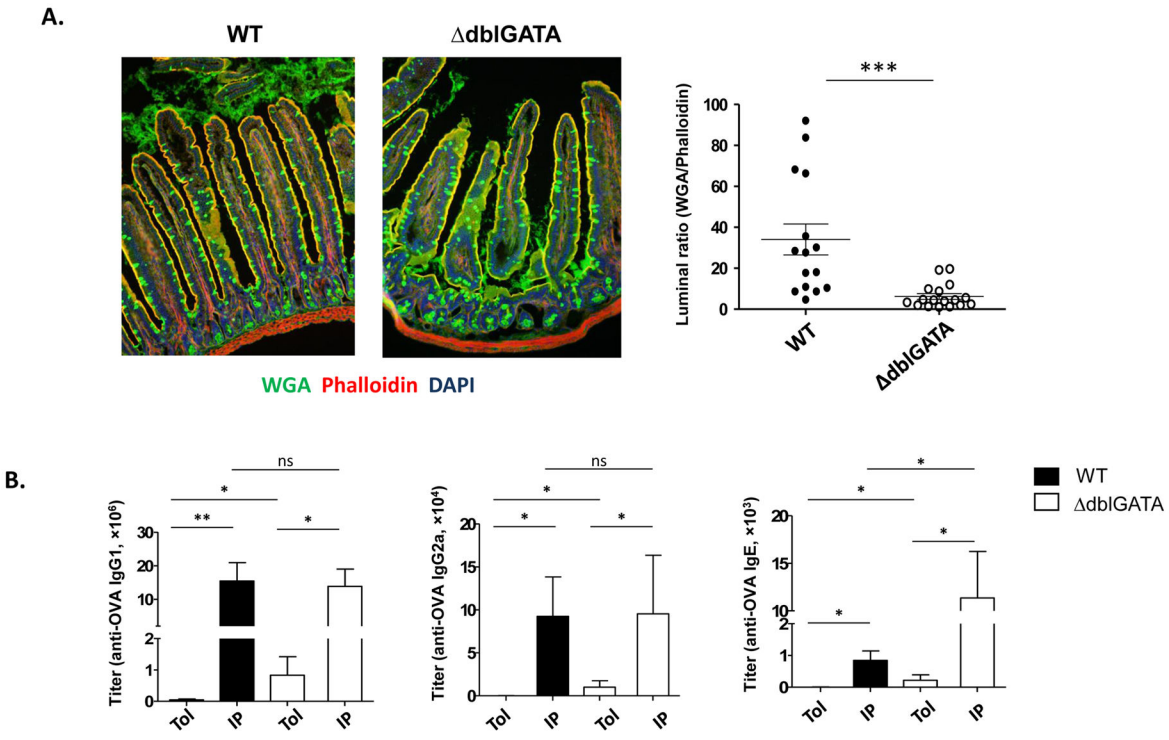

Figure 4. Decreased mucus secretion and less efficient induction of oral tolerance in ΔdblGATA mice

(A) The mucus layer of wild-type (WT) and ΔdblGATA were visualized by immunofluorescence staining of small intestine with wheat germ agglutinin (WGA; green) and phalloidin (red). Sections were ounterstained with DAPI (blue). Original magnification × 40. The WGA-positive and phalloidin-positive luminal areas were measured using Imaris software. Three sections were examined in each group (WT, black circles; ΔdblGATA, white circles) and 5 to 6 fields were selected from 1 section. Data are mean ± standard error of the mean (SEM) values. ***$P < 0.001$ (Student's *t*-test). (B) Ovalbumin (OVA)-specific serum immunoglobulin (Ig) G1, IgG2a, and IgE titers were compared between OVA-fed, sensitized mice (Tol) and sensitized mice without OVA (IP) in WT and ΔdblGATA mice. The data are representative of more than three independent experiments. Data are mean ± standard error of the mean (SEM) values. *$P < 0.05$, **$P < 0.01$ (Student's *t*-test).

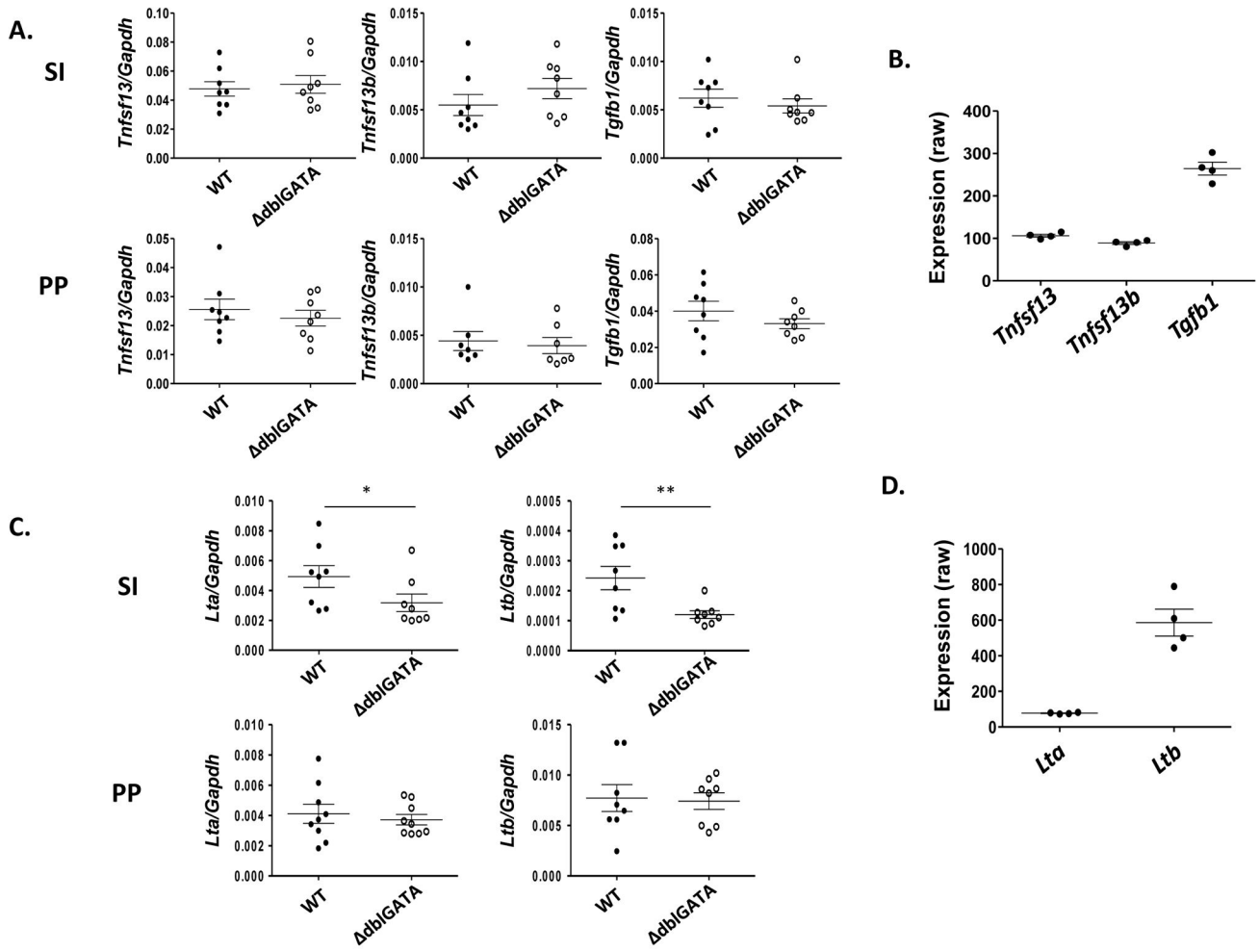

Figure 5. Expression of lymphotoxin α (*Lta)* and β (*Ltb*) are decreased in the small intestine of ΔdblGATA mice

(A) Levels of a proliferation-inducing ligand (*Tnfsf13*), B cell–activating factor of the tumor necrosis factor (*Tnfsf13b*), and transforming growth factor β (*Tgfb1*) mRNA in the small intestine (SI) and Peyer's patches (PP) of wild-type (WT; black circles) and ΔdblGATA (white circles) mice. (B) Eosinophils isolated from the small intestine of WT mice were subjected to genome-wide mRNA microarray using Affymetrix mouse ST 1.0 chip. The raw expression values of *Tnfsf13*, *Tnfsf13b*, and *Tgfb1* were displayed, with a threshold value of 400 units regarded as significant expression. (C) Levels of *Lta* and *Ltb* mRNA in the small intestine and PP of WT and ΔdblGATA mice. (D) The Affymetrix raw expression value of *Lta* and *Ltb* in eosinophils isolated from the small intestine of WT mice. All data, except for microarray analysis, are representative of two or more independent experiments. Data are mean ± standard error of the mean (SEM) values. **P* < 0.05, ***P* < 0.01 (Student's *t*-test).

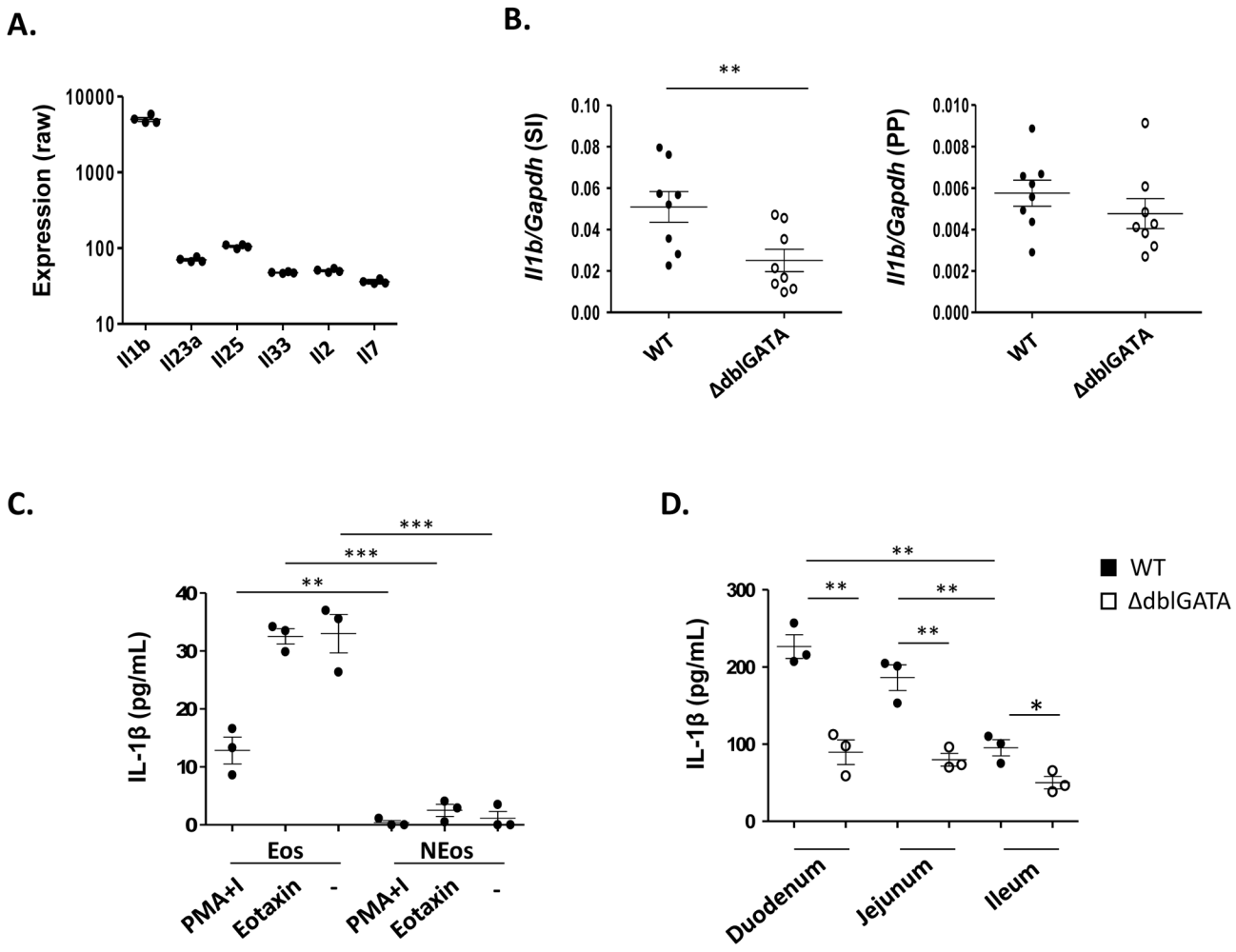

Figure 6. Small intestinal eosinophils actively express IL-1β

(A) The Affymetrix raw expression value of interleukin (IL) 1β (*Il1b*), IL-23 (*Il23a*), IL-25 (*Il25*), IL-33 (*Il33*), IL-2 (*Il2*), and IL-7 (*Il7*) in eosinophils isolated from small intestinal lamina propria of wild-type (WT) mice, with a threshold value of 400 units regarded as significant expression. (B) *Il1b* mRNA levels in the small intestine (SI) and Peyer's patches (PP) of WT (black circles) and ΔdblGATA (white circles) mice. (C) IL-1β production was measured from cultures of small intestinal eosinophils (Eos) or non-eosinophils (NEos, $SSC^{low}CD45^{+}MHC\ II^{+}CD11b^{-}CD11c^{-}$) subsets with the indicated stimuli for 24 hours. (D) IL-1β was detected with the indicated small intestinal segments of WT and ΔdblGATA mice. 1 mg of protein extracted from each of the segment was loaded for ELISA. All data, except for microarray analysis, are representative of two or more independent experiments. Data are mean ± standard error of the mean (SEM) values. $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's *t*-test).

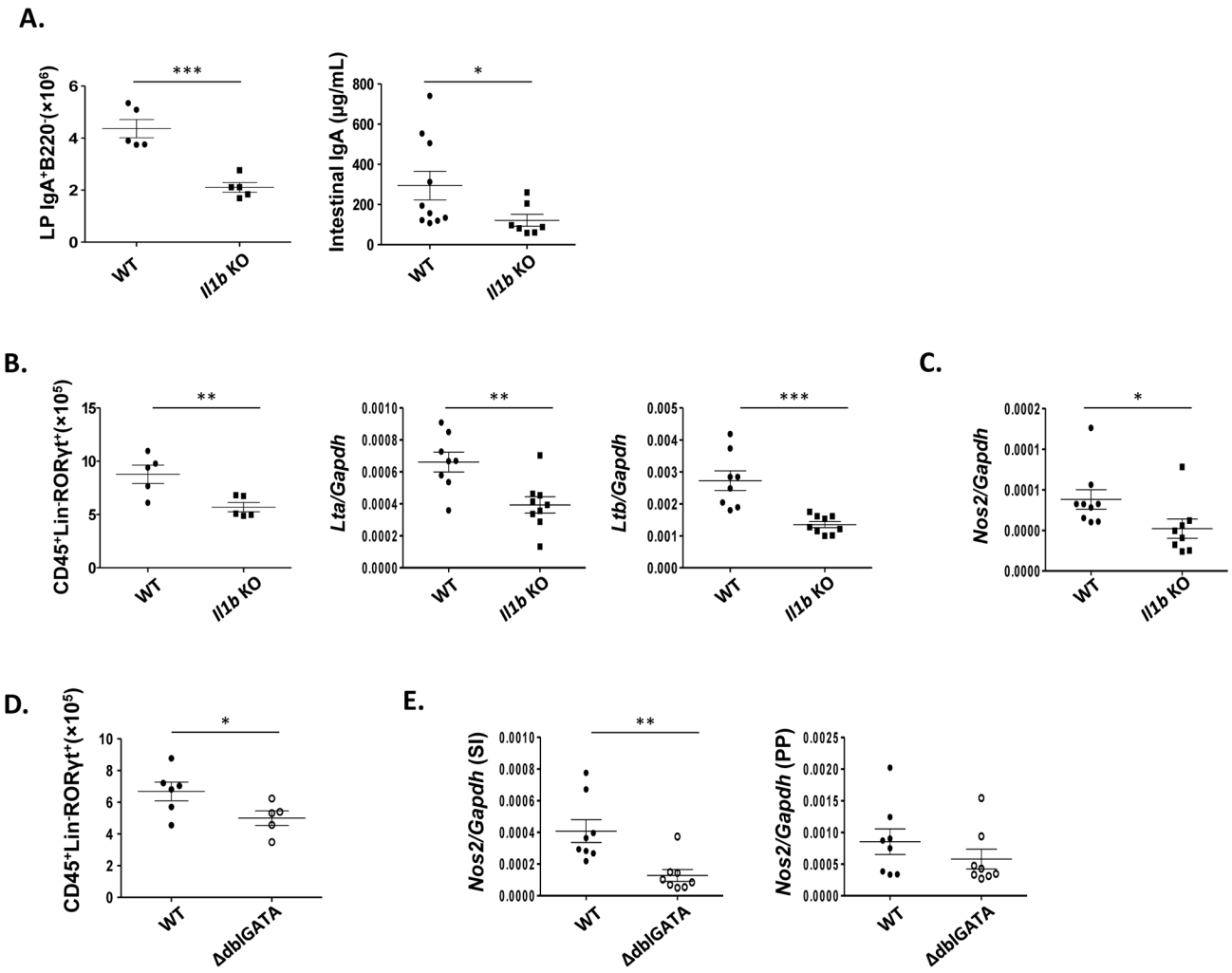

Figure 7. The level of retinoic acid-related orphan receptor gamma t–positive (ROR- γt$^+$) innate lymphoid cells (ILCs) and inducible nitric oxide synthase (iNOS, *Nos2*) expression in interleukin (IL) 1β–deficient (*Il1b* knock out [KO]) and ΔdblGATA mice

(A) Numbers of small intestinal lamina propria (LP) IgA$^+$ cells and intestinal IgA levels in wild-type (WT; black circles) and *Il1b* KO (black squares) mice. (B) ROR- γt$^+$ ILC numbers and expression of lymphotoxin α (*Lta)* and β (*Ltb*) mRNA in the small intestine of WT and *Il1b* KO mice. Lineage marker (Lin) was the combination of CD3, B220, CD11c, and Gr-1. (C) *Nos2* mRNA expressions in the small intestine of WT and *Il1b* KO mice. (D) ROR- γt$^+$ ILC numbers in the small intestine of WT and ΔdblGATA (white circles) mice. (E) *Nos2* mRNA expression in the small intestine (SI) and PP of WT and ΔdblGATA mice. All data are representative of two or more independent experiments. Data are mean ± standard error of the mean (SEM) values. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (Student's *t*-test).

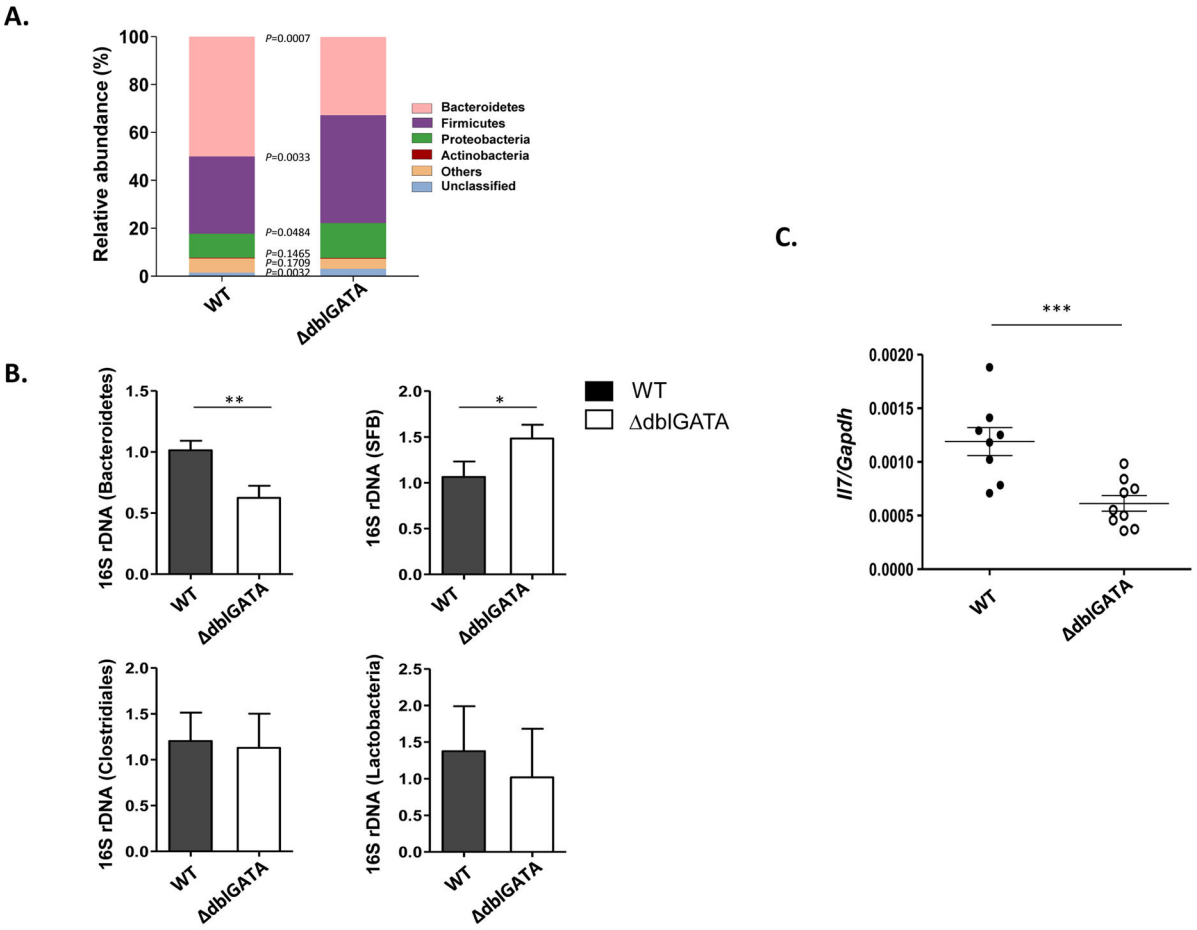

Figure 8. Changes in the microbiota of ΔdblGATA mice

(A) Sequencing analysis of microbiota composition from the stool of wild-type (WT) and ΔdblGATA mice. Representative microbiota composition is presented (n = 9 mice per group). (B) Real-time polymerase chain reaction of microbiota in the stool of WT and ΔdblGATA mice. (C) Interleukin 7 (*Il7*) mRNA expression in the small intestine of WT (black circles) and ΔdblGATA (white circles) mice. All data, except for sequencing analysis, are representative of two or more independent experiments. Data are mean ± standard error of the mean (SEM) values. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (Student's *t*-test).