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

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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 1 β (IL-1 β), inducible nitric oxide synthase, lymphotoxin (LT) α , and LT- β , and reduced levels of retinoic acid-related orphan receptor gamma t-positive (ROR- γ t⁺) 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 (transforming growth factor β). GI eosinophils expressed a relatively high level of IL-1 β , and IL-1 β -deficient mice manifested the altered gene expression profiles observed in eosinophil-deficient mice and decreased levels of IgA⁺ cells and ROR- γ t⁺ 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-1 β 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 the

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small intestine.^{10,11} Recently, lymphotoxin (LT) α (*Lta*) and LT- β (*Ltb*) produced by retinoic acid–related orphan receptor gamma t–positive (ROR- γ t⁺) 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- α , a cognate receptor for CD47, and signal regulatory protein- 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 (mice expressing cytotoxic diphtheria toxin in a promoter of the *Epx*) and Δ dblGATA (mice carrying a targeted deletion of a double palindromic GATA-1 binding site in the *Gata1* promoter)), 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-1 β), *Lta*, *Ltb*, and *Nos2* expression, as well as ROR- γ t⁺ ILCs. In line with reduced *Il1b* in eosinophil-deficient mice and with small intestinal eosinophils being a major source of *Il1b*, IL-1 β –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-1 β –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 Δ dblGATA, *Ccr3* KO, *Ccl11/Ccl24* DKO, and PHIL, had a marked decrease in small intestinal eosinophils defined by CD11b^{high}CD11c^{int} markers (Figure 1a).¹⁷ A

significant reduction of IgA in the serum and intestinal lavage was observed in Δ dblGATA, *Ccr3* KO, and *Ccl11/Ccl24* DKO mice (Figure 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 cytotoxic diphtheria toxin A.¹⁸ Significantly, decreased serum and intestinal IgA levels were also observed in PHIL mice (Figure 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 (post-CSR) IgA⁺ B cells)¹⁰ in the PP of these mice (Figure 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 Δ dblGATA mice also indicated impaired IgA class switching (Supplementary Figure S1A and B online). However, the presence of eosinophils in the culture of total LP cells had no supportive effect on the survival of IgA⁺ cells (Supplementary Figure S1C). In addition, it is unlikely that the B cells of Δ dblGATA are defective in IgA class switching as the expression of activation-induced deaminase and IgA germinal transcript were not impaired in B cells of Δ dblGATA under IgA CSR-inducing conditions (Supplementary Figure S1D and E). In the mesenteric lymph nodes (MLN), no differences were observed in the frequency and number of post-CSR IgA⁺ cells (Figure 1c; 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 Δ dblGATA, *Ccr3* KO, and *Ccl11/Ccl24* DKO mice (data not shown). In addition, integrin α 4 β 7 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 wild-type (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 Δ dblGATA, *Ccr3* KO, *Ccl11/Ccl24* DKO, and PHIL mice (Figure 2a); but no decrease in the frequency of CD4⁺, CD8⁺, or CD19⁺ cells or DCs or 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 (Supplementary Figure S2). Similarly, there was no significant reduction in mononuclear cell populations in these mice (Figure 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 (Figure 2b and c). As total cell numbers of PP were 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

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), Δ dblGATA, CC chemokine receptor 3-deficient (*Ccr3* knockout (KO)), eotaxin-1/2 double-deficient (*Ccl11/Ccl24* double knockout (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 side-scatter (SSC) subset. **(b)** IgA levels in intestinal lavage and serum of WT and eosinophil-deficient mice (Δ dblGATA, *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, Δ dblGATA, *Ccr3* KO, *Ccl11/Ccl24* DKO, and PHIL mice. The IgA⁺ cell number was calculated by multiplying the total cell number of each tissue by the frequency of IgA⁺ cells in that tissue. All data are representative of two or more independent experiments. Data are mean $\pm$ s.e.m. values. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ (Student's *t*-test).

PP were modestly increased in Δ dblGATA and *Ccr3* KO mice (**Supplementary Figure S2**). Δ dblGATA, *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 Δ dblGATA mice

To prove that the IgA deficiency observed in Δ dblGATA 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 Δ dblGATA 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 WT mice and transferred into recipient Δ dblGATA mice. Analysis of the small intestine of reconstituted Δ dblGATA showed significantly increased side-scatter high (SSC^{high}) and CD11b^{high}CD11c^{int} intestinal eosinophils although the degree of increase was modest (**Supplementary Figure S3A**). After 14 days of transfer, recipient mice showed increased numbers of small intestinal IgA⁺B220⁻ cells (**Figure 3a**) and intestinal IgA secretion (**Figure 3b**). We also observed significant restoration of weight and total cell number of PP in recipient mice (**Supplementary Figure S3B**), though IgA⁺B220⁺ number in PP of the recipient mice were not restored to the level of WT mice (**Figure 3a**). The failure of adoptively transferred LP cells isolated from the small intestine of Δ dblGATA to restore IgA levels (**Supplementary Figure S4A**) suggests that the increased IgA in Δ dblGATA following adoptive transfer of WT LP cells was eosinophil dependent. However, it is hard to suggest that

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), Δ dblGATA, CC chemokine receptor 3-deficient (*Ccr3* knockout (KO)), eotaxin-1/2 double-deficient (*Ccl11/Ccl24* double knockout (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 staining of PP in WT and eosinophil-deficient mice. Arrowheads indicate PP in the small intestine. Original magnification $\times 10$. All data are representative of two or more independent experiments with $n \geq 3$ mice. Data are mean $\pm$ s.e.m. values. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Student's *t*-test).

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 (Figure 3c and Supplementary Figure S3C).

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

A significant decrease in IgA synthesis in eosinophil-deficient mice was accompanied by reduced mucus secretion in the small intestine of Δ dblGATA mice as demonstrated by wheat germ agglutinin stains (Figure 4a) and by periodic acid-Schiff stains (Supplementary Figure 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 Δ dblGATA mice (Supplementary Figure 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, Δ dblGATA 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 Figure 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 Δ dblGATA mice compared with the WT mice ($P = 0.0151$, $P = 0.0416$, and $P = 0.0375$, respectively, Figure 4b). Also, as for OVA-specific IgE, the IP group of the Δ dblGATA 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 ($P = 0.4867$; Figure 4b). Taken together, our observations demonstrate that although oral tolerance successfully developed in Δ dblGATA mice, the degree was inefficient compared to WT mice, consistent with the decrease

Figure 3 Effect of adoptively transferred small intestinal lamina propria (LP) cells on immunoglobulin A (IgA) synthesis in Δ dblGATA mice. **(a)** Numbers of IgA⁺ cells in the LP of small intestine and Peyer's patches (PP) of wild-type mice (WT; black circles), Δ dblGATA (eosinophil-deficient; white circles), and Δ dblGATA mice after adoptive transfer of small intestinal LP cells isolated from WT mice (Δ dblGATA WT LP adoptive Tf; white circles). ** $P < 0.01$ and *** $P < 0.001$ (Student's *t*-test). **(b)** IgA levels in serum of Δ dblGATA mice were analyzed over the indicated time period after adoptive transfer of WT LP cells. IgA levels in intestinal lavage of WT, Δ dblGATA mice, and Δ dblGATA mice 14 days after adoptive transfer of WT LP cells. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ (Student's *t*-test). **(c)** Naive 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 $\pm$ s.e.m. values. *** $P < 0.001$ (Student's *t*-test). ns, not significant.

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 Δ dblGATA 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 Δ dblGATA mice. As shown in **Figure 5a**, levels of

Tnfsf13, *Tnfsf13b*, and *Tgfb1* mRNA in the small intestine and PP of Δ dblGATA 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 (**Figure 3c**). Consistent with these results, gene expressions for *Tnfsf13*, *Tnfsf13b*, and *Tgfb1* were not considerable in isolated eosinophils from the small intestine (**Figure 5b**), thus suggesting another mechanism for the decrease in IgA in eosinophil-deficient mice. In addition, expression of matrix metalloproteinase 9 and thrombospondin 1 mRNAs, responsible for the activation of latent form of TGF- β ,^{23,24} were

Figure 4 Decreased mucus secretion and less efficient induction of oral tolerance in Δ dblGATA mice. **(a)** The mucus layer of wild-type (WT) and Δ dblGATA were visualized by immunofluorescence staining of small intestine with wheat germ agglutinin (WGA; green) and phalloidin (red). Sections were counterstained with DAPI (blue). Original magnification $\times 40$. The WGA-positive and phalloidin-positive luminal areas were measured using Imaris software. Three sections were examined in each group (WT, black circles; Δ dblGATA, white circles) and five to six fields were selected from one section. *** $P < 0.001$ (Student's *t*-test). **(b)** Ovalbumin (OVA)-specific serum immunoglobulin (Ig) G1, IgG2a, and IgE titers were compared among OVA-fed, sensitized mice (Tol) and sensitized mice without OVA (IP) in WT and Δ dblGATA mice. The data are representative of more than three independent experiments. Data are mean $\pm$ s.e.m. values. * $P < 0.05$ and ** $P < 0.01$ (Student's *t*-test). ns, not significant.

not decreased in the small intestine of Δ dblGATA mice, although their expressions were considerable in the small intestinal eosinophils (**Supplementary Figure S6A**).

Recently, it has been reported that LT controls IgA production in the small intestine¹² 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 Δ dblGATA mice. As shown in **Figure 5c**, *Ltb* mRNA and *Lta* mRNA, to a lesser extent, were significantly decreased in the small intestine but not in the PP of Δ dblGATA mice. Consistent with this, RNA microarray data from sorted WT small intestinal eosinophils indicate a considerable level of *Ltb* mRNA and barely expressed *Lta* (**Figure 5d**). As the difference of *Lta* between WT and Δ dblGATA mice was reduced when the expression of *Lta* was normalized to leukocyte-specific protein tyrosine phosphatase, receptor-type C (**Supplementary Figure S6B**), the significant decrease of *Lta* in the small intestine of Δ dblGATA mice may be reflective of the decrease of total LP leukocytes in these mice (**Figure 2a**). However, considering the low level of *Lta* mRNA in the WT small intestinal eosinophils, the major population of cells missing in the WT small intestine of Δ dblGATA (**Figure 1a**), we suggest that the distribution of LT- α in other leukocyte subsets could be considerable as LT- α is a soluble protein, whereas LT- β is a membrane-bound molecule associated with leukocytes.²⁶

Small intestinal eosinophils express IL-1 β

Small intestinal eosinophils were found to robustly express a high level of *Il1b* (**Figure 6a**) as determined by the raw signal of RNA microarray of fluorescence-activated cell sorting (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 and not in the PP of Δ dblGATA mice, thus suggesting eosinophils as a major source for IL-1 β production in the small intestine (**Figure 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 the precursor form of IL-1 β ,²⁹ and the production of IL-1 β by small intestinal eosinophils was confirmed by detection of secreted IL-1 β protein from cultured small intestinal LP eosinophils (**Figure 6c**). Accordingly, small intestinal segments of WT mice showed significantly higher IL-1 β levels than those of Δ dblGATA mice, and the expression of IL-1 β in the WT small intestine was decreased from the proximal to the distal segment compatible with intestinal distribution of eosinophils as reported previously (**Figure 6d**).³⁰

IL-1 β regulates SIgA production

Notably, IL-1 β can stimulate proliferation of ROR- γ t⁺ ILCs^{31,32} and also enhance iNOS expression,^{33,34} an enzyme

Figure 5 Expression of lymphotxin α (*Lta*) and β (*Ltb*) are decreased in the small intestine of Δ dblGATA 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 Δ dblGATA (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 Δ dblGATA 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 $\pm$ s.e.m. values. * $P < 0.05$ and ** $P < 0.01$ (Student's *t*-test).

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 **Figure 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 (**Supplementary Figure S7A**). *Il1b* KO mice also showed decreased ROR- γ ⁺ 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, who also had decreased PP weight (**Figure 7b** and **Supplementary Figure S7B**). *Il1b* KO mice also showed decreased *Nos2* expression in the small intestine (**Figure 7c**). Collectively, these findings demonstrate a novel contribution of IL-1 β for the regulation of intestinal immune system and suggest that decreased IL-1 β stimuli in the small intestine are responsible for defective IgA production in Δ dblGATA mice. Consistent with findings in *Il1b* KO mice, Δ dblGATA mice showed decreased numbers of ROR- γ ⁺ ILCs in the LP of the small intestine, which was not restored by adoptive transfer of LP cells isolated from the small intestine of *Il1b* KO mice (**Figure 7d** and **Supplementary Figure S4C**). As a control, expression of c-Kit (a.k.a. CD117) and IL-7R α , which are

surface phenotype markers for ILCs, were comparable between Δ dblGATA and WT mice (**Supplementary Figure S7C**). In addition, we observed a significant decrease in *Nos2* expression in the small intestine but not in the PP of Δ dblGATA mice (**Figure 7e**).

Changes in the microbiota of Δ dblGATA 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 Δ dblGATA revealed a marked increase in Firmicutes-containing segmented filamentous bacteria and a decrease in Bacteroidetes in Δ dblGATA mice (**Figure 8a**), which could be reversed by adoptive transfer of WT LP cells isolated from the small intestine (**Supplementary Figure S8A**). Real-time PCR of selected intestinal commensals verified increased segmented filamentous bacteria and reduced Bacteroidetes in Δ dblGATA mice irrespective of whether they were separated, from or housed with WT mice for >3 weeks (**Figure 8b**; **Supplementary Figure S8B**). Intestinal commensal bacteria affect the development and maturation of organized lymphoid tissue.³⁶ As we observed impaired PP development in Δ dblGATA, we investigated whether aberrant intestinal

Figure 6 Small intestinal eosinophils actively express interleukin 1 β (IL-1 β). (a) The Affymetrix raw expression value of IL-1 β (*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 Δ dblGATA (white circles) mice. (c) IL-1 β production was measured from cultures of small intestinal eosinophils (Eos) or non-eosinophil (NEos, SSC^{low}CD45⁺MHC II⁺CD11b⁻CD11c⁻) subsets with the indicated stimuli for 24 h. (d) IL-1 β was detected with the indicated small intestinal segments of WT and Δ dblGATA 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 $\pm$ s.e.m. values. * P <0.05, ** P <0.01, *** P <0.001 (Student's *t*-test).

microbiota in Δ dblGATA 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 epithelium of the intestine.³⁷ As shown in **Figure 8c**, *Il7* expression was decreased in the small intestine of Δ dblGATA 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 Δ dblGATA 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 intestine,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 in IgA in Δ dblGATA mice is

partially restored by adoptively transferred small intestinal LP cells of WT mice but not by LP cells of Δ dblGATA (**Supplementary Figure S4A**), the acquired immune defect associated with eosinophil deficiency likely results in IgA reduction in Δ dblGATA mice. B cell-depleted LP cells of WT mice also had effects on the increase in IgA in Δ dblGATA mice, thus we suggest that B cells of the recipient mice (but not donor IgA⁺ B cells) account for the restoration of IgA following adoptive transfer (**Figure 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 *Tnfrsf13*, *Tnfrsf13b*, and *Tgfb1* genes for cytokines that directly induce IgA class switching were found to be comparable in the small intestine and PP of Δ dblGATA 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 (**Supplementary Figure S9B**), which are reported to produce IL-6 and directly induce IgA class switching in a TLR5-dependent way.⁴¹ In addition, though Chu *et al.* reported expression of TLR2, 4, 5, 7, and 9 in small intestinal eosinophils, the CD11b⁺ DC is the major subset

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* knockout (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 Peyer's patches (PP) of WT and ΔdblGATA mice. All data are representative of two or more independent experiments. Data are mean ± s.e.m. values. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 (Student's *t*-test).

expressing TLR5 in the isolated small intestinal 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⁺ DCs were not included in eosinophils isolated from the small intestine (Supplementary Figures S3C and S9C), and isolated eosinophils expressed insignificant levels of *Tnfsf13*, *Tnfsf13b*, and *Tgfb1*. Indeed, intestinal *Il1b*, *Lta*, *Ltb*, and *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 (Supplementary Figure S4B and C). The decrease in 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, which was 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 (Supplementary Figure 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 segmented filamentous bacteria and decrease in Bacteroidetes,^{42,43} which are present in ΔdblGATA mice. Notably, Chu *et al.* observed changes in segmented filamentous bacteria and Bacteroidetes that were in the opposite direction of what we observed.³⁹ 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.

Figure 8 Changes in the microbiota of Δ dblGATA mice. **(a)** Sequencing analysis of microbiota composition from the stool of wild-type (WT) and Δ dblGATA mice. Representative microbiota composition is presented ($n=9$ mice per group). **(b)** Real-time PCR of microbiota in the stool of WT and Δ dblGATA mice. **(c)** Interleukin 7 (*Il7*) mRNA expression in the small intestine of WT (black circles) and Δ dblGATA (white circles) mice. All data, except for sequencing analysis, are representative of two or more independent experiments. Data are mean $\pm$ s.e.m. values. * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ (Student's *t*-test). SFB, segmented filamentous bacteria.

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 Δ dblGATA 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 state¹⁶ 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 Δ dblGATA 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-1 β . LT produced by ROR- γ t⁺ 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-1 β promotes proliferation and LT expression of ROR- γ t⁺ ILCs,^{31,32} it is reasonable to suggest that the selective decrease in IL-1 β in the small intestine of Δ dblGATA mice is responsible for the reduction of ROR- γ t⁺ ILCs and LT expression. Our findings are also supported by the decrease in *Nos2*, an essential mediator for LT-dependent IgA production,^{12,49} in the small intestine of *Il1b* KO and Δ dblGATA 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-1 β

production by eosinophils is increased by SIgA,⁵⁰ accordingly *Il1b* mRNA expression was significantly higher in eosinophils isolated from the small intestine 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 (Supplementary Figure S10), suggesting a positive feedback loop between IL-1 β -expressing eosinophils and IgA for the immune homeostasis in the gut.

The decrease in 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 Δ dblGATA 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 significant differences between the Δ dblGATA and WT mice in the frequencies of CD103⁺ DCs and regulatory T cells, cells that are responsible for the development of oral tolerance^{51,52} (Supplementary Figure S11). Considering the critical role of TGF- β in the development of regulatory T cells,⁵³ normal frequencies of regulatory T cells in Δ dblGATA 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- γ t⁺ ILC number and in expression of *Lta/Ltb* and *Nos2* indicate the eosinophils' role in the modulation of LT signaling-mediated IgA class switching. In addition, the decrease in IL-1 β in the small intestine of eosinophil-deficient mice and the reduced production of IgA in *Il1b* KO mice suggest that IL-1 β 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 on eosinophil-derived APRIL and BAFF.

METHODS

Mice. *Ccl11/Ccl24* DKO and *Ccr3* KO mice were generated as previously described.⁴⁰ Δ dblGATA were provided by Dr Stuart H Orkin (Howard Hughes Medical Institute, Chevy Chase, MD), PHIL mice were provided by James J Lee (Mayo Clinic, Rochester, MN), and *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% fetal calf serum (FCS), 20 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 100 U ml⁻¹ penicillin, 100 μ g ml⁻¹ streptomycin, 1 mM sodium pyruvate, 10 mM EDTA, and 10 μ g ml⁻¹ polymyxin B) for 30 min 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⁻¹ collagenase A (Roche, Mannheim, Germany) and 10 μ g ml⁻¹ DNase I (Roche) in RPMI 1640/10% FCS with continuous stirring at 37 °C for 30 min. 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 min at 37 °C. After washing, the cells were subjected to density-gradient centrifugation in 40%/75% Percoll (GE Healthcare, Piscataway, NJ). The cells harvested from the interface were washed and used as leukocytes in assays. For adoptive transfer assay, 1 $\times$ 10⁷ isolated small intestinal LP cells were transferred into Δ dblGATA 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, San Jose, CA) for 15 min at 4 °C, the cells were stained with antibodies against various markers. Anti-mouse B220 (RM3-6B2), CD11c (HL3), and ROR- γ t (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 (San Diego, CA). 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- γ t staining. Each sample was acquired with a FACSCalibur (BD Biosciences), and the data were processed with FlowJo software (Tree Star, Ashland, OR).

Cell culture. Cecal bacterial antigen was prepared as previously described.³⁹ To induce IgA class switching, 1 $\times$ 10⁵ of SSC^{low} CD45⁺ MHC II⁺ IgM⁺ CD19⁺ B cells isolated from the small intestine of WT mice were cultured with 100 μ g ml⁻¹ cecal bacterial antigen or APRIL (100 ng ml⁻¹) plus BAFF (50 ng ml⁻¹). 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-1 β secretion, sorted small intestinal eosinophils were cultured for 24 h in the presence of IL-5 (10 ng ml⁻¹). In some experiments, phorbol myristate acetate (20 ng ml⁻¹) and ionomycin (1 μ M) or eotaxin-1 (25 ng ml⁻¹) were added for the stimulation of eosinophils.

Enzyme-linked immunosorbent assay. For determination of total IgA, 96-well plates were coated with anti-mouse IgA (C10-3, BD Pharmingen, San Diego, CA) and blocked with PBS/bovine serum albumin. Diluted serum or intestinal washes were incubated, and biotinylated anti-mouse IgA (C10-2, BD Pharmingen) was added. After reacting with streptavidin-horseradish peroxidase (HRP), the plates were developed with 3,3',5,5'-tetramethylbenzidine (TMB) substrates (BD Pharmingen), and the absorbance was read at 450 nm. A mouse IL-1 β ELISA kit (R&D Systems, Minneapolis, MN) was used according to the manufacturer's instructions to measure the levels of IL-1 β in cell culture supernatants and small intestinal segments of WT and Δ dblGATA 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 min and permeabilized in 0.2% Triton X-100 (Fisher Scientific, Waltham, MA)/PBS for

10 min. The slides were blocked with 1% bovine serum albumin/PBS for 1 h at room temperature and incubated with Alexa Fluor 488 wheat germ agglutinin (WGA, Molecular Probes, Eugene, OR) and Alexa Fluor 568 Phalloidin (Molecular Probes) for 30 min. Sections were washed three times with PBS after each antibody incubation. After a 10-min 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, Center Valley, PA). WGA-positive luminal signals were quantified using Imaris software (Bitplane, Zurich, Switzerland).

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, St. Louis, MO).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, Hilden, Germany) and subsequently column-purified with an RNeasy mini kit (Qiagen). RNA (500 ng) was treated with DNase I (New England Biolabs, Ipswich, MA), and cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). 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 **Supplementary 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 FACSria (BD Biosciences). Total RNA from sorted eosinophils was extracted by standard TRIzol RNA isolation (Invitrogen, Carlsbad, CA) and subsequently column-purified with an RNeasy mini kit (Qiagen). mRNA integrity was validated by the Agilent 2100 bio-analyzer (Agilent Technologies, Clara, CA). Eosinophil mRNA was amplified and labeled with the WT-Ovation Pico RNA amplification system (NuGen, San Carlos, CA) and subjected to the GeneChip Mouse Gene ST 1.0 Array chip (Affymetrix, Santa Clara, CA). 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 (*Prp2*)) and eosinophil non-expressed glucagon (*Gcg*; **Supplementary Figure 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 **Supplementary Table S1**. For deep sequencing, 16S rRNA was amplified using a combination of the universal bacterial/archaeal primers 515F and 806R.⁵⁸ The 16S rRNA sequence analysis was performed via MiSeq Reporter v2.3.32 (Illumina, San Diego, CA).

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

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at <http://www.nature.com/mi>

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DISCLOSURE

M.E.R. is a consultant for Immune Pharmaceuticals, Celsus Therapeutics and Receptos and has an equity interest in each. He is an inventor of eosinophilic esophagitis-related patents owned by Cincinnati Children's Hospital Medical Center, some of which have been licensed to Diagnostivus. He has a royalty interest in reslizumab, a drug being developed by Teva Pharmaceuticals. T.W. is a co-inventor of eosinophilic esophagitis-related patents owned by Cincinnati Children's Hospital Medical Center, some of which have been licensed to Diagnostivus.

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