Original research

Immunoglobulin A–specific deficiency induces spontaneous inflammation specifically in the ileum

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ABSTRACT

Objective Although immunoglobulin A (IgA) is abundantly expressed in the gut and known to be an important component of mucosal barriers against luminal pathogens, its precise function remains unclear. Therefore, we tried to elucidate the effect of IgA on gut homeostasis maintenance and its mechanism.

Design We generated various IgA mutant mouse lines using the CRISPR/Cas9 genome editing system. Then, we evaluated the effect on the small intestinal homeostasis, pathology, intestinal microbiota, cytokine production, and immune cell activation using intravital imaging.

Results We obtained two lines, with one that contained a <50 base pair deletion in the cytoplasmic region of the IgA allele (IgA tail-mutant; $IgA^{tm/tm}$) and the other that lacked the most constant region of the IgH α chain, which resulted in the deficiency of IgA production ($IgA^{-/-}$). $IgA^{-/-}$ exhibited spontaneous inflammation in the ileum but not the other parts of the gastrointestinal tract. Associated with this, there were significantly increased lamina propria $CD4^+$ T cells, elevated productions of IFN-γ and IL-17, increased ileal segmented filamentous bacteria and skewed intestinal microflora composition. Intravital imaging using Ca^{2+} biosensor showed that $IgA^{-/-}$ had elevated Ca^{2+} signalling in Peyer's patch B cells. On the other hand, $IgA^{tm/tm}$ seemed to be normal, suggesting that the IgA cytoplasmic tail is dispensable for the prevention of the intestinal disorder.

Conclusion IgA plays an important role in the mucosal homeostasis associated with the regulation of intestinal microbiota and protection against mucosal inflammation especially in the ileum.

Significance of this study

What is already known on this subject?

- Immunoglobulin A (IgA) is the most abundantly expressed antibody (Ab) in both humans and mice. IgA deficiency has been reported to increase susceptibility to colon injury in mice, and is associated with various diseases in humans such as inflammatory bowel diseases (IBD), allergies, autoimmune diseases and recurrent infections.

What are the new findings?

- IgA deficiency in mice exhibited spontaneous inflammation in the ileum but not in the colon.
- Gut microbiota were affected significantly in the ileum by IgA deficiency.
- Ca^{2+} signalling in B cells in Peyer's patches was augmented by IgA deficiency.
- IgA deficiency impacts the ileum rather than the colon.

How might it impact on clinical practice in the foreseeable future?

- Our findings underlie a novel target for the diagnosis and treatment of patients with Crohn's disease and/or IgA deficiency through the mechanism by which ileitis may be induced.

INTRODUCTION

It is suggested that immunoglobulin A (IgA), the most abundantly expressed antibody (Ab) in both humans and mice, is known to be important for mucosal defence.[1–5] In humans, IgA deficiency is associated with various diseases such as inflammatory bowel diseases (IBD), allergies, autoimmune diseases and recurrent infections.[6] Further understanding of the role of IgA would benefit from animal model(s) that mimic human pathophysiology. Although a previous study of the IgA-deficient mice did not show a remarkable phenotype,[7] a more recent study found that IgA is crucial for gut homeostasis, especially in neonatal mice, where IgA deficiency increased susceptibility to colon injury.[8] Other studies had suggested the importance of secreted Ig in the lumen of the gut. A study of activation-induced cytidine deaminase (AID)–deficient mice, which exhibit impaired Ig class switching and hyper-somatic mutation, suggested that IgA is required to maintain the gut microbiota.[9] Other studies with AID^{G23S} mutant mice, which also exhibit impaired hyper-somatic mutation, confirmed that high-affinity Abs are crucial for gut microbiota maintenance.[10] A lack of programmed cell death protein 1 (PD-1), which

mediates a T cell–B cell interaction that affects Ig production, can lead to impaired high-affinity Ab production and dysbiosis.[11] In summary, high-affinity Abs appear to be crucial for gut flora maintenance.

The membrane-bound forms of class-switched IgG, IgE and IgA contain longer cytoplasmic tails than that of IgM/IgD. In IgG and IgE, these tails are composed of 28 amino acids (aa) and found to be crucial for Ab production.[12 13] In contrast, IgA has a relatively short cytoplasmic tail of 14 aa,[14] and its role in IgA responses has not been defined.

Our research group has recently established a conditional expression of Ca^{2+} biosensor, named Yellow Cameleon 3.60 (YC3.60), in a transgenic mouse line that facilitates the analyses of both dynamics and signal transduction in living animals.[15] We previously used these mice to demonstrate abnormal Ca^{2+} signalling as an indicator of predisposition to autoimmune disease at the very early phase of disease development. Furthermore, we also established an intravital imaging system to evaluate Peyer's patches (PP) and epithelial layer in intestine.[15–17]

Several novel genome editing technologies have recently been developed. Among these, the CRISPR/Cas9 system comprises a powerful strategy for the generation of genetically engineered mice.[18] One advantage of this method is the ability to introduce multiple allelic mutations during a one-step experiment. In this study, we aimed to generate various IgA mutations in mice, including deletion of either the entire Ig heavy chain (H) α or the cytoplasmic domain of IgA, using the CRISPR/Cas9 system in order to clarify the role of IgA. Moreover, we analysed CRISPR/Cas9-mediated IgA mutant mice using conventional and novel experimental techniques, including our newly established intravital imaging system.[15–17] Here, we show spontaneous pathology in the intestines of mice that are IgA deficient.

MATERIALS AND METHODS

Mice

Various IgA mutant mouse lines were obtained using the CRISPR/Cas9 genome editing system. Details were described in online supplemental materials and methods. The CD19-Cre/YC3.60^{flox} mouse line was described previously.[15]

Antibiotic (ABx) treatment of the mice was performed as follows. Mice were administrated ABx via drinking water containing neomycin (1.0 mg/mL), ampicillin (1.0 mg/mL), metronidazole (1.0 mg/mL) and vancomycin (0.5 mg/mL) for 3 weeks. Alternatively, mice were administered vancomycin (0.5 mg/mL) for 1 week.

All mice were maintained in the animal facility of Tokyo Medical and Dental University (TMDU) under specific pathogen-free conditions in accordance with the animal care guidelines of TMDU.

Flow cytometry

Cells were analysed on a CyAn ADP (Beckman Coulter, Brea, CA),[19] MACSQuant (Miltenyi Biotec, Bergish Gladbach, Germany) or FACSCanto II (BD Biosciences, San Jose, CA) flow cytometer. The specific antibodies (Abs) used are described in online supplemental materials and methods.

Measurement of immunoglobulin levels

Serum Ig levels were measured as described previously,[15] using enzyme-linked immunosorbent assays (ELISAs). Alternatively, 100 mg of ileum and faeces were suspended in 1 mL of phosphate-buffered saline (PBS) and their supernatants were subjected to ELISAs. The results were subjected to an unpaired Tukey-Kramer's test for statistical analysis. P value <0.05 was considered statistically significant.

Histopathological assessment

Male and female mice were euthanised at 6–10 weeks of age, and organs and tissues were collected for histopathological assessment. All specimens were fixed with 4% paraformaldehyde (Wako, Tokyo, Japan) for 24 hours and subsequently embedded in paraffin. Later, samples were cut into 4 μm thick sections and stained with hematoxylin and eosin (H&E). The stained sections were analysed without prior knowledge of genotype. The severity of inflammation in small and large intestines was defined using modified versions of previously described scoring systems.[17 20 21] Details are described in online supplemental materials and methods.

Scanning electron microscopy

Scanning electron microscopy was performed using the modified version of a previously described protocol[22] and described in online supplemental materials and methods.

Cell isolation

Single cells were isolated from spleens (SPL), mesenteric lymph nodes (MLN) and PP using 40 µm cell strainers (BD Biosciences) and ACK solution for red blood cell depletion. Lamina propria lymphocytes (LPL) from either small or large intestine were also isolated as previously described.[17 23]

Cytokine assay

To measure cytokine production, 5×10^5 lymphocytes from SPL, MLN or intestinal LPL were cultured in 100 µL of complete RPMI 1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 500 U/mL penicillin, 100 µg/mL streptomycin (Sigma-Aldrich), 10 mM HEPES, 1% nonessential aa and 50 µM β-mercaptoethanol (Life Technologies, Carlsbad, CA) in the presence of 5 µg/mL plate-bound anti-CD3ε (145-2 C11) and 2 µg/mL soluble anti-CD28 (37.51) Abs on flat-bottomed 96-well plates at 37°C in 5% CO_2 for 48 hours; LPL were additionally treated with 10 µg/mL gentamycin (Nacalai Tesque, Kyoto, Japan). Culture supernatants were harvested and analysed to assess the production of cytokines using ELISAs specific for interferon (IFN)-γ, interleukin (IL)−4, IL-10 (BD Biosciences) and IL-17A (R&D Systems, Minneapolis, MN) according to the manufacturers' instructions. Data are expressed as means±standard error of the mean (SEM), and statistical significance was determined using Tukey-Kramer's test. A p value <0.05 was considered significant.

DNA extraction, preparation of 16S rRNA gene amplicon sequence library and metagenomic analysis

Details are described in online supplemental materials and methods.

Intravital microscopy

Intravital imaging of PP was performed using A1 laser scanning confocal microscope with a ×20 objective and NIS-Elements AR software (Nikon, Tokyo, Japan) as described previously.[15] The yellow fluorescent protein (YFP):cyan fluorescent protein (CFP) ratio was obtained at an excitation wavelength of 458 nm. Data are expressed as means±standard deviation (SD), and statistical significance was determined using Student's t-test. P values <0.05 were considered significant.

RESULTS

Generation of mice lacking either the cytoplasmic domain or the entire heavy chain in IgA genes

To generate IgA-deficient and tail-mutant mouse lines, we designed guide RNAs for the IgE and IgA tails (online supplemental figure S1) and injected these and Cas9 mRNA into C57BL/6 zygotes. Fourteen pups were born from 261 embryos that had been transferred into foster mothers. At the end, we were able to obtain eight surviving mice, which produced offspring. They were analysed by sequencing PCR products of the corresponding region between IgE and IgA tails (online supplemental figure S2A,B). All of these mice harboured at least one mutant allele, indicating that the CRISPR/Cas9-mediated system had worked efficiently. Most of these mice harboured multiple mutant alleles, and two (lines 4 and 8) possessed all four mutant alleles. Although most mutations were short deletions or insertions, lines 5 and 12 harboured long deletions. Line 5 lacked a sequence of >5 kilobase pairs (Kb) between the 3′ region of IgHε and IgHα, resulting in an entire deletion of the IgHα constant region (online supplemental figure 3A) with a slight mutation in the IgE carboxy (C) terminus. Line 12 lacked the cytoplasmic tail of IgA and consequently possessed additional aa residues (online supplemental figure 3B). We generated the homozygous IgHα deletion ($IgA^{-/-}$) and IgA tail-mutant ($IgA^{tm/tm}$) mice derived from lines 5 and 12, respectively.

Characterisation of IgA mutant mouse lines

The cytoplasmic tails of IgHγ and IgHε were previously shown to exert important functions.[12 13] Therefore, in addition to the mice with deficiency of the entire IgHα ($IgA^{-/-}$), we also analysed another mice lacking the cytoplasmic region ($IgA^{tm/tm}$). We initially examined serum levels of different Ig classes and found that $IgA^{-/-}$ had similar or slightly higher levels of IgM, IgG and IgE when compared with the wild-type (WT) mice (figure 1A), and as expected, it did not harbour detectable levels of IgA. In contrast, $IgA^{tm/tm}$ and WT mice had similar serum IgA levels (figure 1B), which suggests that the tail sequence is dispensable for serum IgA production.

We also analysed ileal and faecal IgM and IgG in $IgA^{-/-}$ mice whether they compensate IgA deficiency in gut lumen. Ileal IgG was significantly increased in $IgA^{-/-}$ mice, although ileal IgM was not altered (figure 1C). Furthermore, both of faecal IgM and IgG were increased in $IgA^{-/-}$ mice (figure 1D). These results indicate that other classes of Igs compensate IgA deficiency in the gut.

We further analysed T cells and B cells in lymphoid tissues of $IgA^{-/-}$ mice (figure 2). B cell populations were not altered in the SPL and PP, but increased in the MLN of $IgA^{-/-}$ (figure 2A-C). $CD21^{+}CD23^{-}$ marginal zone B cells of $IgA^{-/-}$ were also increased (figure 2D). Furthermore, $B220^{+}GL7^{+}$ germinal centre B cells were increased in the MLN of $IgA^{-/-}$, but $B220^{-}CD138^{+}$ plasma cells and $B220^{+}CD86^{+}$ B cells were not altered (figure 2E-G). On the other hand, $CD4^{+}$ T cells and $CD8^{+}$ T cells were not altered in $IgA^{-/-}$. $CD4^{+}CD69^{+}$ T cells were also not altered (figure 2H). $CD4^{+}CD44^{+}CD62L^{-}$ effecter memory T cells were slightly increased while $CD4^{+}CD44^{-}CD62L^{+}$ naive T cells were not altered in PP (figure 2I,J). Furthermore, follicular helper T (Tfh) cells were not altered (figure 2K,L). Therefore, some B and T populations were slightly altered in some lymphoid tissues as a result of IgA deficiency. On the other hand, in $IgA^{tm/tm}$ mice, B populations were substantially the same as those of WT mice (online supplemental figure S4).

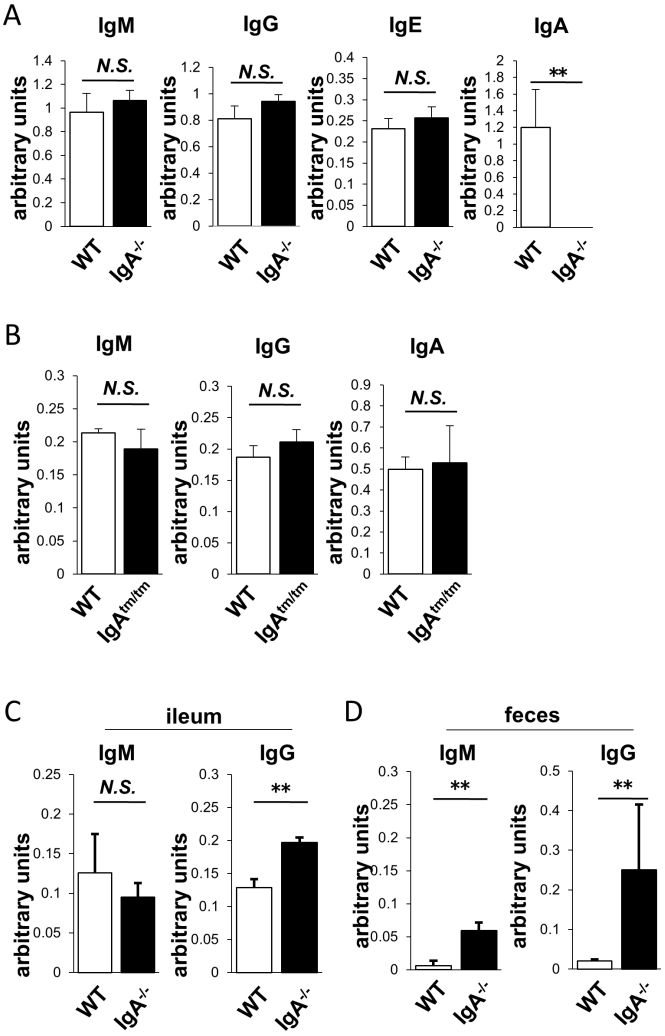

Figure 1 Ig levels in $IgA^{-/-}$ and $IgA^{tm/tm}$. (A) Serum IgM, IgG, IgE and IgA levels in WT (open) and $IgA^{-/-}$ (closed). (B) Serum IgM, IgG and IgA levels in WT (open) and $IgA^{tm/tm}$ (closed). (C) Ileal IgM and IgG levels in WT (open) and $IgA^{-/-}$ (closed). (D) Fecal IgM and IgG levels in WT (open) and $IgA^{-/-}$ (closed) (n>3). Values are presented as means±SD. *N.S.*, not significant. $^{**}p<0.01$.

$IgA^{-/-}$ mice exhibited inflammatory disorder in the small intestine

We next performed a histological assessment of the intestines in $IgA^{-/-}$ and $IgA^{tm/tm}$ mice. Compared with WT (figure 3A), histopathological analysis of $IgA^{-/-}$ revealed several inflammatory lesions including mild lymphocyte infiltrations with slightly thickened wall (figure 3B), cryptitis and/or crypt abscesses (figure 3C), and reduced goblet cells (figure 3D) specifically in the ileum. These pathological findings were significant based on histopathological quantification of these changes defined by the scoring system in the online supplemental materials and methods section. $IgA^{-/-}$ treated with antibiotics (ABx) ameliorated these histological scores (figure 3E), suggesting that microbiota may mediate these abnormalities. Interestingly, these pathological abnormalities were not observed in the other parts of the gastrointestinal tract including the jejunum (data not shown) and the colon (figure 3F) of $IgA^{-/-}$. We also analysed younger $IgA^{-/-}$ mice and found the pathological abnormalities in 6-week-old mice (online supplemental figure S5). The pathological abnormalities appear to be in an age-dependent manner. In contrast,

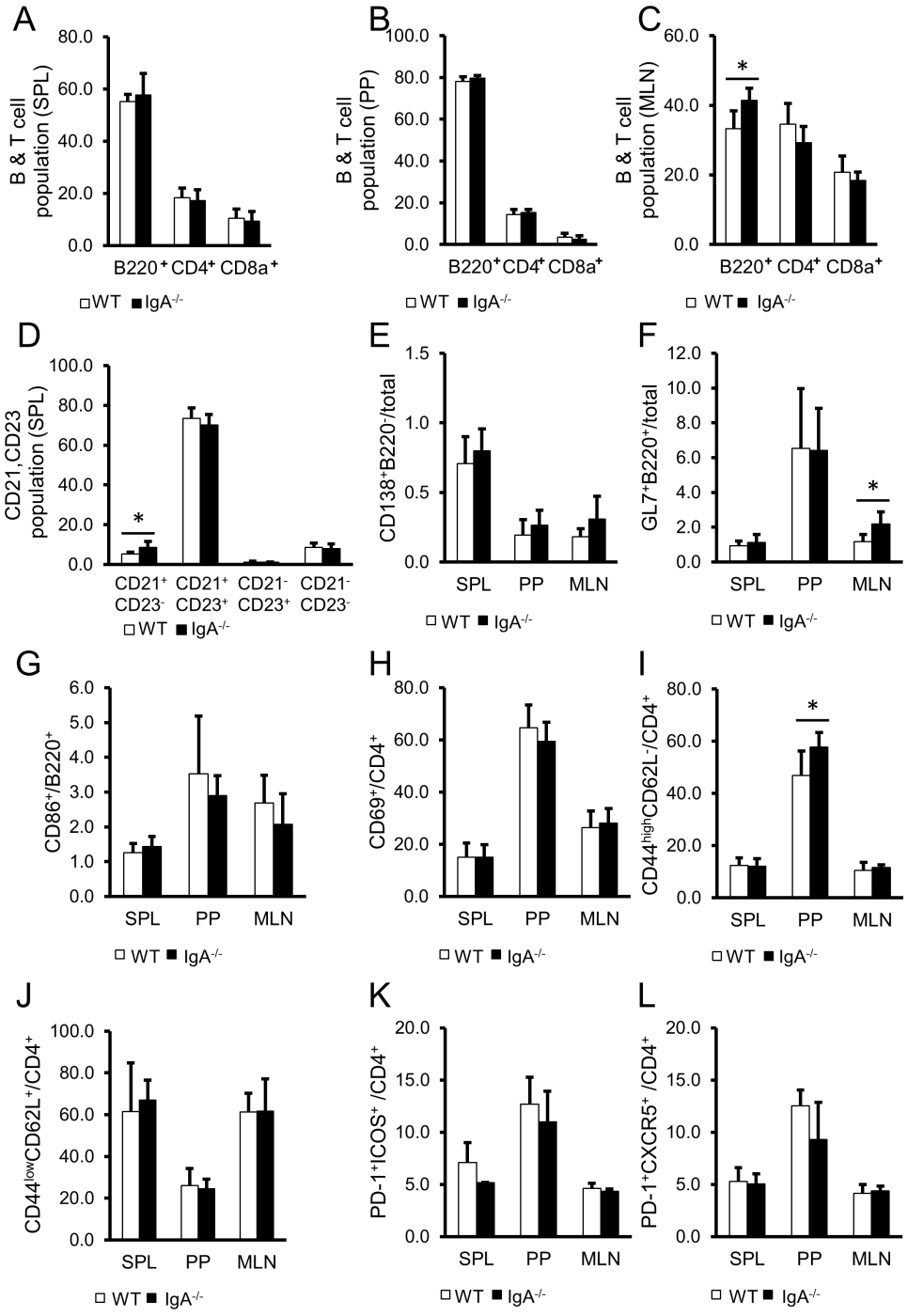

Figure 2 Analysis of lymphoid tissue cell populations in the spleen (SPL), Peyer's patches (PP) and mesenteric lymph nodes (MLN) in WT (open) and IgA$^{-/-}$ (closed). (A-C) Percentage of either B220$^+$, CD4$^+$ or CD8a$^+$ cells in SPL (A), PP (B) and MLN (C). (D) Percentage of CD21$^+$CD23$^-$ marginal zone B cells and CD21$^+$CD23$^+$ follicular B cells in CD19$^+$ population at SPL. (E-L) Percentage of B220$^-$CD138$^+$ plasma cells (E), and B220$^+$GL7$^+$ germinal centre B cells (F) in total cells, CD86$^+$ B cells in B220$^+$ population (G), CD69$^+$ T cells (H), CD44^{high}CD62L$^-$ effecter memory T cells (I), CD44^{low}CD62L$^+$ naive T cells (J), PD-1$^+$ICOS$^+$ Tfh cells (K), PD-1$^+$CXCR5$^+$ Tfh cells (L) in CD4$^+$ population . Data are expressed as means±SD. WT: n=7, IgA$^{-/-}$: n=8. *p<0.05 (t-test).

these pathological abnormalities were not observed in IgA$^{tm/tm}$ mice (data not shown).

CD4$^+$ T cells were increased in the ileal lamina propria of IgA$^{-/-}$ mice

We next analysed the properties of lymphocytes isolated from the ileal tissues. CD4$^+$ T-cell populations were increased in the ileal lamina propria of IgA$^{-/-}$ (figure 4A,B). Next, we evaluated the ability of these T cells to produce cytokines including IFN-γ, IL-4, IL-10 and IL-17. Production of IFN-γ and IL-17 were significantly increased in the ileal lamina propria CD4$^+$ T cells from IgA$^{-/-}$ (figure 4C,D). A tendency towards decreased IL-4 production was also observed in IgA$^{-/-}$ (figure 4E). IgA$^{-/-}$ and WT mice exhibited similar levels of IL-10 production (figure 4F). ABx treatment lowered the cytokine production in IgA$^{-/-}$, suggesting that microbiota were responsible for the

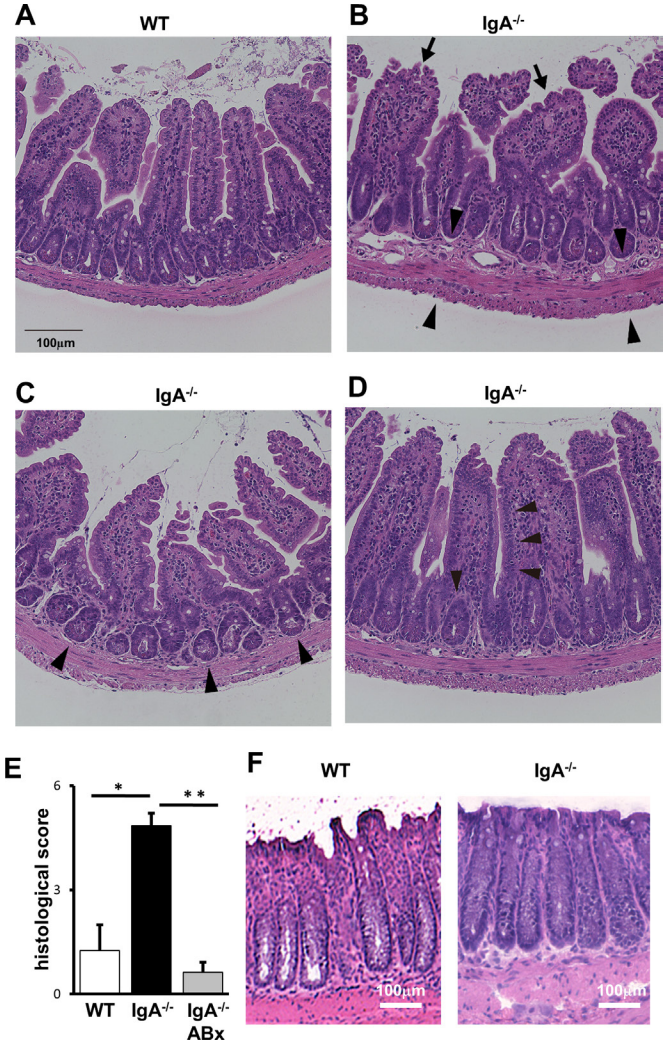

Figure 3 Pathological changes in the ileum tissues of IgA$^{-/-}$. Tissues of WT (A) and IgA$^{-/-}$ (B–D) were fixed and stained with H&E. Normal appearance at ileum of WT (A), mild infiltration with mononuclear cells in the lamina propria (arrows in B), thickened submucosal, muscular and serosal layers (arrowheads in B), crypt abscesses (arrowheads in C), reduced goblet cells (arrowheads in D) at ileum of IgA$^{-/-}$ are indicated. Representative results are shown (n>7). (E) Pathological evaluation of the ileum. WT (left), IgA$^{-/-}$ (middle) and antibiotic (ABx)-treated IgA$^{-/-}$ (right) mice were subjected for the analysis. Scores were determined as described in online supplemental materials and methods. Values are presented as means±SEM. *p<0.05. **p<0.01. (F) Histological evaluation of the colons from WT (left) and IgA$^{-/-}$ (right). Representative results are shown (n>7).

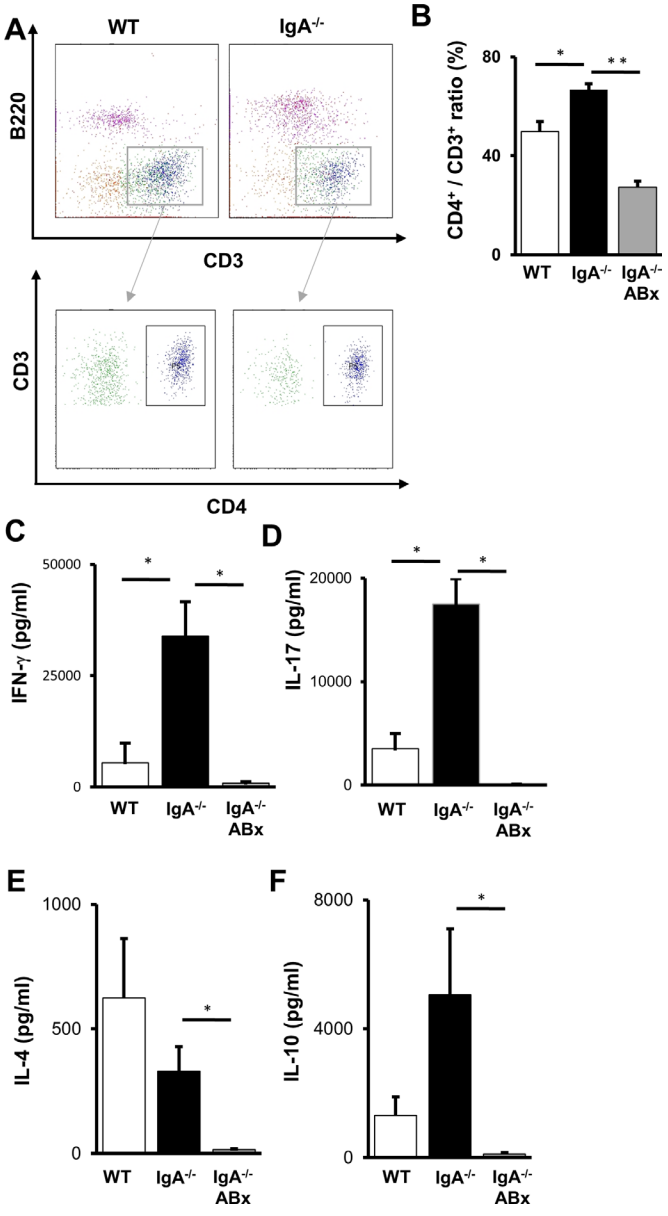

Figure 4 Analysis of ileal lamina propria CD4$^+$ T cells of IgA$^{-/-}$. (A) Flow cytometric analysis of the ileal lamina propria lymphocytes of WT (left) and IgA$^{-/-}$ (right). B220$^-$CD3$^+$ cells were gated and then opened by CD3 and CD4. Representative data from each group are shown. (B) The ratios of stained CD3$^+$ and CD4$^+$ T cells from the lamina propria of WT (left), IgA$^{-/-}$ (middle) and ABx-treated IgA$^{-/-}$ (right). Data are expressed as means±SEM. (n>3). *p<0.05. **p<0.01. (C–F) Cytokine production from the ileal lamina propria CD4$^+$ T cells of WT (left), IgA$^{-/-}$ (middle) and ABx-treated IgA$^{-/-}$ (right) after stimulation with anti-CD3e and anti-CD28 Abs. The concentrations of IFN-γ (C), IL-17 (D), IL-4 (E) and IL-10 (F) in culture supernatants were determined (n>3). Values are presented as means±SEM (n>3). N.S., not significant. *p<0.05.

cytokine induction. In summary, IgA deficiency also affected cytokine production by the CD4$^+$ T cells located at the ileum.

Microbial composition in the gut was skewed by IgA deficiency

To assess the influence of small intestinal inflammation on gut microbiota composition in IgA$^{-/-}$, we initially evaluated segmented filamentous bacteria (SFB), which has been reported to be associated with helper T (Th)17 inflammation.[24] Scanning electron microscopic analysis revealed profoundly increased SFB in the ileum of IgA$^{-/-}$ similar to those of recombination activating gene 2 (Rag2)–deficient mice as a positive control, whereas these microbes were rarely detected in WT (figure 5A). However, almost no SFB were observed in the IgA$^{-/-}$ when treated with ABx. In addition, SFB was not increased in the ileum of IgA$^{tm/tm}$ mice (online supplemental figure S6). These studies suggest SFB may be one of factors associated with the inflammation observed in the ileum of IgA$^{-/-}$.

We further analysed the bacterial composition in the small intestines and faeces using 16S rRNA gene amplicon sequencing (online supplemental figures S7, S8 and table 1). In the small intestine, WT and IgA$^{-/-}$ differed significantly with respect to

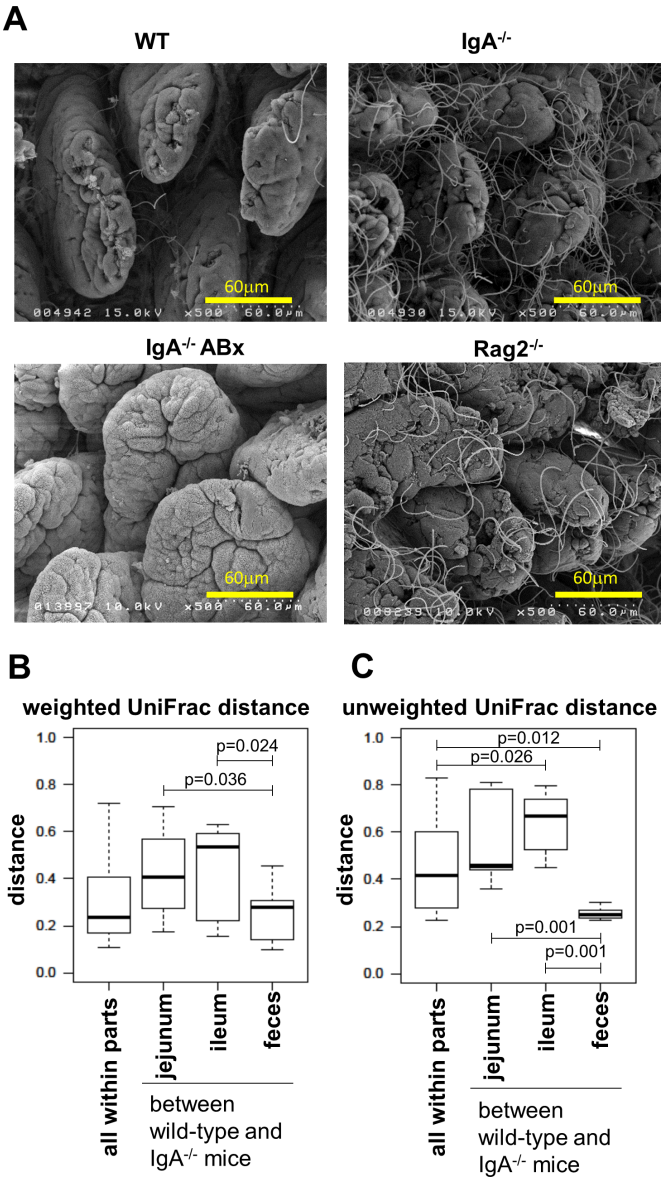

Figure 5 Analysis of microbiota in the gut. (A) Analysis of SFB on the ileal mucosa. The ileal samples from WT (top left), IgA$^{-/-}$ (top right), ABx-treated IgA$^{-/-}$ (bottom left) and Rag2$^{-/-}$ (bottom right) mice were subjected to scanning electromicroscopic analysis. Representative results from each group are shown (n>3). (B and C) Weighted (B) and unweighted (C) UniFrac distances between WT and IgA$^{-/-}$ mice. The extracted samples from either the mixture of small intestine and colon, the isolated jejunum, ileum or faeces were subjected to metagenomic analysis as described in online supplemental materials and methods.

three taxa in the jejunum (table 1 and online supplemental figure S8) and six taxa in the ileum. In faeces, *Desulfovibrio*, *Sutterella* and *Adlercreutzia* species were more frequently detected in IgA$^{-/-}$, whereas *Faecalibacterium* and *Lachnobacterium* species and *Oscillospira guilliermondii* were more frequently observed in WT. The differences observed with *Adlercreutzia* and *Sutterella* were significant in all of the jejunum, ileum and the faeces between WT and IgA$^{-/-}$. However, *Sturella* and *Desulfovibrio*, and *Adlercureutzia* belonging to Proteobacteria and Actinobacteria phyla, respectiviely, may not be coated by IgA significantly.[25 26]

Next, UniFrac distance was calculated to estimate the magnitude of the effect observed in the context of IgA deficiency. A weighted UniFrac analysis showed that the distance between WT and IgA$^{-/-}$ was slightly greater in the jejunum and ileum compared with that in the faeces (p=0.036 and p=0.024, respectively; figure 5B and online supplemental figure S9A). An unweighted UniFrac analysis also observed a smaller intergroup distance for faeces compared with that for the small intestine (p=0.001; figure 5C and online supplemental figure S9B). Furthermore, the absolute number of bacteria appear to increase in the IgA$^{-/-}$ ileum (online supplemental figure S10). These results indicate that IgA deficiency causes distinctive changes in the bacterial communities associated with the small intestine, especially in the ileum.

Furthermore, administration of vancomycin which kills Gram-positive bacteria significantly decreased the pathological abnormalities in IgA$^{-/-}$ mice (online supplemental figure S11). However, some extent of the pathological abnormalities remained, suggesting that both Gram-positive and Gram-negative bacteria contributed to its pathology.

Intravital imaging of PP B cells in IgA$^{-/-}$ mice revealed abnormal Ca^{2+} signalling

We also investigated the impact on B-cell function in the context of IgA deficiency using a conditional Ca^{2+} biosensor YC3.60 transgenic model, which we have previously generated. This transgenic animal model has been shown by us to be able to detect subtle changes in Ca^{2+} signalling *in vivo* with high sensitivity before the onset of a clinical disorder of systemic autoimmune and IBD models.[15 17] To evaluate the effects of IgA deficiency on Ca^{2+} signalling in B cells, we crossed IgA$^{-/-}$ with CD19-Cre/YC3.60^{flox} mice and analysed Ca^{2+} signalling in PP B cells using intravital imaging. In the IgA$^{-/-}$/CD19-Cre/YC3.60^{flox} mice, some of PP B cells exhibited constitutively increased intracellular Ca^{2+} concentrations (figure 6A,B) despite the lack of salient differences in the size of B-cell population and in the numbers of T cells and B cells (figure 2). Furthermore, Ca^{2+} signalling was significantly elevated in randomly selected fields in PP of IgA$^{-/-}$/CD19-Cre/YC3.60^{flox} compared with that of the control (Ctrl) CD19-Cre/YC3.60^{flox} mice (figure 6C). In contrast, IgA$^{tm/tm}$/CD19-Cre/YC3.60^{flox} mice, Ca^{2+} signalling was comparable with that of CD19-Cre/YC3.60^{flox} mice (data not shown). In summary, the PP B cells in the context of IgA deficiency demonstrated significant activation.

DISCUSSION

In this study, we generated IgA$^{-/-}$ and IgA$^{tm/tm}$ mice through a single-step CRISPR/Cas9 system experiment. Interestingly, IgA$^{-/-}$ showed significantly increased SFB in the ileum and skewed microflora composition in the jejunum, ileum and faeces. Furthermore, spontaneous ileitis was also observed in IgA$^{-/-}$ in association with significantly increased IFN-γ-producing and IL-17-producing CD4^{+} T cells in the lamina propria. These abnormalities were restored by ABx treatment. Intravital imaging of PP in IgA$^{-/-}$ ileum revealed an increase in B-cell activation based on the evidence for constitutively elevated Ca^{2+} concentrations.[15 17] In contrast, IgA$^{tm/tm}$ mice exhibited a comparable phenotype with that of WT. Together, these results indicate that IgA is crucial to homeostatic maintenance in the gut, especially in the ileum, although the cytoplasmic tail sequence does not appear to be crucial.

Although most animal models of IBD manifest as colitis, there are some notable exceptions. The IgA$^{-/-}$ mouse model we report here is reminiscent of that observed in *Xbp1*$^{-/-}$ mice, which also develop spontaneous ileitis.[27] The XBP1 gene has

Table 1 Taxonomic groups of significant differences at species level

Phylum	Class	Order	Family	Genus	Species	WT (%)	$IgA^{-/-}$ (%)	Stamp p value	maaslin2 p value	maaslin2 q value	Gram
Jejunum											
Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	*Sutterella*		0.2 erell	1.2 erell	0.017	0.009716	0.466376	(–)
Bacteroidetes	Bacteroidia	Bacteroidales	S24-7			18.4 roidal	50.6 roida	0.022	0.141442	0.741488	(–)
Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	*Adlercreutzia*		0.1 rcreu	0.4 rcreu	0.029	0.021543	0.513532	(+)
Ileum											
Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	*Adlercreutzia*		0.0 rcreu	0.3 rcreu	0.018	0.032124	0.600348	(+)
Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	*Desulfovibrio*	C21_c20	0.1 c20vi	0.7 c20vi	0.034	0.128786	0 .600348	(–)
Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	*Sutterella*		0.0 erell	0.7 erell	0.037	0.052296	0.600348	(–)
Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae			0.0 obact	0.2 obact	0.043	0.151946	0.600348	(+)
Faeces											
Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	*Desulfovibrio*		ND	0.0 lfovi	0.004	0.868091	0.931884	(–)
Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	*Sutterella*		0.2 erell	0.5 erell	0.004	0.029101	0.641393	(–)
Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	*Adlercreutzia*		0.1 rcreu	0.3 rcreu	0.009	0.072939	0.641393	(+)
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Oscillospira*	guilliermondii	0.6 lierm	0.3 lierm	0.031	0.839028	0.929958	(+)
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Faecalibacterium*		0.1 aliba	0.0 aliba	0.043	0.223489	0.641393	(+)
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	*Lachnobacterium*		0.1 nobac	0.0 nobac	0.046	0.446701	0.694329	(+)
Bacteroidetes	Bacteroidia	Bacteroidales	Paraprevotellaceae	(*Prevotella*)		0.2 otell	0.6 otell	0.049	0.173527	0.641393	(–)

ND, not detected.

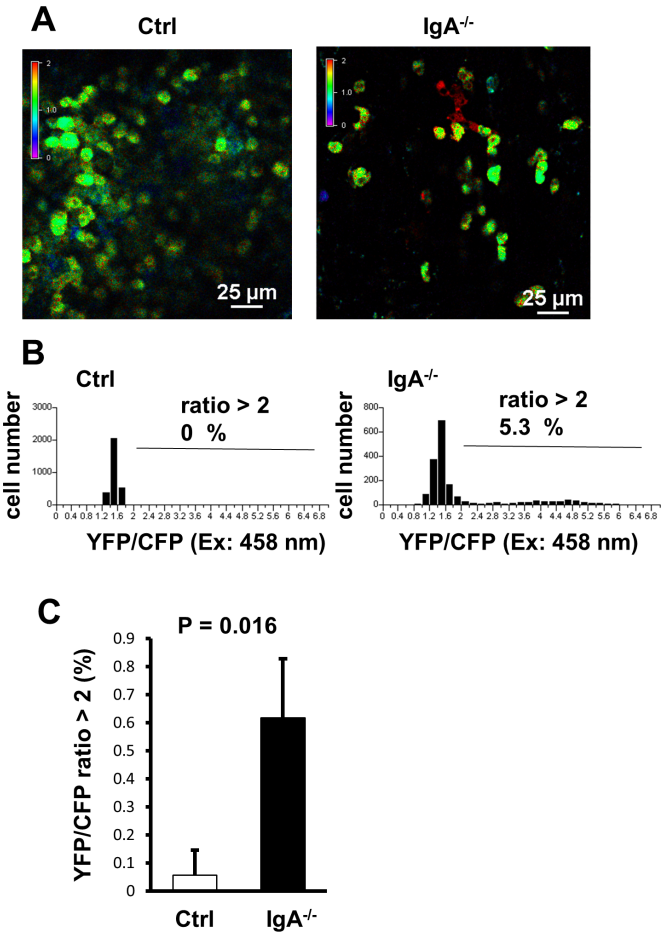

Figure 6 Intravital imaging of Ca^{2+} signalling in Peyer's patch (PP) B cells. (A) Representative images of Ca^{2+} signalling in the PP of the control (Ctrl, left) and $IgA^{-/-}$ (right) CD19-Cre/YC3.60^{flox} mice. Intravital imaging analysis of PP was performed under a confocal microscopy. Time-lapse images were obtained at every 2 s. Representative images reflecting the Ca^{2+} concentration based on the YFP:CFP ratio (excitation: 458 nm) are shown (n=3). (B) Distribution of the time-integrated intracellular Ca^{2+} concentrations of the PP B cells from Ctrl (left) and $IgA^{-/-}$ (right) CD19-Cre/YC3.60^{flox} mice (n=20, frame=147 for Ctrl; n=20, frame=89 for $IgA^{-/-}$). Percentages of cells with YFP/CFP ratios >2 are indicated. (C) YFP/CFP ratiometric intensities (excitation: 458 nm) of the PP B cells from Ctrl (left) and $IgA^{-/-}$ (right) CD19-Cre/YC3.60^{flox} mice. Randomly selected fields from three mice were analysed. $p<0.05$ (*t*-test).

been identified as a genetic risk factor for both types of IBD, Crohn's disease (CD) and ulcerative colitis.[27] CD is one form of IBD characterised by immune-related disorder involving the entire alimentary tract especially the small intestine, although its aetiology is uncertain. More than half of the patients with CD exhibit pathological condition involving the terminal ileum. The *Xpb1*$^{-/-}$ model is also IgA dependent in that loss of IgA results in proximal extension of the inflammation into the duodenum.[28] In addition, the SAMP1/Yit mice have been defined as the model for spontaneous CD enteritis.[27 29] Excess IFN-γ production in the pathological site was also observed in SAMP1/Yit mice.[29] Similar to *Xbp1*$^{-/-}$ mice and SAMP1/Yit mice, $IgA^{-/-}$ mice in our study showed spontaneous inflammation specifically in the small intestine and most notably in the ileum, which was associated with increased pro-inflammatory cytokine production, including IFN-γ and IL-17, from the infiltrating lamina propria CD4$^+$ T cells in the ileum. Thus, the $IgA^{-/-}$ mice appear to be a potential model for CD. In fact, several clinical studies have reported that selective IgA deficiency in humans is correlated with increased risk for IBD, especially CD.[30 31] Interestingly, coeliac disease can also involve inflammation in the small intestine and previous reports have suggested a correlation between selective IgA deficiency and coeliac disease.[32 33] However, a recent review of several prior studies on selective IgA deficiency in humans suggested a weaker association with coeliac disease than that of CD.[34]

A previous report demonstrated that IgA deficiency led to an increase in the serum levels of other Ig classes, such as IgM and IgG.[7] Our findings are consistent with this and also provide evidence that is associated with spontaneous ileitis occurred. Previous associations between IgA deficiency and human IBD have not focused on specific anatomical sites. It is notable that the pathological disorder in the small intestine of $IgA^{-/-}$ observed in our study closely correlated with the areas of greatest IgA expression in the whole GI tract.[25] Specifically, there are estimated to be 10-fold to 15-fold more IgA$^+$ plasma cells in the small intestinal lamina propria relative to that observed in the colonic lamina propria.[25] Consistent with previous studies,[7 8] we did not observe any pathological changes in the alimentary tract, including colon, of $IgA^{-/-}$ mice, despite previous predictions.[8] In this study, we analysed the small intestine using recently established several techniques including direct microinjection of C57BL/6 zygotes, CRISPR/Cas9 genome editing system, metagenomic analysis with 16S rRNA sequencing and intravital microscopy system with a recently established biosensor involving fluorescence resonance energy transfer technology. Importantly, we were able to obtain the mouse lines with a pure strain background directly without need for back-crossing into C57BL/6. It should be noted that the IgA-deficient mice in previous reports were established using conventional procedures at that time, and thus, such mice may have slightly mixed background with different strains such as 129/Sv.[7] Therefore, this may reflect the differences in the pathologic phenotypes between ours and previous reports.[7 8]

Our analyses revealed remarkably increased SFB in the terminal ileum of $IgA^{-/-}$ compared with that of WT. A previous study with AID-deficient mice also implied an aberrant expansion of SFB,[9] even though there was no direct evidence showing that this increase of SFB would be caused by specific lack of IgA, because AID deficiency may also result in decreased production of all Ig classes, except IgM and IgD, in all organs including the intestines. However, the increased SFB and skewed microflora composition in the small intestine of $IgA^{-/-}$ in our study are consistent with the hypothesis of this previous report, and thus, this strongly suggests that IgA secretion in the gut suppresses these bacterial populations. On the other hand, spontaneous ileitis was not described in the same previous report[9] as another previous study with conventional IgA-deficient mice,[7] and therefore our current study is the first to show such pathological phenotype caused by IgA specific deficiency. A recent study demonstrated an abundance of IgA in the small intestine but not in the colon.[25] It also showed production of IgA specifically against SFB in a T cell–dependent manner. Therefore, IgA deficiency is suggested to lead to an expansion of SFB specifically in the small intestine. Furthermore, SFB has been reported to induce Th17 cells in the ileal lamina propria.[24] Indeed, our study also showed an increased IL-17 production from the ileal lamina propria CD4$^+$ T cells of $IgA^{-/-}$ mice. In addition, CD4$^+$ T-cell production of another pro-inflammatory cytokine, INF-γ, which is not induced by SFB efficiently[24] was also significantly

increased in the IgA$^{-/-}$ mice. Furthermore, IgA$^{-/-}$ mice showed slight histological abnormalities in the SFB-free condition by vancomycin treatment. Taken together, these findings suggest that IgA deficiency leads to increased SFB and other bacteria in association with CD4^{+} T-cell activation, even though how these changes culminate in ileal inflammation is still unknown.

We also observed more Gram-negative bacterial taxa (22 taxa) in IgA$^{-/-}$ compared with that of WT (8 taxa). Presumably, IgA deficiency may have provided a symbiotic milieu for Gram-negative bacteria, and this is consistent with a previous report.[8] Notably among these microbes, *Sutterella* species, which are increased in IgA$^{-/-}$ mice, were also previously found to correlate with human autism.[35] And interestingly, IgA has also been suggested to correlate with human autism.[36 37] Furthermore, another microbial species, *Proteobacteria* S24-7, a commensal bacteria that is coated with IgA in a T cell–dependent and T cell–independent manner was increased in IgA$^{-/-}$ mice.[25] It seems that IgA deficiency may therefore cause an expansion of these bacteria in the gut.

In this study, we show that the cytoplasmic tail of IgA is dispensable for IgA Ab production. The cytoplasmic tail of IgA (14 aa) is shorter than those of IgG and IgE (28 aa) but longer than those of IgM and IgD (3 aa).[14] Although the cytoplasmic tails of IgG and IgE are known to be crucial for Ab production,[12 13] IgA production is not dependent on the presence of the tail, as shown in our current study with IgA$^{tm/tm}$ mice, suggesting that this element may differ substantially in function (as well as in length and sequence) from those of IgG and IgE. Therefore, our study here identifies this as an important distinguishing characteristic of IgA relative to IgG and IgE.

We also observed B cells with constitutively increased intracellular Ca^{2+} levels in the PP of IgA$^{-/-}$ mice, and this is reminiscent of our findings in the B cells associated with autoimmune-prone *lpr*/*lpr* mice and oxazolone-induced colitis model[15 17] We previously demonstrated a significant increase in B cells exhibiting constitutively elevated intracellular Ca^{2+} concentrations in lpr/lpr mice despite an absence of any clinical findings. In IgA$^{-/-}$ mice, abnormally augmented Ca^{2+} signalling was observed in B cells from the PP, which coincides with ileal inflammation. The appearance of constitutively activated B cells is consistent with the inflammation observed.

In summary, our study highlights the role of IgA in the gut. Our findings indicate that IgA deficiency elicits the expansion of some commensal bacterial strains and promotes inflammation in the small intestine. This suggests that IgA is an important regulator of commensal bacteria, especially in the small intestine. Further analysis of commensal bacterial expansion is needed to precisely define the role of IgA in gut homeostasis.

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