Immunology Letters 211 (2019) 53–59

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/immlet

Peyer's patches contain abundant isotype-switched B cells with activated phenotypes and are inductive sites for T-independent anti-DNA IgA

Masaaki Hashiguchi*, Yuji Kashiwakura, Hidefumi Kojima, Yumiko Kanno, Tetsuji Kobata

Department of Immunology, Dokkyo Medical University School of Medicine, Japan

ARTICLE INFO

Keywords:
Peyer's patch
IgA
B cells

ABSTRACT

Peyer's patches (PPs) are inductive sites for IgA production; however, the induction mechanism of IgA remains largely unknown. We found that the activated phenotypes of isotype-switched PP B cells were more abundant than those of splenic B cells. Immunoglobulins (Igs) from PP B cells reacted to several substances, including DNA and diet extract. Hybridomas established from PP B cells of untreated mice revealed that IgA mainly react with DNA. PP-deficient mice revealed that PPs were dispensable for a total intestinal IgA amount but were required for intestinal anti-diet extract and anti-DNA IgA. Antibiotic-treated mice and CD4$^+$ T cell-depleted mice demonstrated that the intestinal anti-DNA IgA was induced by microbiota in a T-independent manner. Interestingly, the oral administration of IgA led to the expansion of intestinal bacteria in a reactivity-independent manner. Our findings suggest that PPs are unique and efficient inductive sites for IgA, particularly against T-independent antigens.

1. Introduction

The intestinal tract is the frontline for pathogenic organisms and commensal bacteria. IgA is a major immunoglobulin (Ig) produced in mucosal tissues including the intestine [1–4]. Intestinal IgA is induced by T-dependent (TD) or T-independent (TI) pathway. Many reports demonstrate that an orally administered protein antigen elicits antigen-specific IgA, which is TD [5–7]. Some parts of intestinal IgA are natural polyreactive antibodies [8,9]. Other parts binds to microbiota [10]. These IgAs are T-independently induced. Germ-free animals contain few IgA [10,11], which leads to that microbiota intensively induces IgA production. Although IgA has been thought to act mainly for protection against pathogens, recent work has revealed that IgA contributes to immune homeostasis. IgA-deficient mice and human study have shown that IgA plays an important role in controlling the bacterial community [12,13].

Isotype-switching from IgM to IgA is mediated by several factors. TGF-β1 is the most well studied cytokine involved in isotype-switching to IgA; mice deficient for TGF-βRII, a catalytic subunit of TGF-βR, show impaired IgA production [14,15]. In combination with TGF-β1, IL-21 largely enhances the IgA isotype switch [16,17]. Moreover, B-cell activating factor of the tumor necrosis factor family (BAFF), TNFSF13b, and a proliferation-inducing ligand (APRIL), TNFSF13, are known to enhance IgA production in response to TI antigens [1,18,19]. In addition to cytokines, retinoic acid also induces IgA production in combination with TGF-β [20,21]. These are abundantly expressed in the intestine, which may account for the high levels of IgA in the intestine.

Peyer's patches (PPs) are, at least in part, inductive sites for IgA production [22]. PPs are unique lymphoid tissues scatteredly located in the small intestine (SI). PPs are organized from three major regions: B cell follicle, follicle-associated epithelium (FAE) and subepithelial dome (SED). PPs contain immunocytes, including antigen-presenting cells such as dendritic cells (DCs), T cells and B cells, required for mounting immune reactions. Moreover, M cells, specialized epithelial cells for the uptake of bacteria and viruses from the lumen, are located between FAE [23,24]. Indeed, the germinal center (GC) is constitutively formed in PPs even at the quiescent stage. Consistently, most PP lymphocytes have been previously found to be activated [25,26] and the responses of PP cells are distinct from those of systemic cells. Our previous report demonstrated that naïve CD4$^+$ T cells in PPs secrete a higher amount of IL-6 in response to antigens [27]. PP innate lymphoid cells (ILCs) constrain commensal bacteria in PPs [28]. PPs contain more GC B cells

---

Abbreviations: BSA, bovine serum albumin; DC, dendritic cell; ds, double stranded; ELISA, enzyme-linked immunosortbent assay; GC, germinal center; HAT, hypoxanthine-aminoprerin-thymidine; HRP, horse radish peroxidase; Ig, Immunoglobulin; ILC, innate lymphoid cell; ILF, isolated lymphoid follicle; MLN, mesenteric lymph node; LP, lamina propria; LPS, lipopolysaccharide; OVA, ovalbumin; PP, Peyer's patch; SI, small intestine; TD, T-dependent; Tfh, follicular helper T cell; TI, T-independent; TNP, trinitrophenyl

* Corresponding author at: Department of Immunology, Dokkyo Medical University School of Medicine, 880 Kitakobayashi, Mibu, Tochigi, 321-0293, Japan
E-mail address: mhashi@dokkyomed.ac.jp (M. Hashiguchi).

https://doi.org/10.1016/j.imlet.2019.05.015
Received 7 May 2019; Received in revised form 21 May 2019; Accepted 26 May 2019
Available online 27 May 2019

and PP B cells show more activated phenotypes compared to splenic B cells [26]. Moreover, BAFF and APRIL treatment *in vivo* downregulates PP B GC formation and class-switching [26]. Thus, PPs play distinct roles in immunological reactions.

In this study, we sought to further characterize PP B cells and their produced IgA. We analyzed the expressions levels of surface molecules and transcription factors and found that isotype-switched PP B cells as well as un-class-switched IgM$^+$ B cells were more abundantly activated. We also examined the reactivities of IgA produced by PP B cells by *in vitro* culture and establishing hybridomas and found that anti-DNA is a major target of intestinal IgA. We utilized PP-deficient mice induced by an *in utero* anti-IL-7R mAb injection to investigate the roles of PPs in the production of IgA. The anti-DNA IgA was induced by microbiota in a T-independent manner. Furthermore, the administration of IgA mAb up-regulated bacterial numbers in the intestine in an antigen-independent manner. Thus, our findings demonstrate that PPs are inductive sites for TI anti-DNA IgA.

2. Materials and methods

2.1. Mice

C57BL/6 (B6) mice were purchased from CLEA Japan (Tokyo, Japan) and B6.Cg-*Rag2*$^{\mathrm{tmFwa}}$ (*Rag2*-deficient) mice were obtained from the Central Institute for Experimental Animals (Kawasaki, Japan). All mice were maintained in the Research Animal Center of Dokkyo Medical University under specific pathogen-free conditions. Both male and female mice between the ages of 8 and 16 weeks at the start of experiments were used. All research involving animals was performed in accordance with the guidelines of the Committee for the Care and Use of Laboratory Animals, Dokkyo Medial University (#0950).

2.2. Cell preparation and cell culture

PPs and spleens were minced with scissors, stirred in 1 mg/ml collagenase IV and 10 μg/ml DNaseI at 37 °C for 30 min, and passed through a 70-μm filter to obtain a single-cell suspension. Splenocytes were depleted of erythrocytes using a 0.15 M ammonium chloride solution. PP cells and splenocytes for the analysis of surface molecule expression and MLN cells were mechanically prepared by grinding using microslides. LP cells from the SI were prepared as previously described [29]. Cells were cultured for 7 days and culture supernatants were used for ELISA.

2.3. Preparation of intestinal contents

SI and colon contents were collected, weighed and dissolved in PBS at 10 μl/mg of content. Mixtures were centrifugated at 12,000xg for 5 min. The resulting supernatants were saved for further analysis.

2.4. Enzyme-linked immunosorbent assay (ELISA)

The amounts of Igs in culture supernatants were determined by isotype-specific ELISA, then 1 μg/ml was used to examine antigen specificity. Antigen (10 μg/ml of antigen [BSA, insulin, LPS, human (h) IgG, and double stranded (ds) DNA from Sigma; E. coli, lysed in 1% Triton-X100 and diluted for 10-fold with water], except for 100 μg/ml of diet extract)-coated or goat anti-mouse Ig (BioLegend)-coated 96-well plates (Iwaki, Tokyo, Japan) were treated with 1% bovine serum albumin to block non-specific binding and incubated with the standards and appropriately diluted samples, followed by horse radish peroxidase (HRP)-conjugated isotype-specific Abs. The antibodies used are listed in Supplementary Table S1. Binding was assessed by 3,3’,5,5’-tetramethylbenzidine or o-phenylenediamine dihydrochloride coloring.

2.5. qRT-PCR and qPCR

Total RNA was isolated using TRIzol (Invitrogen). cDNA was synthesized from total RNA using Superscript III or IV reverse transcriptase (Invitrogen), and qPCR was performed using Thunderbird SYBR qPCR mix (Toyobo, Japan, Tokyo) and an CFX Connect Real-Time PCR Detection System (Bio-Rad). Data were normalized to *Actb* gene expression levels. The specific primers used are listed in Supplementary Table S2.

For 16 s qPCR analysis, DNA was prepared form intestinal bacteria and qPCR analyses were performed as described above.

2.6. Flow cytometry

The culture supernatant from the 2.4G2 (anti-CD16/CD32) hybridoma was used to block nonspecific binding. Fluorochrome-conjugated mAbs and streptavidin conjugates were purchased from BioLegend or eBioscience. The mAbs used for flow cytometry are listed in Supplementary Table S3. Isotype and fluorochrome-matched mAbs were used for control staining. Flow cytometry experiments were performed using a FACSCalibur flow cytometer, and data analyses were performed using FlowJo (TreeStar, Ashland, OR) software.

2.7. Generation of hybridomas

PP, LP, and MLN cells were fused with P3U1 myeloma cells according to standard protocols. These were then selected using the hypoxanthine-aminopterin-thymidine (HAT) medium. Specificity was confirmed by Ag-binding by ELISA. Positive cells were cloned using a limiting dilution method. The anti-trinitrophenyl group (TNP) IgA (MTIgA-1) was established from the MLNs of TNP-ovalbumin (OVA)-immunized B6 mice. TNP-specific control rat IgG2b (RTIgG2b-f) was established by fusing rat popliteal and inguinal LN cells from a TNP-OVA immunized SD rat with P3U1. mAbs were purified from the ascites of *Rag2*-deficient mice administered intraperitoneally with the hybridoma using caprylic acid. Purities were confirmed by SDS-PAGE followed by CBB staining.

2.8. In vivo treatment

To kill intestinal bacteria, a cocktail of antibiotics (1 mg/ml ampicillin, 1 mg/ml neomycin, 0.5 mg/ml vancomycin, and 1 mg/ml metronidazole) was continuously administered via drinking water for 2 weeks. The clearance of bacteria in feces were confirmed by the absence of colony on LB plate culture.

To obtain PP-deficient mice, pregnant female B6 mice (E14.5) were injected with 1 mg anti-IL-7R mAb (A7R34 [30]) intravenously. The deficiency in the offspring was confirmed by the absence of PPs upon macroscopic observation.

To deplete the CD4$^+$ cells in mice, 0.5 mg of anti-CD4 mAb (GK1.5, ATCC; prepared from the ascites using caprylic acid) was administered ip 4 times (once every 3 days). For the control, rat IgG2b, κ (RTIgG2b-f) was used.

2.9. Statistical analysis

The significance of the results was determined using two-tailed Mann–Whitney *U*-tests or unpaired two-tailed Student’s *t*-tests with Prism software (GraphPad software). Values of $p < 0.05$ were considered significant.

3. Results

3.1. Isotype-switched B cells with activated phenotypes are abundant in PPs

Our previous study demonstrated that the activated phenotypes of

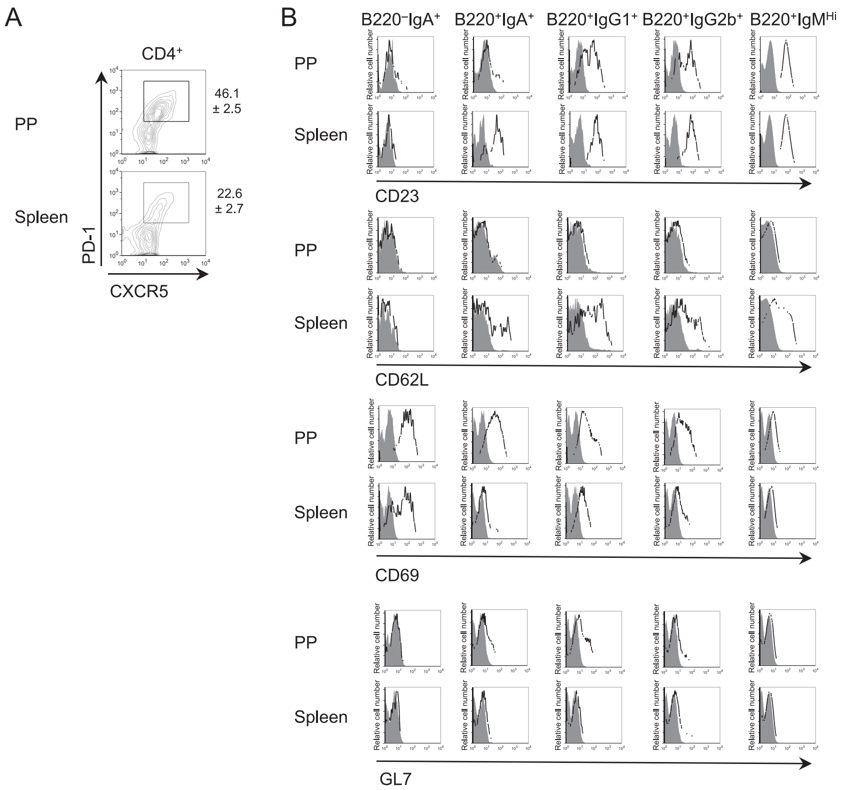

Fig. 1. Isotype-switched PP B cells show more activated phenotypes. (A) PPs contain more Tfh. PP and splenic cells were examined for PD-1 and CXCR5 expression on CD3$^+$CD4$^+$ cells. The numbers represent the mean ± SD of triplicates. Data are representative of three independent experiments. (B) PP and splenic B cells were gated on B220$^-$ IgA$^+$, B220$^+$ IgA$^+$, B220$^+$ IgG1$^+$, B220$^+$ IgG2b$^+$, or B220$^+$ IgM^{Hi} cells, and analyzed for CD23, CD62L, CD69 and GL7 expression. Shaded histograms denote isotype control staining. Data are representative of two to three independent experiments.

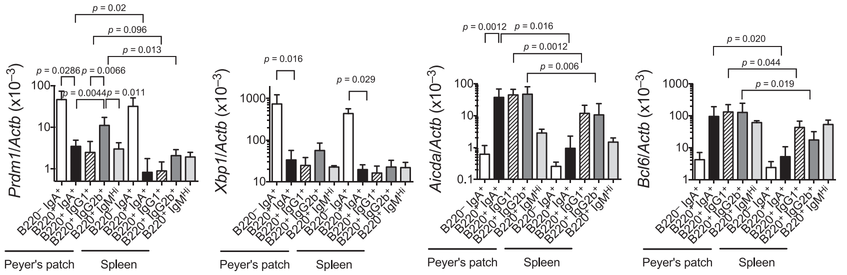

Fig. 2. Isotype-switched B cells in PPs express altered levels of transcription factors. B220$^-$ IgA$^+$, B220$^+$ IgA$^+$, B220$^+$ IgG1$^+$, B220$^+$ IgG2b$^+$, and B220$^+$ IgM^{Hi} cells were sorted from PP or spleen and examined for expression of *Prdm1*, *Xbp1*, *Aicda* or *Bcl6* by qRT-PCR. The data were normalized with *Actb* levels and expressed as the mean + SD of three to seven independent preparations. Statistical significance was determined by unpaired two-tailed Student's *t*-test.

PP B cells are abundant and that PPs contain more GC B cells than splenic B cells [26]. This suggests that more follicular helper T (Tfh) cells are present in PPs. As expected, CD4$^+$ cells with PD-1^{hi} CXCR5^{hi}, which represent Tfh, were more abundant in PPs than splenic cells (Fig. 1A). This abundancy may help in the activation of isotype switching and Ab production. Prior to analyzing the phenotypes of PP B cells, each Ig frequency was examined. The frequencies of IgM$^+$ cells in both PP and spleen were high (Supplementary Fig. 1). In PP B cells, after IgM, IgA was the second highest, and followed by IgG2b and IgG1. In splenic B cells, after IgM, IgG2b was the second highest. The frequency of IgA$^+$ cells in splenic cells was around one-tenth that of PP B cells. B220$^-$ plasma cells were observed clearly only on IgA. The amount of Ig produced *in vitro* corelated with the frequencies of each Ig on both PP and splenic B cells (Supplementary Fig. 2). Next, we examined the phenotypes of isotype-switched B cells in PPs and spleens (Fig. 1B). Almost all B220$^+$ splenic B cells and all IgM^{Hi} and half of the IgG1$^+$ or IgG2b$^+$ PP B cells expressed CD23, a marker for follicular B cells, whereas only some fraction of PP IgA$^+$ cells were positive for CD23. Around half of each isotype from splenic B cells were found to express CD62L, an activation negative marker. PP B cells, however, did not express this marker. Consistent with CD62L expression, CD69, an activation positive marker, was reversely expressed on PP B cells. Similarly, GL7, a germinal center marker, was expressed on more isotype-switched PP B cells. These findings demonstrate that isotype-switched PP B cells show a greater frequency of activated phenotypes than splenic B cells, which suggests that enhanced GC formation upregulates isotype-switching. B220$^-$ IgA$^+$ and B220$^+$ IgA$^+$ cells from lamina propria (LP) showed similar phenotypes for CD62L, CD69, and GL7 to those of PPs (Supplementary Fig. 2), which implies that PP B cells migrate to LP.

3.2. Isotype-switched PP B cells express higher levels of transcription factors

We assumed that the activated phenotypes of PP B cells are brought about by higher expression levels of the relevant transcription factors. Each isotype was cell-sorted from PPs or spleens and the mRNA levels of the transcription factors related to plasma cell differentiation and isotype switching were quantified by qRT-PCR (Fig. 2). We confirmed that B220– IgA$^+$ plasma cells from both PPs and spleens expressed higher levels of mRNA for *Prdm1*, a transcript for BLIMP-1, a critical molecule for plasma cell differentiation. The respective isotypes of PPs expressed relatively higher levels of *Prdm1* than those of spleens. Interestingly, IgG2b$^+$ PP B cells expressed higher levels of *Prdm1* than the other isotypes in PPs. Although the statistically significant differences were not observed, these profiles showed similar tendencies on the expression levels of *Xbp1*, a molecule required for plasma cell differentiation. All isotype-switched PP B220$^+$ cells expressed higher levels of *Aicda*, a transcript for AID, than those of spleen. Notably, the level of

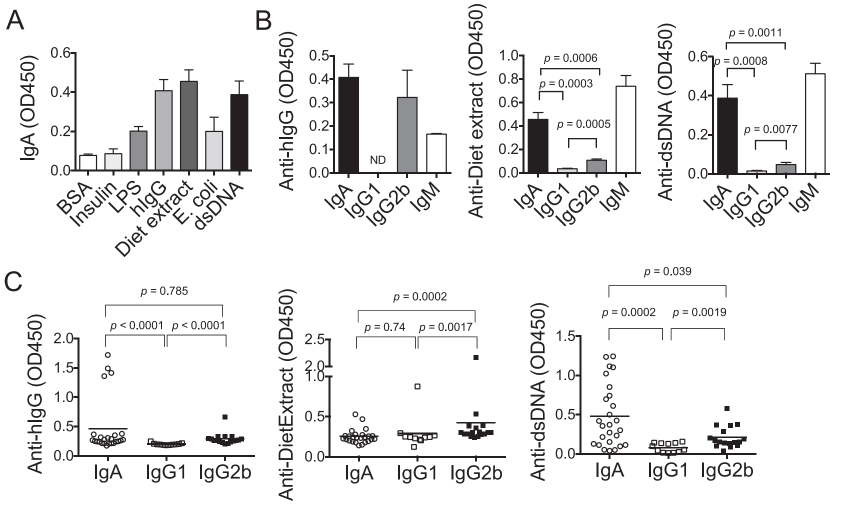

Fig. 3. Reactivities of PP Igs. (A-C) Culture supernatants of PP cells (A and B) or hybridomas established from PP cells of untreated B6 mice (1 μg/ml of IgA [*n* = 25], IgG1 [*n* = 11], and IgG2b [*n* = 17]) (C) were subjected to ELISA. Statistical significance was determined by unpaired Student's *t*-test (A) or two-tailed Mann-Whitney *U*-test (B). ND; not done.

B220$^+$IgA$^+$ cells from PPs was much higher than those from spleen. Similarly, Bcl6, a marker of the GC, was expressed at higher levels in isotype-switched PP B220$^+$ cells. IgA$^+$ B220$^+$ PP cells showed particularly higher expression levels of Bcl6 than those of spleens. Taken together, these results suggest that PP B cells are transcriptionally distinct from splenic B cells, which may be induced by a larger number of Tfh in PPs.

3.3. Reactivities of PP B cells

The study of the reactivities of IgA produced in the intestine is gradually increasing. As a results, part of IgA in the SI is known to react to bacteria [10]. However, the specificity and differences between isotypes remain unknown. To examine the reactivities of PP B cells, culture supernatants of PP cells were subjected to ELISA analysis for binding to bovine serum albumin (BSA), insulin, lipopolysaccharide (LPS), hIgG, diet extract, *E. coli* lysate, or dsDNA. PP IgA strongly reacted to hIgG, diet extract, and dsDNA and only weakly reacted to LPS and *E. coli* lysate (Fig. 3A). The three antigens with the greatest reactivity were selected and assayed. PP Ig showed weak binding to hIgG with IgA and IgG2b, and IgM binding to a lesser extent (Fig. 3B). PP IgA, IgM, and, to a lesser extent, IgG2b bound to diet extract. dsDNA was detected with IgA and IgM. Splenic Ig showed relatively similar profiles compared to PP, with the exceptions of higher IgM titers (Supplementary Fig. 4). On the other hand, IgA from SI strongly reacted to dsDNA and weakly reacted to hIgG and diet extract (Supplementary Fig. 5A). This profile was quite similar to that of IgA from LP (Supplementary Fig. 5B). This accounts for the fact that LP is a direct effective site for intestinal IgA production. IgA from mesenteric lymph node (MLN) showed less reactivity but its level of activity was comparable to the antigens tested (Supplementary Fig. Fig. 5B).

B cell hybridoma is established generally by immunized B cells. It is conventionally known but minimally reported [31] that the fusion efficiency is dependent on activation status. PP contains activated B cells in a homeostatic state. Hybridoma can be established from the PP B cells of unprimed mice [32]. Therefore, to further evaluate the reactivities of PP B cells, the establishing hybridoma method was applied. For this, 25 IgA-producing, 11 IgG1-producing, and 17 IgG2b-producing hybridomas were established from the PP cells of untreated B6 mice. We failed to establish any hybridoma from the splenocytes of untreated mice. This suggested that PP B cells are more activated than splenic B cells. First, culture supernatants were isotypically pooled and their reactivities were examined by ELISA. The pooled samples of IgA showed binding to hIgG, diet extract, and dsDNA but not to either BSA, insulin, LPS, or *E. coli* lysate (data not shown). Then, individual culture supernatants were subjected to ELISA for their binding to selected materials. Several IgA from the hybridomas showed strong binding to hIgG (Fig. 3C). Certain IgAs reacted to diet extract, however, statistically, larger numbers of IgG2b-producing hybridomas reacted to diet extract in comparison. Surprisingly, dsDNA was strongly bound to by many IgA and weakly by some IgG2b from the hybridomas. They also bound to single stranded DNA (data not shown). Only one IgG1 from the hybridomas showed reactivity to diet extract. The levels reactivities are summarized in Supplementary Fig. 6.

We established 20 IgA-producing hybridomas and 1 IgM-producing hybridoma from LP cells, and 2 IgA-producing and 1 IgG2a-producing hybridomas from MLN and compared their reactivities with PP hybridomas. The IgA reactivities of LP hybridomas were comparable to that of IgA from PP hybridomas, and some LP hybridoma IgA showed higher reactivities than PP hybridoma IgA to DNA (Supplementary Fig. 7). We failed to determine the reactivities of the IgM LP hybridoma and all MLN hybridomas.

3.4. PPs and microbiota but not CD4+ cells are required for anti-DNA IgA

Next, we investigated what is required for the induction of intestinal IgA. In order to determine whether PPs are required for anti-DNA IgA generation, PP-deficient offsprings were generated by injecting pregnant mice with anti-IL-7R mAb at E14.5 [33]. PP deficiency did not impair the amount of intestinal IgA (Fig. 4A). However, the IgA titers of anti-diet extract and anti-DNA decreased significantly (Fig. 4B). These reductions were not correlated with the amount of anti-bacterial IgA

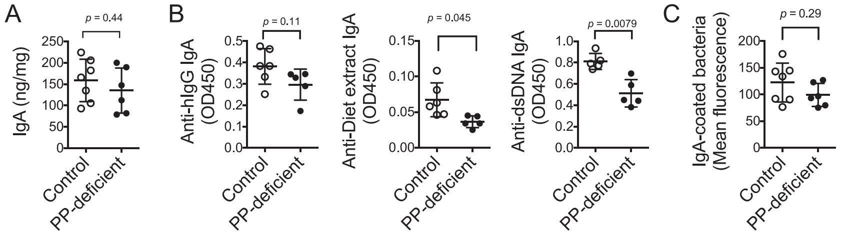

Fig. 4. PPs are required for production of IgA against dietary antigen and DNA in the intestine. (A-C) Mice deficient for PP were induced by the maternal injection of anti-IL-7R mAb at E14.5. The amount of IgA (A) or specific IgA (B) in the contents of SI were determined by ELISA, and IgA-coating levels of small intestine bacteria were examined with anti-IgA-PE on flow cytometry (C). Data are representative of three independent experiments with six to seven mice per group. Statistical significance was determined by two-tailed Mann-Whitney *U*-test.

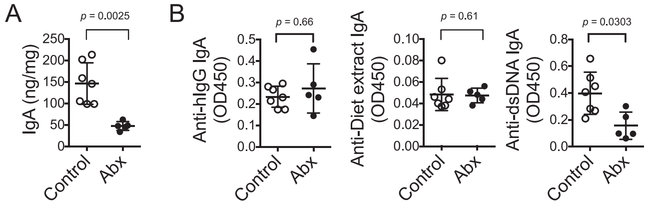

Fig. 5. Microbiota is required for anti-DNA IgA induction in the intestine. (A, B) B6 mice were treated with antibiotics (Abx; ampicillin, neomycin, vancomycin and metronidazole) for two weeks, and IgA in SI contents were analyzed to determine the amount of IgA (A) and the reactivities to diet extract, hIgG, and dsDNA (B). Equal amounts (5 μg/ml) of IgA were used for the determination of the reactivities. Data are representative of two independent experiments with six to seven mice per group. Statistical significance was determined by two-tailed Mann-Whitney *U*-test.

(Fig. 4C). These results suggest that microbiota induce IgA against their DNA in PPs, and that they evoke bacterial surface molecule-reactive IgA not in PP but possibly in isolated lymphoid follicles (ILFs) or LP.

DNA was the major target of IgA, which suggests that the DNA comes from the microbiota. B6 mice were treated with a cocktail of antibiotics (ampicillin, neomycin, vancomycin and metronidazole) in drinking water for 2 weeks. The contents of the SI were investigated for the amount and the specificities of IgA by ELISA. As expected, antibiotic treatment largely lowered the amount of IgA (Fig. 5A). Equal amounts of IgA indicated that antibiotic treatment did not alter the levels of anti-diet extract or anti-hIgG IgA (Fig. 5B). However, the antibiotic treatment lowered the levels of anti-DNA IgA. These results suggest that microbiota induces anti-DNA IgA in the intestine.

To confirm that anti-DNA antibodies are TI, CD4$^+$ cells were depleted via the administration of specific mAb. Depletion was confirmed by flow cytometry; the percentages of CD4$^+$ cells of control and anti-CD4 mAb-injected mice were 18.6 ± 0.15 and 2.22 ± 0.88 in splenocytes and 13.17 ± 1.50 and 3.66 ± 0.63 in PP cells, respectively. In the mice, the amount of SI IgA and the IgA titers to diet extract, human immunoglobulin, and DNA were comparable between the control and the CD4$^+$-depleted mice (Fig. 6). These results demonstrate, as expected, that anti-DNA IgA does not require CD4$^+$ T cells.

3.5. IgA regulates intestinal bacteria

Next, we examined the role of anti-DNA IgA in the intestine. Several types of IgA, including control anti-TNP IgA, were purified from the established hybridomas and administered via drinking water to *Rag2*-deficient mice (lacking Igs) for 2 weeks. Intestinal bacteria numbers were assessed by their *16 s* levels by qPCR. Surprisingly, IgA administration, including control, substantially increased the number of bacteria (Fig. 7A). The composition taxa of the bacteria appeared to be unchanged, since the ratio of Bacteroidetes and Firmicutes was comparable by qPCR (data not shown). These mAbs weakly but substantially bound to LI bacteria in an unidentified manner, although the binding strength was different for each mAb (Fig. 7B). A recent article demonstrated that certain *Bacteroides* preferentially bind to IgA to colonize in the intestine [34,35]. These results imply that bacteria upregulate anti-DNA IgA to stabilize their localization.

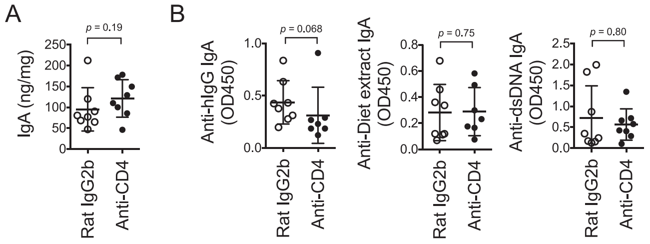

Fig. 6. Anti-DNA IgA is independent of CD4$^+$ cells. (A, B) B6 mice were injected ip with isotype control (rat IgG2b) or anti-CD4 mAb (GK1.5) every 3 day for 5 times, and IgA in the contents of SI were analyzed for the amount of IgA produced (A) and the reactivities to diet extract, hIgG, and dsDNA (B). Equal amounts (5 μg/ml) of IgA were used for the determination of the reactivities. Data are representative of two independent experiments with seven to eight mice per group. Statistical significance was determined by two-tailed Mann-Whitney *U*-test.

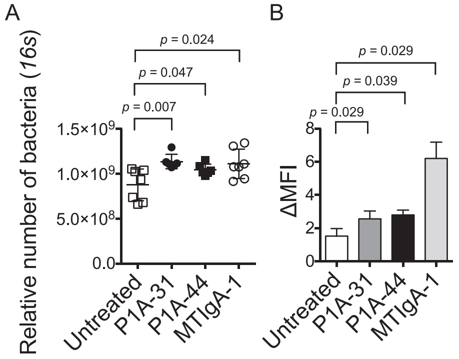

Fig. 7. Intestinal IgA modulates bacteria. (A) *Rag2*$^{-/-}$ mice were either untreated or orally administered IgA (MTIgA-1, P1A-31 or P1A-44; 25 μg/ml) for two weeks. The *16 s* levels of the large intestinal bacteria were examined by qPCR (*n* = 6 to 7 per group). (B) Bacteria from 10 mg large intestine content were incubated either with 1 μg IgA (MTIgA-1, P1A-31, or P1A-44) or alone, followed by anti-IgA-PE, then analyzed on flow cytometry. The values indicate the relative mean fluorescent intensities to background (ΔMFI) + SD of quadruplicate samples. Data are representative of two independent experiments. Statistical significance was determined by two-tailed Mann-Whitney *U*-test.

4. Discussion

Isotype-switched PP B cells clearly show activated phenotypes with activation markers and transcription factors. PPs constantly uptake not only diet but also bacteria and viruses from the lumen [23,24]. Most protein antigens are TD; diet-derived proteins and proteins from microorganisms presented by antigen-presenting cells, such as DCs, to T cells may induce GCs. Given that both antibiotic treatment and a germ-free environment results in a drastically reduced size of PPs, microorganism protein should induce GCs in PPs. Although we did not evaluate the contribution of TD Igs to SI Igs, microorganisms, at least in part, should play a boosting role in B cell activation. In contrast, GC formation does not seem to affect TI Ig production. The microorganisms possess huge amounts of TLR ligands, which activate the immune system. Therefore, microorganism-derived substances are likely to be largely involved in TI Ig production. These findings suggest that PPs are appropriate sites for the induction of immune responses to both TD and TI antigens. Although PP B cells are the more activated phenotype, not all isotype-switched B cells were activated; some still look like splenic B cells. On the other hand, LP IgA$^+$ cells showed highly activated phenotypes. These findings suggest that highly activated B cells may exit from PPs and migrate into LP.

It has been reported that mice lacking PPs shows an unaltered intestinal total IgA amount but an impaired protein antigen-specific IgA level [6,36]. Thus, TD antibody production occurs in PPs. On the other hand, the production of TI antibody induced in PPs remain largely unknown. A limited number of studies have been published on this topic. Mice deficient for *Cd40*, which is required for T-B cognate interaction, were found to only show partly attenuated IgA levels [37]. Anti-DNA IgA, believed to be produced T-independently, is produced by PP B cells [32]. This suggests that TI IgA occurs in PPs. However, due to the small number of reports regarding TI IgA generation in PPs, the current consensus is that TI IgA is largely induced in the ILF or LP. TI antibody is believed to be produced by B-1 cells [38]. In particular, B-

1b cells, which exhibit a phenotype of CD5$^-$ CD11b$^+$, account for IgA production [39]. However, this subset of cells is mainly found in the murine peritoneal cavity. B-2-deficient L2 transgenic mice demonstrated that the contribution of B-1 cells to IgA in PPs and LP is negligible [40]. As such, the relevance of B-1 cells remains be fully elucidated. As per our preliminary results, we failed to detect typical B220^{Lo} CD5$^-$ CD11b$^+$ IgA$^+$ B-1b cells but found a small population of B220^{Hi} CD5$^+$ CD11b$^-$ IgA$^+$ unclassified B cells in the PPs. Although we have not been able to examine the function of the subset, these cells might account for TI IgA.

Anti-DNA Igs were the majority in the SI. The gastrointestinal environment preserves tremendous numbers of commensal bacteria and considerable numbers of dietary compounds. PPs constantly uptakes components and even whole bacteria and viruses via M cells [23]. These organisms are composed of nucleotides. Therefore, a number of nucleotide fragments are present in PPs. Bacterial DNA contains a CpG motif that acts as an adjuvant via ligation to TLR9 [41]. Thus, the anti-DNA antibody is induced by bacterial DNA as a “complete antigen”, containing an adjuvant. PP follicular dendritic cells abundantly express TGF-β1 and BAFF to induce TI IgA [42]. Therefore, the location and cytokine milieu facilitate TI IgA isotype switching in PPs.

Nucleic acid has different characteristics from the proteins tested in the specificities of Igs. This leads to a thought that although ELISA results showed comparable reactivities, the molar density of epitopes of DNA may be much higher than the others. This raises the possibility of the lower avidities to DNA. This accordingly casted doubt on the specificity. PP B cells are more activated, and activation or inflammatory condition alter the glycosylation [43]. Therefore, IgA from PP B cells may be more glycosylated than IgG. These might reflect carbohydrate-medicated DNA binding. However, a recent paper showed that recombinant Ig with human Fc and a variable region of PP IgA still bind to DNA [9]. Therefore, we conclude that PP IgA antigen specifically reacts DNA.

PP IgA plasma cells show completely activated phenotypes, whereas the splenic equivalent is weakly expressed. Among the isotypes in B220$^+$ cells, IgA$^+$ cells are the most prominent, followed by IgG2b$^+$-cells. PPs contain more Tfh-like cells, and T cells and B cells with activated phenotypes than other systemic organ, such as the spleen. This suggests that larger amounts of cytokines are present PPs which activate isotype-switched B cells. In terms of transcription factors, IgG2b$^+$ PP B cells show higher levels of *Prdm1* and *Xbp1*, key factors for plasma cell differentiation. We were able to distinguish between the plasma cells/plasmablasts and the pre-plasmablasts/memory cells in IgA on the basis of B220 expression. In contrast, plasma cells/plasmablasts and pre-plasmablasts/memory cells may be more difficult to distinguish on IgG2b$^+$ cells; IgG2b-secreting plasma cells may still express B220.

The main function of IgA in the intestine was believed to be the provision of protection against pathogen invasion [4]. However, the physiological roles of IgA have recently started to be defined. IgA controls intestinal bacteria [12]. However, certain bacteria are able to utilize IgA for their colonization of the intestine. Commensal *Bacteroides fragilis* requires IgA for stable colonization [34]. The IgA coating is regulated through commensal colonization factors and polysaccharide C. Commensal *B. thetaiotaomicron* is coated with IgA in an antigen specificity-independent manner [35]. The binding of IgA to bacteria was, at least in part, carried out by the carbohydrates on the bacteria [34,35,44]. Our results demonstrate that microbiota induces anti-DNA IgA. Anti-DNA IgA is supposed not to act live but to damaged and dead bacteria. Thus, commensal bacteria induce possibly unharmful but functional IgA for their coating and colonization. Although we failed to identify a clear effect on the bacterial taxonomic ratio, we observed a tendency for Bacteroides to increase and Firmicutes to decrease after the oral administration of IgA mAb (data not shown). As such, certain taxa may efficiently utilize IgA to compete with one another.

In summary, here we demonstrated that PP B cells are distinct from splenic B cells, and that PPs possess an efficient Ig induction mechanism. These findings could be used further elucidate the mechanisms of these cells in order to develop vaccines against certain pathogens.

Conflict of interest

The authors have no commercial and financial conflicts of interest.

Acknowledgments

This work was supported by JSPS KAKENHI (24580196 and 15K07438).

We thank Ms. Y. Tasaku, Ms. Y. Nonaka, and Ms. Y. Nitta for technical assistant, cell sorting and secretarial assistant, respectively.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imlet.2019.05.015.

References

[1] A. Cerutti, M. Rescigno, The biology of intestinal immunoglobulin A responses, Immunity 28 (2008) 740–750, https://doi.org/10.1016/j.immuni.2008.05.001.

[2] P. Brandtzaeg, Secretory IgA: Designed for Anti-Microbial Defense, Front. Immunol. 4 (2013) 222, https://doi.org/10.3389/fimmu.2013.00222.

[3] C. Gutzeit, G. Magri, A. Cerutti, Intestinal IgA production and its role in host-microbe interaction, Immunol. Rev. 260 (2014) 76–85, https://doi.org/10.1111/imr.12189.

[4] A.J. Macpherson, B. Yilmaz, J.P. Limenitakis, S.C. Ganal-Vonarburg, IgA Function in Relation to the Intestinal Microbiota, Annu. Rev. Immunol. 36 (2018) 359–381, https://doi.org/10.1146/annurev-immunol-042617-053238.

[5] N. Lycke, J. Holmgren, Long-term cholera antitoxin memory in the gut can be triggered to antibody formation associated with protection within hours of an oral challenge immunization, Scand. J. Immunol. 25 (1987) 407–412.

[6] M. Yamamoto, M.N. Kweon, P.D. Rennert, T. Hiroi, K. Fujihashi, J.R. McGhee, et al., Role of gut-associated lymphoreticular tissues in antigen-specific intestinal IgA immunity, J. Immunol. 173 (2004) 762–769.

[7] E. Gardby, J. Wrammert, K. Schon, L. Ekman, T. Leanderson, N. Lycke, Strong differential regulation of serum and mucosal IgA responses as revealed in CD28-deficient mice using cholera toxin adjuvant, Journal of immunology (Baltimore, Md.: 1950) 170 (2003) 55–63, https://doi.org/10.4049/jimmunol.170.1.55.

[8] J. Benckert, N. Schmolka, C. Kreschel, M.J. Zoller, A. Sturm, B. Wiedenmann, et al., The majority of intestinal IgA$^+$ and IgG$^+$ plasmablasts in the human gut are antigen-specific, J. Clin. Invest. 121 (2011) 1946–1955, https://doi.org/10.1172/JCI44447.

[9] J.J. Bunker, S.A. Erickson, T.M. Flynn, C. Henry, J.C. Koval, M. Meisel, et al., Natural polyreactive IgA antibodies coat the intestinal microbiota, Science 358 (2017), https://doi.org/10.1126/science.aan6619 eaan6619.

[10] A.J. Macpherson, D. Gatto, E. Sainsbury, G.R. Harriman, H. Hengartner, R.M. Zinkernagel, A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria, Science 288 (2000) 2222–2226.

[11] S. Hapfelmeier, M.A. Lawson, E. Slack, J.K. Kirundi, M. Stoel, M. Heikenwalder, et al., Reversible microbial colonization of germ-free mice reveals the dynamics of IgA immune responses, Science 328 (2010) 1705–1709, https://doi.org/10.1126/science.1188454.

[12] K. Suzuki, B. Meek, Y. Doi, M. Muramatsu, T. Chiba, T. Honjo, et al., Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut, Proceedings of the National Academy of Sciences of the United States of America 101 (2004) 1981–1986, https://doi.org/10.1073/pnas.0307317101.

[13] J. Fadlallah, H. El Kafsi, D. Sterlin, C. Juste, C. Parizot, K. Dorgham, et al., Microbial ecology perturbation in human IgA deficiency, Sci. Transl. Med. 10 (2018), https://doi.org/10.1126/scitranslmed.aan1217.

[14] S. Borsutzky, B.B. Cazac, J. Roes, C.A. Guzman, TGF-β receptor signaling is critical for mucosal IgA responses, J. Immunol. 173 (2004) 3305–3309.

[15] B.B. Cazac, J. Roes, TGF-β receptor controls B cell responsiveness and induction of IgA in vivo, Immunity 13 (2000) 443–451.

[16] G.Y. Seo, J. Youn, P.H. Kim, IL-21 ensures TGF-β1-induced IgA isotype expression in mouse Peyer’s patches, J. Leukoc. Biol. 85 (2009) 744–750, https://doi.org/10.1189/jlb.0708450.

[17] A.T. Cao, S. Yao, B. Gong, R.I. Nurieva, C.O. Elson, Y. Cong, Interleukin (IL)-21 promotes intestinal IgA response to microbiota, Mucosal Immunol. 8 (2015) 1072–1082, https://doi.org/10.1038/mi.2014.134.

[18] M.B. Litinskiy, B. Nardelli, D.M. Hilbert, B. He, A. Schaffer, P. Casali, et al., DCs induce CD40-independent immunoglobulin class switching through BLyS and APRIL, Nat. Immunol. 3 (2002) 822–829, https://doi.org/10.1038/ni829.

[19] E. Castigli, S. Scott, F. Dedeoglu, P. Bryce, H. Jabara, A.K. Bhan, et al., Impaired IgA class switching in APRIL-deficient mice, Proceedings of the National Academy of Sciences of the United States of America 101 (2004) 3903–3908, https://doi.org/

10.1073/pnas.0307348101.

[20] G.Y. Seo, Y.S. Jang, H.A. Kim, M.R. Lee, M.H. Park, S.R. Park, et al., Retinoic acid, acting as a highly specific IgA isotype switch factor, cooperates with TGF-beta1 to enhance the overall IgA response, J. Leukoc. Biol. 94 (2013) 325–335, https://doi.org/10.1189/jlb.0313128.

[21] J.M. Lee, Y.S. Jang, B.R. Jin, S.J. Kim, H.J. Kim, B.E. Kwon, et al., Retinoic acid enhances lactoferrin-induced IgA responses by increasing betaglycan expression, Cell. Mol. Immunol. 13 (2016) 862–870, https://doi.org/10.1038/cmi.2015.73.

[22] S.W. Craig, J.J. Cebra, Peyer's patches: an enriched source of precursors for IgA-producing immunocytes in the rabbit, J. Exp. Med. 134 (1971) 188–200.

[23] N.A. Mabbott, D.S. Donaldson, H. Ohno, I.R. Williams, A. Mahajan, Microfold (M) cells: important immunosurveillance posts in the intestinal epithelium, Mucosal Immunol. 6 (2013) 666–677, https://doi.org/10.1038/mi.2013.30.

[24] A. Reboldi, J.G. Cyster, Peyer's patches: organizing B-cell responses at the intestinal frontier, Immunol. Rev. 271 (2016) 230–245, https://doi.org/10.1111/imr.12400.

[25] S. Fagarasan, S. Kawamoto, O. Kanagawa, K. Suzuki, Adaptive immune regulation in the gut: T cell-dependent and T cell-independent IgA synthesis, Annu. Rev. Immunol. 28 (2010) 243–273, https://doi.org/10.1146/annurev-immunol-030409-101314.

[26] M. Hashiguchi, Y. Kashiwakura, Y. Kanno, H. Kojima, T. Kobata, Tumor necrosis factor superfamily member (TNFSF) 13 (APRIL) and TNFSF13B (BAFF) down-regulate homeostatic immunoglobulin production in the intestines, Cell. Immunol. 323 (2018) 41–48, https://doi.org/10.1016/j.cellimm.2017.10.009.

[27] M. Hashiguchi, S. Hachimura, A. Ametani, T. Sato, H. Kojima, Y. Kumagai, et al., Naive CD4$^+$ T cells of Peyer's patches produce more IL-6 than those of spleen in response to antigenic stimulation, Immunology letters 141 (2011) 109–115, https://doi.org/10.1016/j.imlet.2011.09.001.

[28] M. Hashiguchi, Y. Kashiwakura, H. Kojima, A. Kobayashi, Y. Kanno, T. Kobata, Peyer's patch innate lymphoid cells regulate commensal bacteria expansion, Immunology letters 165 (2015) 1–9, https://doi.org/10.1016/j.imlet.2015.03.002.

[29] M. Ikutani, T. Yanagibashi, M. Ogasawara, K. Tsuneyama, S. Yamamoto, Y. Hattori, et al., Identification of innate IL-5-producing cells and their role in lung eosinophil regulation and antitumor immunity, J. Immunol. 188 (2012) 703–713, https://doi.org/10.4049/jimmunol.1101270.

[30] T. Sudo, S. Nishikawa, N. Ohno, N. Akiyama, M. Tamakoshi, H. Yoshida, et al., Expression and function of the interleukin 7 receptor in murine lymphocytes, Proceedings of the National Academy of Sciences of the United States of America 90 (1993) 9125–9129.

[31] K. Kuus-Reichel, A. Beebe, C. Knott, Isolation of hybridoma forming cells from immune spleens by unit gravity sedimentation, Hybridoma 10 (1991) 529–538, https://doi.org/10.1089/hyb.1991.10.529.

[32] M. Shimoda, Y. Inoue, A. Ametani, J. Fujiwara, N.M. Tsuji, J. Kurisaki, et al., Anti-DNA IgA autoantibodies are spontaneously generated in mouse Peyer's patches, Immunology 95 (1998) 200–207.

[33] H. Yoshida, K. Honda, R. Shinkura, S. Adachi, S. Nishikawa, K. Maki, et al., IL-7 receptor α^+ CD3– cells in the embryonic intestine induces the organizing center of Peyer's patches, Int. Immunol. 11 (1999) 643–655.

[34] G.P. Donaldson, M.S. Ladinsky, Sanders JG Yu KB, B.B. Yoo, W.C. Chou, et al., Gut microbiota utilize immunoglobulin A for mucosal colonization, Science 360 (2018) 795–800, https://doi.org/10.1126/science.aaq0926.

[35] A. Nakajima, A. Vogelzang, M. Maruya, M. Miyajima, M. Murata, A. Son, et al., IgA regulates the composition and metabolic function of gut microbiota by promoting symbiosis between bacteria, J. Exp. Med. 215 (2018) 2019–2034, https://doi.org/10.1084/jem.20180427.

[36] J. Kunisawa, I. Takahashi, A. Okudaira, T. Hiroi, K. Katayama, T. Ariyama, et al., Lack of antigen-specific immune responses in anti-IL-7 receptor α chain antibody-treated Peyer's patch-null mice following intestinal immunization with microencapsulated antigen, Eur. J. Immunol. 32 (2002) 2347–2355, https://doi.org/10.1002/1521-4141(200208)32:8<2347::AID-IMMU2347>3.0.CO;2-V.

[37] P. Bergqvist, E. Gardby, A. Stensson, M. Bemark, N.Y. Lycke, 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. 177 (2006) 7772–7783.

[38] A. Meyer-Bahlburg, B-1 cells as a source of IgA, Ann. NY Acad. Sci. 1362 (2015) 122–131, https://doi.org/10.1111/nyas.12801.

[39] B. Roy, A.M. Brennecke, S. Agarwal, M. Krey, S. Duber, S. Weiss, An intrinsic propensity of murine peritoneal B1b cells to switch to IgA in presence of TGF-β and retinoic acid, PLoS One 8 (2013) e82121, , https://doi.org/10.1371/journal.pone.0082121.

[40] B. Roy, S. Agarwal, A.M. Brennecke, M. Krey, O. Pabst, S. Duber, et al., B-1-cell subpopulations contribute differently to gut immunity, Eur J Immunol 43 (2013) 2023–2032, https://doi.org/10.1002/eji.201243070.

[41] D.S. Pisetsky, Immune activation by bacterial DNA: a new genetic code, Immunity 5 (1996) 303–310.

[42] K. Suzuki, M. Maruya, S. Kawamoto, K. Sitnik, H. Kitamura, W.W. Agace, et al., The sensing of environmental stimuli by follicular dendritic cells promotes immunoglobulin A generation in the gut, Immunity 33 (2010) 71–83, https://doi.org/10.1016/j.immuni.2010.07.003.

[43] A. Bondt, S. Nicolardi, B.C. Jansen, T.M. Kuijper, J.M.W. Hazes, Y.E.M. van der Burgt, et al., IgA N- and O-glycosylation profiling reveals no association with the pregnancy-related improvement in rheumatoid arthritis, Arthritis Res Ther 19 (2017), https://doi.org/10.1186/s13075-017-1367-0 160-160.

[44] A. Mathias, B. Corthesy, Recognition of gram-positive intestinal bacteria by hybridoma- and colostrum-derived secretory immunoglobulin A is mediated by carbohydrates, J. Biol. Chem. 286 (2011) 17239–17247, https://doi.org/10.1074/jbc.M110.209015.