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T Cell-Independent IgA Class Switch Recombination Is Restricted to the GALT and Occurs Prior to Manifest Germinal Center Formation

Peter Bergqvist, Anneli Stensson, Nils Y. Lycke, and Mats Bemark

Recently, we reported that CD40−/− mice, exhibiting exclusively T cell-independent IgA class switch recombination (CSR), demonstrated near normal levels of IgA plasma cells in the gut lamina propria (LP), despite the complete lack of germinal centers (GCs). In this study, we have extended our analysis focusing on how to reconcile these findings using flow cytometry and molecular markers for IgA CSR. In agreement with our previous results with small intestinal LP, the colon LP was found to host IgA CSR only when lymphoid follicles were present. Thus, no IgA CSR was observed in the nonorganized colon LP. By contrast, the Peyer’s patch (PP) was the dominant IgA CSR site in both CD40−/− and wild type (WT) mice, and they both hosted similar levels of mRNA expression for B cell activating factor of the TNF family, a proliferation inducing ligand, and inducible NO synthase, potential switch-factors for IgA. Unexpectedly, we found that PP B cells undergoing IgA CSR were GL7-intermediate. Levels of mRNA expression for B cell activating factor of the TNF family, a proliferation inducing ligand, and inducible NO synthase, potential switch-factors for IgA. Unexpectedly, we found that PP B cells undergoing IgA CSR were GL7-intermediate. These cells had not undergone somatic hypermutations (SHMs), whereas GL7-high cells in WT PP, which exhibited GCs, were heavily mutated. Moreover, IgA plasma cells in the LP of CD40−/− mice demonstrated few mutations in their Ig V regions, whereas WT LP B cells from different sites showed extensive SHMs, which were also clonally related. Therefore, IgA CSR can occur in PP at a stage preceding manifest GC (GL7-intermediate), whereas SHM require GC formations (GL7-high). These findings reconcile that IgA CSR can occur in PP in the absence of GC with the fact that CD40−/− mice host near normal levels of IgA plasma cells in the LP. The Journal of Immunology, 2010, 184: 3545–3553.

A unique feature of the mucosal membrane is the large production of IgA Abs, which are generated in response to the commensal flora or invading pathogens (e.g., food Ags) (1). Despite the importance of this defense system, our understanding of the mechanisms responsible for IgA B cell differentiation and their microanatomic localization is still incomplete (2–5). Although oral immunizations with T cell-dependent Ags can elicit strong gut IgA responses, it is the IgA formation against T cell-independent Ags that has attracted much attention in recent studies (6–12). In particular, IgA Ab formation against the commensal flora has been studied intensely, and results from germ-free mice indicate that such Abs are a dominant proportion of the total IgA found in the gut (13–16). Whether IgA class switch recombination (CSR) against T cell-independent and T cell-dependent Ags occurs at the same or different sites is currently debated (17). Conflicting reports have essentially described two scenarios for where and how naïve B cells undergo IgA CSR, with the classical model ascribing IgA CSR to the organized GALT, whereas recent reports have identified the nonorganized lamina propria (LP) of the small and large intestine as potential sites for IgA CSR (10, 11, 18–20).

Similar to CSR in peripheral lymph nodes or the spleen, the former relies on the formation of germinal centers (GCs) and is dependent on CD40 and TGFβ, whereas the latter occurs in the absence of GC, is independent of CD40, and is supported by production in situ of inducible NO synthase (iNOS), B cell activating factor of the TNF (BAFF), and a proliferation inducing ligand (APRIL) by epithelial cells, dendritic cells (DCs), or stromal cells (21–26). Whereas IgA production is near normal in the absence of CD40, it is dramatically impaired in APRIL- or BAFF-deficient mice (27–30). Moreover, the action of these molecules is independent of CD40 and TGFβ (9, 11, 21, 25). iNOS-deficient mice were also found to be impaired in IgA CSR, a mechanism that probably was secondary to a defect in BAFF and/or APRIL produced by DCs in the GALT and nonorganized LP of the intestine (22). Nevertheless, both pathways for IgA CSR require B cell proliferation and expression of activation-induced cytidine deaminase (AID) (31–33). Our recent study in CD40-deficient mice, and also previous studies in ICOS- or CD28-deficient mice, supports the hypothesis that near normal IgA plasma cell levels can be found in the LP in the complete absence of GCs in Peyer’s patches (PPs) (8, 30). We proposed previously that such IgA CSR could have occurred at alternative sites outside of the GALT. However, we failed to find evidence for IgA CSR in the nonorganized LP of the small intestine or in the peritoneal cavity, two of the reportedly strongest candidates for IgA CSR (20, 30, 34). A recent study has, however, pointed to the nonorganized LP of the colon as a possible site for IgA CSR (11). The present study is an extended analysis of alternative sites for IgA CSR in CD40−/− mice, focusing on the nonorganized colon LP for evidence of molecular markers for IgA CSR, which involved using sensitive...
quantitative RT-PCR for detection of α-germline transcripts and AID mRNA and a semiquantitative PCR for the detection of switch α-circle transcript (CT). Moreover, we analyzed in detail the PPs in CD40<sup>−/−</sup> and wild type (WT) mice and determined the relationships between GL7-expression and evidence for IgA CSR and somatic hypermutations (SHMs) in these populations.

**Materials and Methods**

**Mice**

CD40<sup>−/−</sup> and WT mice (both on a C57BL/6 background) were bred and housed at the animal facility Experimentell Biomedizin at the University of Gothenburg under specific pathogen-free conditions.

**RNA extraction and cDNA synthesis**

The PPs and 1-cm pieces of the colon were isolated and directly submerged in 350 μl buffer RLT (Qiagen, Hilden, Germany). The tissue was disrupted and homogenized for 2 min using the tissue lyser (Qiagen) according to the manufacturer’s instructions, followed by RNA extraction using the RNeasy Mini Kit (Qiagen). To isolate RNA from sorted cells or from tissue sections, we used the RNeasy Micro Kit (Qiagen) according to the manufacturer’s instructions. The concentration of the RNA was determined, and 4 μg RNA was used for cDNA synthesis using oligo(dT)₄₇ primers (Fermentas, Burlington, Ontario, Canada) and superscript III reverse transcriptase (RT) polymerase (Invitrogen, Carlsbad, CA). The cDNA synthesis was performed at 50°C for 1 h followed by 15 min at 70°C to inactivate the enzyme.

**Real-time PCR**

All real-time PCR reactions were performed on a Lightcycler 480 (Roche, Basel, Switzerland) using the probes master mix (Roche) according to the instructions. Template (2 μl) was used in all reactions, and the reactions were run in multiplex together with CD79a as a B cell loading control and as triplicates to minimize pipetting errors. To detect germline α-transcripts, the following primers were used: germline_for_qpcr, 5′-CCCA GGC TAG ACA GAG GCA AG-3′; germline_rev_qpcr, 5′-CCGG AAG GGA AGT AAT CGT GA-3′. As probe, we used the Roche universal library probe #27 (Roche). The following primers were used for AID transscripts: mAID_for_qpcr, 5′-TTC TCC TCA CTG GTC T-3′; mAID_rev_qpcr, 5′-GGCT GAG GAA CAA CAA TTC CAC-3′. As probe, we used the Texas Red-labeled oligo 5′-GAG GCC AGG AGG TCT AGA-3′. Cycles were performed in the same tube with the following primers: germline_for_qpcr, 5′-CCCA GGC TAG ACA GAG GCA AG-3′; germline_rev_qpcr, 5′-GGCT GAG GAA CAA CAA TTC CAC-3′. As probe, we used the Roche universal library probe #27 (Roche). The following primers were used for AID transcripts: mAID_for_qpcr, 5′-5′-GAT GCC AGG GGG TCT AGA-3′; mAID_rev_qpcr, 5′-GGCT GAG GAA CAA CAA TTC CAC-3′. As probe, we used a Texas Red-labeled oligo 5′-RED-CGT CAT GCC GAC CCG CAG T-BHQ2-3′. Cycling was performed under the following conditions: denaturation at 95°C for 5 min followed by 45 cycles of PCR (95°C for 15 s, 60°C for 30 s, and 72°C for 1 min). The gene expression was calculated using the ΔΔCT method and normalized against the CD79a expression calculated by the software and normalized against the CD79a expression.

**Immunohistochemistry**

Naïve mice were sacrificed and the small intestine, colon, or the PP were removed, embedded in TissueTek OCT compound, and snap frozen in liquid nitrogen. Frozen sections (7 μm) of the small intestine were prepared on microscope slides using a cryostat (Leica, Wetzlar, Germany). The cryostat sections were stored at −70°C until used. The slides were fixed in 50% acetone for 30 s and 100% acetone for 5 min at 4°C and air dried at room temperature. The tissue was rehydrated in PBS followed by blocking with normal horse serum in PBS (5%) for 15 min in a humidifying chamber. For detection of germlinal centers, the sections were stained for 30–60 min with FITC conjugated anti-GL7 mAb diluted 1:100 (BD Pharmingen, San Diego, CA) and biotinylated anti-B220 diluted 1:500 (BD Pharmingen). The B220 was visualized by adding streptavidin conjugated Texas Red (DakoCytomation, Glostrup, Denmark) 1:100 dilution. Consecutive sections were stained with B220 together with FITC conjugated anti-IgA diluted 1:100 (BD Pharmingen) or FITC conjugated anti-Ki67 diluted 1:5 (BD Pharmingen). The sections were washed in PBS and mounted with DakoCytomation fluorescent mounting medium and visualized using a Leica DMLB microscope.

**Flow cytometry and cell sorting**

PPs were excised and transferred into ice cold PBS supplemented with 0.1% BSA. Single-cell suspensions were prepared by passing the tissue through a 100-μm nylon mesh cell strainer (BD Falcon). The cells were washed and stained with anti-B220-PE/Cy7 (BD Pharmingen) and anti-GL7-FTTC (BD Pharmingen) at 1:300 dilution for 30 min on ice. The cells were then washed twice with PBS 0.1% BSA and sorted or analyzed using a FACSAria or a LSR II (BD Biosciences). The colonies were sorted in PBS - 0.1% BSA and immediately centrifuged at 600 x g and resuspended in 350 μl buffer RLT (Qiagen) for subsequent RNA or DNA extraction using the RNeasy Micro Kit (Qiagen) or the Allprep DNA/RNA Mini Kit (Qiagen).

**Colony hybridization for GL7 mutational analysis**

To analyze the mutational frequency of PP cells, we adopted a method previously described (35). Briefly, we isolated DNA from GL7<sup>−/−</sup>, GL7 intermediate (GL7<sup>MI</sup>), and GL7<sup>high</sup> sorted cells from PPs and performed a genomic PCR, using the following primers: FR3S558, 5′-GCC TGA CAT CGG ACTG C-3′ and IGH<sub>intron</sub>, 5′-CTT CAC TGG TTT CGG CTG AAC-3′. The program for the PCR was 98°C for 1 min followed by 30 cycles of 90°C for 10 s, 63°C for 1 min, and 72°C for 1 min. The program ended with a final extension step at 72°C for 10 min. To minimize mutations inserted by the PCR, we used the proofreading high-fidelity enzyme Phusion (Finnzymes, Espoo, Finland). The PCR generated blunt-ended products that were cloned directly using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen), and the plasmid was transformed into chemically competent *E. coli* and spread and selected using antibiotic resistance and blue/white screening. The selected colonies were patched onto a selective plate, grown overnight, and lifted onto a nitrocellulose membrane (Bio-Rad Healthcare, Hercules, CA). The colonies were sequentially blotted, adding 10% SDS to the membrane, followed by a denaturing step in 1.5 M NaCl and 0.5 M NaOH. The membrane was then neutralized in 1 M Tris-HCl (pH 7.4) and 1.5 M NaCl and finally submerged in 2×SSC solution before the membrane was left to air dry and ultraviolet cross-linked using 700 J/cm<sup>2</sup>. All steps were performed for 5 min each. The membrane was subsequently blocked for 1 h at 42°C in 15 ml blocking solution (0.5 M PO<sub>4</sub> buffer [pH 7.2]) containing 1 mM EDTA and 7% SDS) in a rotating chamber; 5 μmol of the probe JH4: 5′-TAT GCT ATG GAC TAC TGG-3′ was radioactively labeled by adding 5 μl ATP, [γ<sup>33P</sup>]-ATP (5000 Ci/mmol, 10 μCi/ml) EasyTide Label, 250 μCi (Perkin Elmer, Wellesley, MA), 5 μmol T4 PNK (Promega) and up to 50 μl water, followed by a 1 h incubation at 37°C. Free nucleotides were removed by using a ProbeQuant G-50 micro column (GE Healthcare) and the labeled probe was added to the sample and incubated at 42°C overnight. Hybridization was detected on a Pharsys FX molecular imager (Bio-Rad, Hercules, CA), and positive colonies were picked and grown in liquid selective media overnight followed by plasmid preparation using GenElute HP
Isolation of follicle free colon tissue

The mice were sacrificed and the colons were removed. To ensure analysis of follicle-free sections, 2–3-cm pieces of the colon were frozen and sectioned as described (30, 36). Every fifth section was placed on a slide, and the sections in between were stored in buffer RLT (Qiagen) at −70°C. The sections on the slide were stained with Texas Red conjugated anti-IgM (SouthernBiotech, Birmingham, AL) and FITC conjugated anti-IgA (BD Pharmingen) to localize the follicles. If two consecutive sections on the slide were positive or negative for follicles, the sections in between (stored in buffer RLT) were considered to also contain follicles or to be follicle free. RNA was prepared from these sections using the RNeasy Micro Kit (Qiagen) and analyzed by PCR as described above.

Cloning and IgA sequencing

The mice were sacrificed and the small intestine was removed and divided into three pieces: one proximal, one middle, and one distal part. To ensure analysis of isolated lymphoid follicle (ILF)-free tissue, the same procedure as for the colon was used. Subsequent cDNA synthesis was performed with Accuscript RT polymerase (Stratagene, La Jolla, CA) using an IgA-specific primer (5'-TGA CAT TGG TGG GTT TAC-3') followed by PCR using the following primers: Vhdgen: 5'-CTT CCG GAA TTC SAR GTN MAG CTCG SAG SAG TC-3'; IgArev: 5'-TTC CTC GAG AGG GCA GGT GGG AAG TTT A-3'. The PCR was performed using the high fidelity polymerase PFU (Fermentas). The PCR product and the pBSK (Fermentas) were cleaved using XhoI and EcoRI (Fermentas) and purified on a gel using the QIAquick Gel Extraction Kit (Qiagen). The fragments were ligated into the plasmid, and the ligation was transformed into chemically competent DH5α (Invitrogen) that was spread on selective plates for blue/white screening. White colonies were inoculated into 2 ml of selective LB medium grown overnight at 37°C, and the plasmid was purified using the GeneElute Plasmid Miniprep Kit (Sigma-Aldrich) followed by sequencing (Eurofins MWG Operon). The sequences were blasted against the IgBlast database (www.ncbi.nlm.nih.gov/igblast/). Cloning and IgA sequencing

Statistical analysis

The statistical analysis was based on Mann-Whitney U test comparing two populations with a non-Gaussian distribution. Individual p values based on a 95% confidence interval are stated in every figure.

Results

An abundance of gut IgA plasma cells despite the complete lack of germinal centers in CD40−/− mice

In our previous study of CD40−/− mice, we searched for alternative sites for IgA CSR in the GALT and in the nonorganized gut intestinal LP that could reconcile our conflicting findings of near normal levels of IgA plasma cells in the gut LP with a complete lack of GCs in the PP (Fig. 1A, 1B) (30). We observed significant levels of IgA CSR in the PP of CD40−/− mice, whereas all other locations, including the mesenteric lymph node (MLN), lacked IgA CSR activity (30). Because recent reports have provided evidence of IgA CSR against T cell-independent Ags in the LP of the human colon, we analyzed this tissue in greater detail in the CD40−/− mice (11). Similar to the small intestine, the colon of both CD40−/− and WT mice had comparable IgA plasma cell numbers and few IgM+ B cells in the LP (Fig. 1B, 1C). A majority of the latter, IgM+ B cells, were found in the organized lymphoid follicles in the gut intestine (Fig. 1B, 1C). Unexpectedly, whereas WT and CD40−/− mice hosted similar numbers of small ILFs, the CD40−/− mice lacked bigger follicles in the colon and small intestinal LP biopsies (Fig. 2A) (30). In fact, only a small proportion of LP samples contained switch α-CT; from 17 WT and 22 CD40−/− mice, 15% and 16%, respectively, of the colon samples contained switch α-CT (Fig. 2B). To rule out the inclusion of organized lymphoid follicles (ILFs or colonic patches), we undertook extensive sectioning of the biopsies (Fig. 3A). After microscopical analysis for the presence of organized lymphoid tissues, we isolated RNA from the
sections and analyzed these samples for the presence of IgA CSR markers (Fig. 3B). Consistently, we found that AID mRNA was detected only in samples that hosted lymphoid follicles, and that switch α-CT were never found in follicle-free samples of colon LP from either WT or CD40(-/-) mice (Fig. 3C). However, in 22% and 14%, respectively, of the sections that hosted lymphoid follicles, we also detected switch α-CTs as well as AID mRNA (Fig. 3C). Thus, using this refined method, IgA CSR activity was found to be exclusively restricted to the organized colonic lymphoid tissues, whereas no evidence for IgA CSR was found in the nonorganized LP in either strain (Fig. 3C).

PPs are dominant sites for IgA CSR in both CD40(-/-) and WT mice
Because we failed to detect switch α-CT in the small intestinal and colon LP, we analyzed the IgA CSR activity in the PP of CD40(-/-) mice in greater detail. To this end we used the refined quantitative RT-PCR methods that we had developed. We found that α-germline RNA and AID mRNA expression were reduced in the PP of CD40(-/-) compared with those in WT mice (Fig. 4A, 4B). Moreover, only half of the PP samples from the CD40(-/-) mice had switch α-CT, whereas almost 100% of the PP contained switch α-CT in WT mice (Fig. 4C). In fact, a semiquantitative PCR analysis of RNA samples using serial dilutions demonstrated that samples from WT mice contained twice the amount of switch α-CT compared with PP samples from CD40(-/-) mice (Fig. 4D). Therefore, the estimated overall IgA CSR activity in PPs of CD40(-/-) mice, lacking GC formations completely, was ~25% of that in WT PPs. Thus, PP from CD40(-/-) mice hosted significant IgA CSR and, compared with the ILFs in which ~80–85% of the samples were negative for IgA CSR, it appeared that the PP was the dominant site for IgA CSR.

Unaltered presence of IgA switch factors in PP of CD40(-/-) mice
Because T cell-independent IgA CSR has been reported to depend on BAFF, APRIL and/or iNOS production, we next investigated mRNA levels of these factors in PP from CD40(-/-) and WT mice (9, 22, 26). We found comparable mRNA expression of all three factors in both WT and CD40(-/-) mice. Random biopsies from the PP, small intestine (SI) or colon from WT and CD40(-/-) mice showed a high frequency of switch α-CT in PP, but few in the small intestine or colon. A, Examples of gel analysis of switch α-CT in random biopsy specimens taken from the PP, colon, or small intestine. The two differently sized bands correspond to two different splice variants of the transcript (49). B. The compiled data from a large number of random samples (indicated in the center of the pie chart) from the colon of 17 WT and 22 CD40(-/-) mice, illustrating the frequency of switch α-CT, which was found to be ~15% in these biopsy sections from WT or CD40(-/-) mice.

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This is one representative experiment of three giving similar results. Each black dot represents the sample dilution that gave a positive score for with samples from three individual mice and pooled material from 4 PPs and 5 PPs analyzed per mouse. The line in the diagram shows the mean analysis of switch of GL7high cells, as analyzed by FACS (Fig. 6).

To normalize for the number of B cells in each sample, the germline transcripts and AID mRNA expression levels were normalized against the B cell-specific CD79a mRNA expression. The diagram shows a detailed analysis of switch α-CT from the same PPs as in A and B. Each data point represents the frequency of switch α-CT positive PPs in individual mice and 5 PPs analyzed per mouse. The line in the diagram shows the mean frequency of positive PPs. D, A dilution series of PP cDNA was performed with samples from three individual mice and pooled material from 4 PPs. Each black dot represents the sample dilution that gave a positive score for switch α-CT expression. Gray circles indicate no detectable switch α-CT. This is one representative experiment of three giving similar results. p values of significance are given.

strains, indicating that the PP milieu in the CD40−/− mouse was well equipped to support T cell-independent IgA CSR (Fig. 5). Note-worthy, the presence and level of expression of BAFF, APRIL and iNOS mRNA was clearly independent of GC formations as they were found at similar levels in PP of CD40−/− mice as in WT mice. IgA CSR occurs at the GL7 intermediate stage prior to manifest GC formation

The relationship between the IgA CSR that occurred in GC-free PPs of CD40−/− mice and that in GC-rich PPs of WT mice was analyzed next. We made the unexpected finding that a substantial B cell population expressed GL7int levels in both CD40−/− and WT mice, whereas only WT mice hosted a significant population of GL7high cells, as analyzed by FACS (Fig. 6A). Because the GL7int PP B cells were nearly as frequent in CD40−/− as in WT mice, we hypothesized that these cells were responsible for IgA CSR in the PPs of both strains. Because a prerequisite for CSR is cell division, we analyzed the PP B cells for expression of Ki67+ cell division, we analyzed the PP B cells for expression of Ki67+ (Fig. 6B). Furthermore, AID mRNA expression, necessary for CSR, was found in GL7int cells from CD40−/− mice, albeit at lower levels than in WT mice (Fig. 6C). Of note, the strongest expression of AID mRNA by far was found in GL7high PP B cells (not shown). Extending the analysis to include switch α-CT expression, in six experiments we found that GL7neg and GL7high populations failed to demonstrate switch α-CT (Fig. 6D). In contrast, in both CD40−/− and WT PP GL7int B cells, we found significant expression of switch α-CT (Fig. 6D). In a total of 16 experiments, we found evidence of switch α-CT and IgA CSR in 50% of the experiments (not shown). When comparing the degree of SHM in the J558 intron in GL7int and GL7high PP B cells from WT mice, we observed that GL7int cells were mostly unmutated, similar to the GL7neg PP B cells, whereas GL7high cells were highly mutated (Fig. 6E). These results suggest that T cell-independent IgA CSR in PP B cells occurs at the GL7int stage, in the absence of GCs, whereas SHM follows later when the B cells are GL7high, express high levels of AID mRNA, and have manifested GC formation.

The distribution of somatic hypermutations reflects clonal relationships in gut IgA plasma cells

To test our hypothesis that IgA CSR occurred in GL7int PP B cells, we used the different levels of SHM in GL7int and GL7high cells as a marker for the origin of LP IgA plasma cells. If the hypothesis was correct, CD40−/− mice, hosting predominantly GL7int cells, would be expected to have few mutations in LP IgA plasma cells. Therefore, we analyzed random V regions of the IgA cells from spatially separated segments of the LP in CD40−/− and WT mice. We RT-PCR cloned the IgAV regions using a degenerate upstream primer together with a downstream IgA C region primer. If the hypothesis was correct, a marker for the origin of LP IgA plasma cells. If the hypothesis was correct, CD40−/− mice, hosting predominantly GL7int cells, would be expected to have few mutations in LP IgA plasma cells. Therefore, we analyzed random V regions of the IgA cells from spatially separated segments of the LP in CD40−/− and WT mice. We RT-PCR cloned the IgA V regions using a degenerate upstream V region primer together with a downstream IgA C region primer. Importantly, this allowed us to determine the level of mutations associated with SHM, as opposed to mutations resulting from the cloning, because constant regions do not undergo SHM. In WT mice, 84% of the cloned V regions from LP cells carried mutations, with an average of 5.2 mutations per sequenced V region (Fig. 7A; Table I). In contrast, LP IgA cells in CD40−/− mice remained largely unmutated, supporting the finding that IgA CSR had occurred in GL7int B cells before manifest GC in the PP, but that efficient SHM required GC formations. Whereas ~14% of the LP sequences from CD40−/− mice showed mutations, the average number was only 0.3 mutations per sequenced V region. These V region mutations in CD40−/− mice, however, occurred at a higher frequency than mutations restricted to the IgA constant regions (0.9 mutations per 1000 bp versus 0.1 mutations per 1000 bp), indicating that LP IgA cells probably undergo some SHM despite the lack of GC in PP. Nevertheless, in a total of almost 75,000 bp
FIGURE 6. IgA CSR in PP B cells from CD40-/- mice occurs in GL7int cells before manifest GC formation. B cells with differential expression of GL7 were sorted from PP B cells of CD40-/- and WT mice and analyzed for IgA CSR and SHM. A, PP B cells pooled from three mice were labeled with anti-B220 Abs and sorted by flow cytometry into GL7neg, GL7int, and GL7high fractions. Few cells were found in the GL7high population in CD40-/- mice compared with that in WT mice, where the GL7int PP B cells were almost comparable in number between the two strains. B, Evidence of cell proliferation in the PP B cell GL7-fractions was obtained after labeling with anti-Ki67 Abs and FACS analysis. The GL7int population hosted large numbers of proliferating cells. This representative experiment is one of three with similar results. C, The fold change ± SD in AID mRNA expression detected by a quantitative RT-PCR analysis revealed AID expression in the GL7int fraction of both CD40-/- and WT mice. The level of AID mRNA expression was calibrated against WT PP AID mRNA expression, which was set to 1.0 and using CD79 mRNA as the B cell-specific housekeeping gene. D, Subfractions of PP B cells from WT mice were further processed for RNA isolation, and samples were subjected to a one-step nested PCR analysis for switch α-CT, which was found exclusively in GL7int PP B cells in six experiments. A total of 16 experiments were then performed with sorted GL7int PP B cells from WT or CD40-/- mice. Switch α-CT were found in >50% of these experiments. E, Mutational analysis of pooled and sorted GL7int, GL7mid, and GL7high PP B cells isolated from WT mice. DNA was isolated and analyzed for JH4 intron mutations, and the results were given as the number of mutations per sequence. These data are compiled from three identical experiments.

FIGURE 7. Clonally related IgV sequences are found in WT and CD40-/- small intestinal LP IgA+ cells, but few mutations in CD40-/- LP IgA+ cells. Small intestinal LP biopsies from three CD40-/- or WT mice were isolated and used to extract mRNA for further analysis of mutations and clonal relationships between IgH V–Cα regions. cDNA synthesis was performed with a Cα downstream to specifically amplify Cα rearrangements. A, The pie charts show the mutation frequency in WT and CD40-/- mice, where the number of mutations contained in each segment. The total number of sequences analyzed in is given in the center of the chart. B, Analysis of small intestine clonally related IgV sequences with identical CDR3 rearrangements from three spatially separated segments. Mutations are shown with a vertical line and shared mutations are indicated with either an asterisk (*) or pound sign (#). The clonally identical V, D, and J segments are indicated by their name as found using the IgBLAST (www.ncbi.nlm.nih.gov/igblast). The image shows examples in which all three sites in the small intestine share clonality. C, A lineage tree analysis generated from the data from B. Gray cells represent hypothetical intermediate stages.
the GALT of CD40

previous reports on IgA CSR in human and mouse non-organized LP samples lacking follicles. Therefore, our study fails to confirm expressed from LP plasma cells isolated from the intestine of CD40

a stage before manifest GC (i.e., when the cells are at a GL7int stage). PPs, we provide evidence that IgA CSR in PP B cells can occur at

mRNA expression and switch

a

mRNA for further analysis of mutations and clonal relationships between IgH V–C regions. cDNA synthesis was performed with a Co downstream to specifically amplify Co rearrangements. A, The pie charts show the mutation frequency in WT small intestine and colon, where the size of the segments shows the number of sequences containing the number of mutations indicated in each segment. The total number of sequences analyzed is given in the center of the chart. B, Analysis of small intestine or colon clonally related IgV sequences with identical CDR3 rearrangements from WT mice. The image shows an example of clonality between sequences in the colon and in the small intestine. Mutations are shown with a vertical line, and shared mutations are indicated with a pound sign (#).

FIGURE 8. Clonally related IgV sequences are found in the colon and the small intestine in WT LP IgA+ cells. Colon and small intestine LP biopsy specimens from two WT mice were obtained and used to extract mRNA to specifically amplify Co rearrangements. A, The pie charts show the mutation frequency in WT small intestine and colon, where the size of the segments shows the number of sequences containing the number of mutations indicated in each segment. The total number of sequences analyzed is given in the center of the chart. B, Analysis of small intestine or colon clonally related IgV sequences with identical CDR3 rearrangements from WT mice. The image shows an example of clonality between sequences in the colon and in the small intestine. Mutations are shown with a vertical line, and shared mutations are indicated with a pound sign (#).

Discussion

This report unequivocally demonstrates that IgA CSR does not occur in nonorganized lymphoid tissues of the mouse gut LP. We found that IgA CSR was restricted to gut samples containing B cell follicles, whereas we failed to detect any switch α-CT in 35 mice with LP samples lacking follicles. Therefore, our study fails to confirm previous reports on IgA CSR in human and mouse non-organized LP (11, 20). Furthermore, to reconcile that IgA CSR was restricted to the GALT of CD40−/− mice despite the complete lack of GCs in the PPs, we provide evidence that IgA CSR in PP B cells can occur at a stage before manifest GC (i.e., when the cells are at a GL7int stage). In contrast, high levels of SHM in PP were found only in GL7high B cells that were associated with manifest GCs, and an extended analysis of mutations of IgA V-region sequences revealed that high levels of mutations occurred in LP IgA plasma cells in WT mice, but not in CD40−/− mice. Our data indicate that PP B cells in CD40−/− and WT mice undergo IgA CSR at a stage before manifest GC. In contrast, efficient SHM requires GC formation. The observation that clonally related sequences were found in LP at distant sites further supports the hypothesis that LP IgA plasma cell precursors undergo CSR, SHM and expansion before seeding the LP and also argues against local IgA CSR in the nonorganized gut LP being a dominant source for these cells.

Previous studies have suggested that the nonorganized LP is a site for substantial IgA CSR in mice (20). A more recent study by He et al. (11) suggested that IgA CSR could be induced in the nonorganized LP of the human colon in a process that involved recognition of intestinal bacteria by epithelial cells, the release of thymic stromal lymphopoietin, and production of APRIL by gut epithelial cells and DCs. CSR in the colon was not only from IgM to IgA, but also from IgA1 to IgA2. However, other studies in mice and humans do not find any evidence that the nonorganized LP is a prominent site for IgA CSR (30, 37, 38). All of these studies essentially failed to detect molecular evidence for ongoing IgA CSR in the non-organized LP. In addition, we found few IgM cells that could undergo IgA CSR in the nonorganized LP, whereas most IgM cells were found in organized follicles or in the PP. These findings exclude the LP as a primary site for IgA CSR, at least in the mouse. Missing evidence of significant B cell proliferation in the nonorganized LP in humans, or that APRIL can promote cell proliferation, also weakens this interpretation for human tissues (21, 37). Moreover, further arguing against IgA CSR in the nonorganized LP is our finding that clonal relationships existed between IgA plasma cells isolated at distant sites in the gut intestine. Such a presence is best explained by the expansion of B cells that have already undergone IgA CSR and SHM in organized lymphoid tissues before seeding the gut intestinal LP. Studies in humans corroborate this interpretation, in which closely related gene sequences were found at widely separated sites in the gut intestinal LP (5, 37, 39). Our data favor a model in which gut LP plasma cells originate from precursors in the GALT that can support both IgA CSR and SHM, such as the PP, before seeding the nonorganized small intestinal or colon LP. In CD40−/− mice, we believe the dominant site for IgA CSR is the PP, rather than the ILF or the colonic patches, because these mice largely failed to carry colonic patches and only 15–20% of biopsy specimens that hosted organized lymphoid tissue expressed switch α-CT. This hypothesis is further supported by our previous observation that young 3-wk-old CD40−/− mice that have PP, but lack most ILFs in the early phase of bacterial colonization, still exhibit significant levels of IgA plasma cells in the LP of the gut intestine (30).

In our previous study of CD40−/− mice, we reported low IgA CSR-activity in the MLN (<5% of the samples); therefore, we do not consider this site an important contributor to T cell-independent IgA CSR in conventionally reared mice (30). Notably, MLN has been found to be important for IgA CSR in mice treated in utero with LTβR-Ig fusion protein and lacking PP, suggesting a compensatory function under conditions of an
IgA CLASS SWITCH RECOMBINATION PRIOR TO MANIFEST GCs

References

Abstract
Aberrant mucosal barrier (40, 41). In addition, we observed enhanced IgA CSR activity in the MLN of germ-free mice subjected to bacterial colonization and lacking an intact IgA barrier function (P. Bergqvist, unpublished observation). Furthermore, we did not find evidence for IgA CSR in the spleen, bone marrow, peritoneal cavity, or nonorganized LP of the small intestine and, as documented in the current study, nor in the LP of the colon. Therefore, we propose that T cell-independent IgA CSR occurs predominantly in the PP in CD40−/− mice. We found that ~50% of all PP samples from CD40−/− mice contained switch α-CT. The B cell population that expressed switch α-CT and AID mRNA in the PPs was GL7int, undetectable by histologic analysis in CD40−/− mice, whereas the GL7high B cells, detectable by histologic analysis in WT mice, failed to express switch α-CT, but were strongly mutated and expressed high levels of AID mRNA. The higher expression of AID mRNA in WT GL7high cells agrees with the finding that AID expression and CSR in general is division linked and reflects possibly both CSR and SHM in the manifest GC (33). Although GL7 is widely used as a GC marker, no study has correlated the switch α-CT and AID mRNA transcription in relation to GL7 expression (42–45). We found that GL7int B cells exhibited all features associated with successful IgA CSR (i.e., α-germline RNA transcripts, AID mRNA, switch α-CT). The implication of our finding is that T cell-independent IgA CSR occurs in the PP at a stage before manifest GC. The fact that PP from CD40−/− mice hosted comparable or higher levels of gene expression for the potentially important IgA switch-factors, BAFF and APRIL (and perhaps iNOS), is important because it indicates that the PP milieu is well equipped for supporting IgA CSR, even in the absence of GC (22, 26). This information coupled with the evidence of cell proliferation in the GL7int population is the basis for proposing that the PP is the site for IgA CSR also against T cell-independent Ags.

To what extent the GL7int B cells were located in clusters or dispersed in the tissue close to or within the B cell follicles of the PP was not analyzed. However, as these cells hosted Ki67+ proliferating cells, detectable only by FACs and not seen microscopically, we can only speculate that they were dispersed in the tissue. Whether GL7int B cells were located at an extrafollicular or follicular site in the PP is not known. In support of an interfollicular site for IgA CSR in PP, AID mRNA expression accompanied by switch α-CT has been found in secondary lymphoid organs outside the GC, scattered in the T cell zone and within the mantle zone (46–48). Nevertheless, in this study we provide compelling evidence that T cell-independent gut IgA CSR occurs in the PP at a stage before manifest GC formations and that such IgA CSR cells carry largely unmutated V region sequences. We propose that T cell-independent IgA CSR in the GALT normally precedes SHM at a GL7int stage, before manifest GC. Furthermore, our study excludes that IgA CSR occurs in the nonorganized LP of the small intestine or the colon. Clonal relationships were found between IgA cells in the small intestine and colon, further supporting the notion of a common source for IgA CSR before seeding the intestinal LP. The PP appears to be central for IgA CSR in WT and CD40-deficient mice, and it hosts a milieu rich in IgA switch-factors regardless of the presence or absence of GCs.

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