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Gut IgA Class Switch Recombination in the Absence of CD40 Does Not Occur in the Lamina Propria and Is Independent of Germinal Centers

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

Conflicting findings have recently been presented as to the sites and sources of B cells that undergo class switch recombination (CSR) to IgA in the gut. In this study we provide compelling evidence in CD40<sup>−/−</sup> mice demonstrating that IgA CSR can be independent of CD40 signaling and germinal center formation and does not occur in the gut lamina propria (LP) itself. We found that CD40<sup>−/−</sup> mice had near normal levels of gut total IgA despite lacking germinal centers and completely failing to raise specific responses against the T cell-dependent Ags cholera toxin and keyhole limpet hemocyanin. The Peyer’s patches in CD40<sup>−/−</sup> mice expressed unexpectedly high levels of activation-induced cytidine deaminase mRNA and germline α transcripts, but few post-switch circular DNA transcripts, arguing against significant IgA CSR. Moreover and more surprisingly, wild-type mice exhibited no to low IgA CSR in mesenteric lymph nodes or isolated lymphoid follicles. Importantly, both strains failed to demonstrate any of the molecular markers for IgA CSR in the gut LP itself. Whereas all of the classical sites for IgA CSR in the GALT in CD40<sup>−/−</sup> mice appeared severely compromised for IgA CSR, B cells in the peritoneal cavity demonstrated the expression of activation-induced cytidine deaminase mRNA comparable to that of wild-type mice. However, peritoneal cavity B cells in both strains expressed intermediate levels of the germinal center marker GL7 and exhibited no germline α transcripts, and only three of 51 mice analyzed showed the presence of postswitch circular DNA transcripts. Taken together, these findings strongly argue for alternative inductive sites for gut IgA CSR against T cell-independent Ags outside of the GALT and the nonorganized LP. The Journal of Immunology, 2006, 177: 7772–7783.

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4 Abbreviations used in this paper: LP, lamina propria; AID, activation-induced cytidine deaminase; APRIL, a proliferation-inducing ligand; BAFF, B cell-activating factor; CSR, class switch recombination; CT, cholera toxin; DC, dendritic cell; GC, germinal center; ILF, isolated lymphoid follicle; KLH, keyhole limpet hemocyanin; MLN, mesenteric lymph node; PerC, peritoneal cavity; PP, Peyer’s patch; SFC, spot forming cell; SHM, somatic hypermutation; TD, T cell dependent; TI, T cell independent; WT, wild type.

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nodes (MLN) (19, 21). However, lymphotoxin α receptor-deficient mice, lacking both PP and MLN and reconstituted with normal bone marrow, produced near normal gut IgA levels, arguing against PP or MLN as necessary sites for IgA B cell development (22). Still, because dendritic cells (DC) can take-up luminal content through the epithelial barrier and thus transport bacteria to the MLN, they could serve as IgA inductive sites independently of the PP (23). Also, the LP itself has been proposed to be a possible primary site for IgA CSR for TI Ags (24). This is, though, a controversial finding, and two recent independent reports have failed to support the notion that naive B cells can undergo IgA CSR in the nonorganized LP (25, 26).

CSR and somatic hypermutation (SHM) are important processes for refining and shaping the function of humoral immunity. Subsequent to Ag exposure, these events take place in the organized secondary lymphoid tissues and are linked to the formation of the GC (27–29). Whereas CSR to IgG subclasses, except for IgG3 in the mouse, are known to depend on GC formations, less is known about the role of GC in IgA B cell differentiation (2). We and others have reported a number of different gene knockout or transgenic mouse models with a poor correlation between GC in the GALT and the presence of gut IgA and ability to respond to oral immunization (7, 30). For example, IL-4−/−, CD19−/−, or CTLA4-Hg1 transgenic mice respond poorly to oral immunizations with cholera toxin (CT) but demonstrate normal GC reactions in PP and have near normal levels of gut IgA (31, 32). By contrast, CD28−/− or TNFR1−/− mice have no GC in PP but produce near normal levels of gut IgA (7, 30). Thus, the presence of GC in PP does not constitute a requirement for IgA production in the gut LP. In fact, PP-null mice were shown to still have high numbers of IgA plasma cells in the gut LP, suggesting alternative inductive sites for IgA CSR (21).

CSR is a complex process and requires the interplay of a number of different enzymes and genes that are not yet completely understood. Recently, an enzyme called activation-induced cytidine deaminase (AID), which is necessary for CSR and SHM, was discovered (33). Mouse and humans lacking AID fail to produce Abs other than IgM (34), and the mice suffer from hyperplasia of ILF in the LP (35). The role that AID has in CSR is still debated. There are two main hypotheses, disputing whether RNA or DNA is the substrate for AID (36). However, the end result of AID activity is recombination of Ig genes and the production of switch circles that are looped out of the chromosomal DNA (37). These looped-out postswitch circles are present for a short time after CSR, and during this period spliced RNA transcripts between germline I promoters and Cμ can also be detected. Thus, the presence of the postswitch circles and the postswitch circular DNA transcripts represents our best marker for a just completed CSR (37). Together with the expression of germline α transcripts and AID mRNA transcripts, we have very sensitive molecular tools for the detection of CSR IgA. Using these tools, evidence for active CSR was reported to occur in the LP itself (24), but these results have been disputed. The controversy relates to whether or not the isolated cells could have been derived from ILF rather than the LP (24).

Notwithstanding this controversy, there is ample documentation that naïve IgM B cells can undergo IgA CSR under the influence of certain factors acting alone or together. In most species, TGFβ has been shown to induce IgA CSR, and deletions of the TGFβ-1 or the TGFβ receptor type II genes result in severe loss of IgA in mice (38, 39). Yet, TGFβ alone is insufficient to drive IgA CSR in B cells in vitro but requires a combination with, e.g., LPS or CD40 to provide mitogenic stimulation (40). However, recent reports have documented the possibility that IgA CSR could be stimulated by B cell-activating factor (BAFF) and a proliferation-inducing ligand (APRIL) acting through TACI (transmembrane activator, calcium modulator, and cyclophilin ligand interactor) alone or together with cytokines such as IL-10, and, as it appears, even independently of CD40-signaling (41–51). These factors, produced from stromal cells, macrophages, or DC in the tissue, would then act directly on naïve IgM B cells but may still not be produced completely independent of gut T cells, as suggested by Casola et al. (16). Because CD40-CD40L interactions control the expression of costimulatory molecules, the lack of CD40 may also indirectly influence such noncognate T cell functions. Furthermore, whereas CD40L is expressed not only by activated T cells but also by activated DC, the lack of CD40 could potentially have an impact on overall IgA formation. Even TI Ag-driven IgA CSR may, in this way, depend on CD40 signaling (52). Thus, both CD40 and TI Ag-stimulated gut IgA production pathways may require CD40 signaling to be effective.

In the present study we have used wild-type (WT) and CD40−/− mice to analyze to what extent gut IgA CSR is dependent on CD40 signaling and to address the compartments of the GALT from which IgA CSR may emanate. In particular, we have addressed whether IgA CSR occurs in the nonorganized gut LP. We have also investigated the relationship between GC reactions, gut IgA formation, and the expression of the molecular markers for IgA CSR as well as germline α transcripts, AID mRNA and postswitch circular transcripts in the GALT, LP, and PerC.

Materials and Methods

Mice

CD40−/− and WT mice (both on a C57BL/6 background) were bred and housed at the experimental medicine animal facility at Göteborg University (Göteborg, Sweden) under specific pathogen-free conditions. For comparison, we also used IgA−/− and double-deficient CD28/ICOS mice (53). The mice were sacrificed at 6–10 wk of age and used for experiments.

Immunohistochemistry

Naïve CD40−/− and C57BL/6 mice were sacrificed and the intestine, PP, or MLN were removed, embedded in TissueTek OCT compound, and snap frozen in liquid nitrogen. Frozen sections (7 μm) of the small intestine were prepared on microslides using a cryostat (Leica), and the sections were stored at −70°C until used. The slides were fixed in 50% acetic acid for 30 s and in 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 germinal centers, the sections were stained with FITC-conjugated anti-GL7 mAb diluted 1/100 (BD Pharmingen) and biotinylated anti-anti-B220 diluted 1/100 (BD Pharmingen) and were stained for 30–60 min. The B220 was visualized by adding streptavidin-conjugated Texas Red (DakoCytomation) 1/100 dilution. Consecutive sections were stained with stained with B220 together with FITC-conjugated anti-IgA diluted 1/100 (BD Pharmingen) and 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 LSC microscope.

For visualization and counting of ILF, intestines were divided into four parts, rolled up on a toothpick, embedded in TissueTek OCT compound, and snap frozen in liquid nitrogen, and sectioned as described above. The sections were blocked with 0.3% H2O2 in PBS for 5 min, rinsed in PBS, and then blocked with normal horse serum in PBS (5%) for 15 min in a humidifying chamber. To block endogenous biotin, sections were incubated with a avidin/biotin blocking kit (Vector Laboratories) followed by incubation with primary biotinylated anti-B220 for 30–60 min. Following a brief rinse in PBS, peroxidase-conjugated avidin/biotin complexes were prepared and added to the sections according to the manufacturer’s instructions, incubated for 45 min, washed with PBS, and visualized by adding 3-amino-9-ethylcarbazole (Vector Laboratories). The sections were counterstained with hematoxylin, mounted with Faramount (DakoCytomation), and visualized using a Leica LSC microscope.

Cell turnover assessment

BrdU (Sigma-Aldrich) was administered orally by feeding the mice once with 500 μl of BrdU (1 mg/ml) in H2O and simultaneously injecting i.p.
500 μl of BrdU (4 mg/ml) in PBS. Twenty-four hours later the mice were given another BrdU pulse orally. Seventeen hours, 3 days, 1 wk, or 3 wk after BrdU treatment the mice were sacrificed, and the intestine was removed and frozen as described above. Before immunofluorescent staining the sections were treated with 2 M HCl for 30 min at 37°C, neutralized in 0.1 M Na2B4O7 for 5 min. The sections were then stained and mounted as described above using FITC-conjugated anti-BrdU (1/5) and anti-IgA-TXRd (1/100). The number of BrdU-labeled cells in the lamina propria was determined within a fixed area in all sections and visualized in a diagram.

Lamina propria lymphocytes were prepared and BrdU incorporation was visualized using a BrdU flow kit (BD Biosciences) and double stained with anti-IgA according to the instructions and analyzed by FACS. naive intestines from CD40−/− mice and WT mice were removed and washed twice time by time in PBS. The intestine was snap frozen and sectioned as thin as possible. To visualize apoptosis we used the ApopTag in situ TUNEL apoptosis detection kit (Chemicon). The sections were counterstained with Texas Red-conjugated anti-IgA (Southern Biotechnology Associates) diluted 1/100 for 30 min, washed in PBS, and mounted with DakoCytomation fluorescent mounting medium.

**ELISPOT**

A 96-well MultiScreen plate (Millipore) was washed in PBS and coated with 5 μg/ml purified rat-anti-mouse-IgA Abs (BD Pharmingen) or 5 nmol/ml ganglioside GM1 followed by 3 μg/ml CT or 100 μg/ml keyhole limpet hemocyanin (KLH) at 4°C overnight. The plates were washed in PBS, blocked with 0.2% BSA in PBS, and 10,000 purified lamina propria lymphocytes, prepared as previously described (30), were added in duplicate and diluted 1/3 in four serial dilutions directly on the plate and incubated at 37°C overnight. The plate was then washed twice with PBS plus 0.05% Tween 20 and twice with PBS, followed by incubation with alkaline phosphate-conjugated goat anti-mouse-IgA Abs (Southern Biotechnology Associates) overnight at 4°C. The next day the plates were washed twice with PBS plus 0.05% Tween 20 and twice with PBS. The spots were visualized by the addition of the one 5-bromo-4-chloro-3-indolyl phosphate tablet (Sigma-Aldrich) dissolved in 10 ml of H2O, and 100 μl was added to each well to incubate at 37°C for 10 min in the presence of 10 ng/ml CT or 100 μg/ml CT overnight. The plates were then washed twice with PBS plus 0.05% Tween 20, twice in PBS, dried, and analyzed using the ImmunoSpot ELISPOT system (Cellular Technology).

**ELISA**

Ninety-six-well plates (Nunc) were coated with 100 μg/ml KLH (100 μl well), 0.5 mM GM1 followed by 0.5 μg/ml CT at 4°C overnight. For total IgG titers, the 96-well plates were coated with 5 μg/ml anti-mouse-IgG (Southern Biotechnology Associates) and incubated at 4°C overnight. The plates were washed three times with PBS followed by blocking in PBS plus 0.1% BSA for 30 min at 37°C. The PBS/BSA was discarded, serum samples were diluted 1/100 and added to the plates, and a 3-fold serial dilution was made. The gut lavage was diluted 1/10 and a 2-fold serial dilution was made. The samples were incubated at 4°C overnight and washed three times with PBS-Tween 20. The plate was then washed twice with PBS plus 0.05% Tween 20, twice in PBS, dried, and analyzed using the ImmunoSpot ELISPOT system (Cellular Technology).

**Flow cytometry and cell sorting**

Lymphocytes from PP and MLN were prepared by excising the PP from the intestine and passing them through a cell strainer of 100-μm nylon mesh (BD Falcon). Peritoneal cells were isolated by carefully peeling off the skin from the mouse peritoneum, and 8 ml of ice-cold PBS was injected into the peritoneal cavity. After 5 min the peritoneum was cut open and the fluid was retrieved using a transfer pipette. The cells were suspended in PBS supplemented with 0.1% BSA and 0.02% EDTA and stained with anti-B220-PE (BD Pharmingen) and GC-specific anti-GL7-FITC Abs (54) (BD Pharmingen) or anti-IgA-FTTC (Southern Biotechnology Associates). Forty-eight hours after the last BrdU pulse the cells were fixed and permeabilized using the FoxP3 staining set (Bioscience), but the anti Ki67-FITC Ab (BD Pharmingen) was used instead of the anti-Foxp3 Ab. To ensure single cell suspensions, the cells were filtered through a MACS preseparation filter (Miltenyi Biotec). The cells were sorted in a FACSaria device (BD Biosciences) in PBS supplemented with 0.1% BSA and 0.02% EDTA. Sorted cells were spun down at 2000 rpm at 4°C followed by RNA extraction using the RNeasy micro kit (Qiagen). The data were analyzed using the FlowJo software (Tree Star).

**RNA preparation and RT-PCR**

The small intestines were removed and punch biopsies were made from the PP using a 2-mm diameter dermal puncher, isolated, and stored in TRIzol (Invitrogen Life Technologies). RNA was prepared according to the TRIzol protocol, dissolved in 50 μl of UltraPure distilled water (Invitrogen Life Technologies), and stored at −80°C. The RNA concentration was measured and 4 μg of RNA was used for cDNA synthesis using oligo(dT) RETROscript primers (Ambion) and SuperScript III RT polymerase (Invitrogen Life Technologies). MLN were isolated from naive mice and stored in TRIzol, and RNA was prepared as described above.

**ILF-free sections of gut LP**

To ensure analysis of ILF-free sections, 2- to 3-cm pieces of the small intestine were frozen and sectioned as described (55). Every fifth section was placed on a slide and the sections in between were stored in RLT buffer and RNeasy lysis buffer (Qiagen) at −70°C. The sections on the slide were stained with Texas Red-conjugated anti-IgM (Southern Biotechnology Associates) and FITC-conjugated anti-IgA (BD Pharmingen) to localize ILF. If two consecutive sections on the slide were ILF-negative, the sections in between were stored in RLT buffer and considered to be ILF free. RNA was prepared from these sections using the RNeasy micro kit (Qiagen).

**Peritoneal washouts**

Mice were sacrificed and the cells were isolated as described above. The cells were spun down at 2000 rpm at 4°C and RNA was extracted using the RNeasy mini kit (Qiagen).

**Primers**

To detect germline α transcripts the primers Io5′-CCA GGC GTG GAG ATA GAG GTA GAG GTA GTC TGG-3′ and CaR 5′-GAG CTC GTG GGA GTG TCA GTG-3′ were used. Cycling was performed under the following conditions: denaturation at 95°C for 5 min followed by 30 cycles of PCR (95°C for 1 min, 62°C for 1 min, and 72°C for 1 min) followed by a final extension at 72°C for 10 min. For AID transcripts the following primers were used: mAID119 (5′-GAG GGA GTC AAG AAA GTC ACG CTC G-3′), mAID1 (5′-CCA GGC TTT GAA AGT TCT TCT ACG-3′), and, as a cell loading control, CD79a forward (5′-AAC ACA GGG GCT TGT ACT CCT GG-3′) and CD79a reverse (5′-ATC TCC AAT GAG GTT GAC GC-3′). The cycling was performed as described above with 25 cycles and an annealing temperature of 59°C. Circular transcripts were detected by a nested PCR using the outer primers Io4′ (5′-ACC CTG GAT GAC TTC ATG GTT-3′) andSoup4 (5′-CAT CTG GAC TCC TCT GCT CA-3′) and the inner primers Io5′ (5′-CCA GGC ATG GAT GAG ATA GAG GTA G-3′) and CaR (5′-AAT GGT GCG GAG GAA GAA GA-3′). Each PCR was performed as described above with 30 cycles each and an annealing temperature of 56°C for the first PCR and 62°C for the second one.

**Southern blot**

The AID and CD79 PCR products were separated on a 2% agarose gel and the gel was photographed to visualize AID and CD79. To confirm the AID expression, the fragments were transferred to a Hybond − membrane (Amersham Biosciences) by passive diffusion using 0.4 M NaOH overnight. The membrane was dried and cross-linked by UV light and then incubated for 1 h in blocking solution at 45°C. An AID-specific probe, AIDhyb_1 (5′-ATA TGG ACA GCC TTC TGA TGA AAC AA AGA AGT TTC-3′), was labeled by mixing 5 pmol of AIDhyb_1 probe, 5 μl of 10× T4 PNK buffer (Promega), 1 μl of T4 polynucleotide kinase (Promega), 5 μl of Redivue adenosine 5′-[32P]triphosphate, triethylammonium salt (Amersham Biosciences), and water to 50 μl. The mixture was incubated for 1 h at 37°C, and free nucleotides were removed using a ProbeQuant G-50 micro column (Amersham Biosciences). The labeled probe was added to the membrane and incubated at 45°C overnight. Hybridization was detected on autoradiography film (Kodak).

**One-step PCR**

RNA prepared from sorted cells, cells from sectioning, and peritoneal washouts was analyzed using one-step RT-PCR (Invitrogen Life Technologies). The RNA was purified as described above and 2 μl of RNA was used in the RT-PCR. For the CDNA synthesis, gene-specific primers were

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added and the cDNA synthesis temperature was the same as the annealing temperature for the PCR except during the detection of germline α transcripts, when the cDNA synthesis temperature was 59°C and PCR annealing temperature was 62°C.

Statistical analysis

Data were compared using Student’s t test.

Results

CD40–/– mice show near normal formation of gut IgA

Although it is well established that cognate T cell-B cell interactions are required for systemic IgG responses, less is known about CSR to IgA and to mucosal IgA in particular (4, 5). CSR is classically thought to be restricted to GC, which do not develop in the absence of CD40 (56). Therefore, we predicted a dramatically impaired CSR and mucosal IgA formation in CD40–/– mice. However, quite in contrast, we found substantial IgA production in the gut of CD40–/– mice. In fact, labeling frozen sections of the small intestine with anti-IgA revealed almost normal numbers of IgA plasma cells in gut villi (Fig. 1A). Moreover, total IgA levels in gut lavage were comparable with those in WT mice, and the frequency of IgA-producing, spot-forming cells (SFC) in freshly isolated LP cells was not significantly reduced in CD40–/– mice (Fig. 1, B and C). Whereas IgM was increased 3-fold and IgA reduced to 50% of normal serum levels, IgG1 and IgG2a Abs were below detection limits in serum from CD40–/– mice (Fig. 1D).

Next we addressed whether the substantial IgA production observed in the LP of CD40–/– mice readily reflected an ability to respond to TD Ags in the gut. To this end, oral immunizations with KLH supplemented with CT adjuvant were performed (57). We found no IgA or IgG responses to these Ags in CD40–/– mice, whereas WT mice demonstrated strong specific serum IgA and IgG and gut LP IgA production (Fig. 2, A–D). None of the CD40–/– mice responded with specific IgM Abs against CT, and only 5 of 15 responded against KLH, whereas WT mice responded strongly with IgM Abs against both Ags (Fig. 2, C and D). Furthermore, a similar result was obtained in serum after parenteral immunizations with KLH plus CT adjuvant with no specific IgG or IgA production in CD40–/– mice, whereas IgM Abs against KLH, but not CT, were detected in some mice (Fig. 2, E and F). Of note, parenteral immunizations also failed to stimulate specific IgA responses in serum in WT mice (Fig. 2, E and F). Taken together, our data indicated that CSR to IgA, as opposed to IgG-subclasses (except IgG3), can occur independently of CD40. However, as expected, CD40-CD40L interactions are clearly required to mount specific IgA and IgG responses to orally or parenterally administered TD Ags (58).

Lack of IgA inductive sites in the GALT of CD40–/– mice

Given that CD40–/– mice have poor CSR to IgG and an impaired ability to respond to orally administered TD Ags but show near normal levels of total gut IgA, we analyzed various compartments of the GALT for possible inductive sites for IgA CSR. Frozen sections were investigated for the presence of GC, IgA production, and proliferating cells using the Ki67 Ag as a marker. Whereas the PP of WT mice contained distinct GC areas with aggregates of cells brightly staining with GL7-, IgA- and Ki67-specific Abs, PP from CD40–/– mice failed to label with any of these markers (Fig. 3A). FACS analysis of PP from CD40–/– mice showed <1% of the B220+ cells expressing GL7, whereas in WT mice an average of 6% PP B cells were GL7+ (Fig. 3B). In WT PP B cells, roughly 5% were stained with an anti-IgA mAb, whereas in CD40–/– mice <1% of the cells were labeled (Fig. 3B). For comparison, we used the low/background levels of IgA and GL7-labeling B cells in PP from IgA–/– or CD28/ICOS double-deficient mice (no GC-reactions), respectively, as negative controls (53). Another potential site for IgA-switching is ILF along the anti-mesenteric lining of the small intestine (Fig. 1A). The frequency and distribution of ILF in CD40–/– mice were comparable to that in WT mice with increasing numbers toward the distal part of the small intestine (Fig. 4A). A thorough analysis of the ILF revealed a lack of IgA- and Ki67-positive cells in CD40–/– mice (Fig. 4B). ILF from WT mice were also largely devoid of IgA+ cells, and no GL7- or Ki67-labeling areas were found (Fig. 4B), suggesting that these structures do not contribute much to the overall production of IgA, even in the normal gut. In young mice (3 wk of age) from both strains we were unable to detect any ILF, but IgA SFC numbers in gut LP were significant and comparable in CD40–/– and WT mice (Fig. 4, C and D); again supporting the notion that IgA formation in the gut LP of CD40–/– and WT mice does not rely on the presence of ILF but appear to be recruited from other inductive sites. Furthermore, the gut draining MLN in both CD40–/– and WT mice appeared to be poor sites for IgA CSR. Although none of the MLN from CD40–/– mice stained with any of the three markers, a few small and scattered GC were found in WT MLN, failing to support the notion that MLN is an important site for CSR to IgA (19) (Fig. 4E). FACS analysis demonstrated low levels of GL7-expressing cells in WT and CD40–/– mice, not
exceeding the frequency seen in PP of CD40-/- mice (Fig. 4E). Thus, we were unable to find a site in the GALT with ongoing GC formation and CSR to IgA in CD40-/- mice, and even in WT mice the ability to develop such a site appeared to be poor outside of the PP.

A simple explanation for the large number of IgA plasma cells in the LP, despite the impaired ability to respond to TD Ags, could be altered gut plasma cell dynamics in the CD40-/- mice. We failed, however, to identify any difference in plasma cell turnover in the gut LP of CD40-/- mice as compared with that in WT mice, as evidenced by pulse-chase BrdU treatment experiments (Fig. 5A). Following BrdU loading of gut plasma cells we observed no cell turnover in CD40-/- mice, as evidenced by pulse-chase BrdU treatment experiments (Fig. 5A). The figure shows one representative experiment of three. Mean values ± SD are shown (p < 0.01). ND, Not detected.

The BrdU-treated animals showed that both strains had ~1% of IgA+ BrdU+ cells in the small intestine 1 wk after BrdU labeling (data not shown). Moreover, the levels of IgA+ cells undergoing apoptosis within the gut mucosa of both strains were comparable, arguing against a difference in IgA plasma cell turnover between the strains (Fig. 5D). These data suggested that IgA plasma cells do not exhibit altered dynamics in gut LP of CD40-/- as compared with WT mice.

**Molecular screening for IgA CSR in the GALT of CD40-/- mice**

To increase the sensitivity of the analysis for detection of IgA CSR in the GALT of CD40-/- mice, we used RT-PCR for the presence of germline α transcripts, AID mRNA, and postswitch circular DNA transcripts (24, 37). With this technique we confirmed that PP in WT mice are strong sites for IgA CSR with frequent and high mRNA expression of AID and germline α transcripts as well as the presence of postswitch circular DNA transcripts (Fig. 6A). By contrast, PP from CD40-/- mice showed the expression of germline α transcripts and AID mRNA, albeit weaker, but few postswitch DNA-transcripts were found, arguing against PP being a major site for IgA CSR in these mice (Fig. 6A). In fact, we
FIGURE 4. ILF frequency and distribution are comparable in WT and CD40−/− mice. A, The diagram illustrates the distribution of ILF determined by consecutive sectioning along rolled up intestines. The intestine was divided into four parts, rolled up, and snap frozen, followed by an analysis of the presence of ILF by staining sections with anti-B220. Mean values ± SD of three individual experiments are shown. B, The illustration shows three consecutive sections of a typical ILF from CD40−/− and WT mice stained with anti-B220 (red) and anti-GL7, anti-Ki67, and anti-IgA (green). C, Small intestines from 3-wk-old and adult mice (8 wk) were rolled up and stained with anti-B220 and counterstained with hematoxylin. Arrowheads in the photographs of the adult intestines indicate the presence of ILF. D, The relative frequency of IgA-producing cells in the LP of 3-wk-old mice is shown. The diagram shows one representative experiment of three. E, Immunohistochemical analysis of MLN is shown. Naive MLN were excised, snap frozen, and sectioned, followed by staining with anti-B220 (red) and anti-GL7, anti-Ki67, and anti-IgA (green). F, Representative FACS analysis of GL7 expression in MLN from naive CD40−/− and WT mice are shown. n.s., No significance.
detected postswitch DNA transcripts in <25% of 45 PP isolated from 10 different CD40−/− mice, whereas in 47 PP from 10 WT mice >85% demonstrated presence of these transcripts. Of note, given that no GC reactions (GL7- and Ki67-negative) were detected in PP of CD40−/− mice (Fig. 3), it was surprising to find relatively prominent AID mRNA expression (Fig. 6A). An extended analysis of sorted GL7-high, -intermediate, and -negative cells from both strains revealed readily detectable AID mRNA expression in the GL7-intermediate and -high populations, but not in the negative population. Thus, substantial AID mRNA-expression was evident in the GL7-intermediate population, albeit not nearly as strong as in the GL7-high population. (Fig. 6B). Moreover, in agreement with the histological analysis, the MLN of both CD40−/− and WT mice expressed very low levels of any of the molecular markers for IgA CSR (Fig. 6C). Of 21 MLN samples from nine CD40−/− mice analyzed, we detected AID mRNA in 20% and postswitch circular DNA-transcripts in <5%. However, MLN from WT mice also appeared to be poor sites for IgA CSR when compared with PP from the same mice, as we detected weak expression of AID mRNA and postswitch circular DNA transcripts in only 14% of the MLN samples. Although an unlikely site for IgA CSR, we also included the spleen in our analysis but found no germline transcripts, weak or no AID mRNA-expression, and no postswitch DNA transcripts in this tissue in neither CD40−/− or WT mice (Fig. 6D).

The gut LP is not a site for IgA CSR

Previous work has indicated that the gut LP could be a site for TI IgA CSR (24). Because we had failed to explain the relative abundance of IgA in the gut LP of CD40−/− mice, we therefore turned to investigating the LP for evidence of IgA CSR. Gut LP cells showed no postswitch circular DNA transcripts or AID mRNA expression in either WT or CD40−/− mice, clearly rejecting the notion that IgA CSR normally occurs in this nonorganized tissue of the mucosal immune system (Fig. 7). We analyzed 12 individual mice from each strain and two different samples from each intestine and never found AID mRNA expression or postswitch circular DNA transcripts, whereas germline transcripts were clearly detected. Of note, to insure that gut LP samples were devoid of ILF we used the method previously described by Stoel, M. et al. (55) (see Materials and Methods). Importantly, specimens containing ILF occasionally demonstrated AID mRNA expression (not shown).

**FIGURE 5.** Unaltered gut plasma cell turnover in CD40−/− mice. A, Mice were given BrdU by simultaneously feeding it orally for 2 days and injecting it i.p., and the mice were then sacrificed at the indicated time points after challenge. The intestines were removed and stained with anti-IgA (red) and anti-BrdU (green). B, High magnification view of stained sections taken one week after BrdU treatment. C, BrdU-positive cells in the lamina propria at the different time points indicated. Numbers are average BrdU-positive cells from three different sections within one visual field in the microscope. D, Detection of apoptotic cells in the small intestine using a TUNEL assay is shown. Intestines from naive mice were stained for apoptotic cells (green) and IgA-positive cells (red).**
FIGURE 6. Analysis of molecular markers for IgA CSR. RNA was prepared and analyzed by RT-PCR. The figure shows the expression of germline α transcripts (α-GT) measured by RT-PCR, AID transcripts measured by RT-PCR followed by Southern blot analysis and postswitch circle transcripts measured by nested RT-PCR. A, PP from naive CD40−/− and WT mice were analyzed. The CD79 PCR was performed in the same tube as the AID PCR and used as a B cell-specific loading control, and β2m PCR was used as a RNA loading control. Samples without RT added (−RT) and samples from mt mice were used as negative controls, and a PP sample from a WT mouse was used as a positive control. The different sizes in the postswitch circle transcripts correspond to different splice variants of the transcript. B, FACS sorting of PP cells from WT and CD40−/− mice was performed. PP lymphocytes from five individual mice were isolated and pooled and stained with anti-B220, anti-GL7, and anti-CD3 and were then sorted into GL7-negative (GL7−), GL7-intermediate (GL7 int), and GL7-high (GL7 hi) cells. The gates show the different sorted fractions before and after sorting. RNA was isolated from the different fractions and AID expression was analyzed by one-step RT-PCR amplification followed by Southern blotting. CD79 was used as a B cell-specific loading control. C, Expression of germline α transcripts, AID transcripts, and postswitch circle transcripts in MLN of naive CD40−/− and WT mice was performed as described above. D, Expression of germline α transcripts (α-GT), AID transcripts, and postswitch circle transcripts in the spleens of naive CD40−/− and WT mice was performed as described above.
has, to our knowledge, not been previously investigated, although germline \( \gamma^H9251 \) transcripts and postswitch \( \gamma^H9251 \) transcripts have been reported (20). Thus, to this end we isolated PerC B cells and extracted RNA for subsequent RT-PCR analysis for IgA CSR. We found that PerC B cells from the two strains demonstrated high and comparable expression of AID mRNA, but very few samples expressed germline \( \gamma^H9251 \) transcripts or postswitch circular DNA-transcripts in either CD40 \( \gamma^H11002 \) or WT mice (Fig. 8A). In total, 56 mice were analyzed and only three of these (one WT and two CD40 \( \gamma^H11002 \) ) indeed expressed postswitch circular DNA transcripts. FACS sorting of PerC B cells demonstrated that AID mRNA expression was largely restricted to the GL7-intermediate population, in particular in the CD40 \( \gamma^H11002 \) mice (Fig. 8B). Furthermore, we could detect proliferating Ki67\(^{+} \)IgM\(^{bright} \) cells in the PerC of both strains (Fig. 8C). However we could not detect any difference in the frequency of Ki67-labeled cells in the PerC of the two strains, albeit there

FIGURE 7. Expression of molecular markers for ongoing CSR in the lamina propria. RNA from ILF-free lamina propria from CD40 \( \gamma^H11002 \) and WT mice was isolated according to Materials and Methods. Expression levels of germline \( \alpha \) transcripts (\( \alpha\)-GT), AID transcripts, and postswitch circle transcripts was visualized by one-step RT-PCR followed by Southern blot (AID) and nested PCR (postswitch circle transcripts). The CD79 PCR was performed in the same tube as the AID PCR and used as a B cell-specific loading control, and the \( \beta2m \) PCR was used as a RNA loading control.

FIGURE 8. Peritoneal cavity cells express AID but very few postswitch circular transcripts. A, Peritoneal cavities from naive CD40 \( \gamma^H11002 \) and WT mice were washed out with PBS, and RNA was isolated from the collected cells. The expression of germline \( \alpha \) transcripts (\( \alpha\)-GT), AID transcripts, and postswitch circle transcripts was visualized by RT-PCR (\( \alpha\)-GT), RT-PCR followed by Southern blotting (AID), and nested PCR (postswitch circle transcripts). The CD79 PCR was performed in the same tube as the AID PCR and used as a B cell-specific loading control, and the \( \beta2m \) PCR was used as a RNA loading control. B, A FACS analysis of PerC cells is shown. The gates show the populations before and after sorting. RNA was prepared from the sorted fractions and analyzed for AID expression as in A. RNA prepared from a PP of a WT mouse was used as a positive control. GL7\(^{-} \), GL7-negative cells; GL7 int, GL7-intermediate cells; and GL7 hi, GL7-high cells. C, An analysis of the presence of proliferating cells in the PerC and the PP of CD40 \( \gamma^H11002 \) and WT mice is shown. The cells were stained with anti-IgM or anti-B220, permeabilized, fixed, and stained with anti-Ki67. Mean values of IgM\(^{+} \)Ki67\(^{+} \) cells were 7.4 \( \pm \) 2.5% for CD40 \( \gamma^H11002 \) (n = 7) and 8.0 \( \pm \) 3.6% for WT (n = 7) in the PerC.
was a 3- to 4-fold lower level of proliferating Ki67+B220+ cells in the PP in CD40−/− mice as compared with that in WT mice (Fig. 8C).

**Discussion**

The present study of CD40−/− mice corroborated observations of normal to high IgA levels in patients with impaired CD40-signalizing (59). Here we report near normal levels of gut IgA and a 50% reduction in serum IgA, whereas IgG1 and IgG2a were undetectable in CD40−/− mice. Thus, significant IgA CSR can occur in the complete absence of CD40 and GC reactions. Furthermore, we found that CD40−/− mice completely failed to respond to oral (as well as parenteral) immunizations with the TD Ags, CT, and KLH while, in fact, demonstrating near normal levels of total IgA plasma cells in their gut LP. In the search for a site where naïve B cells would undergo IgA CSR in these mice, we were unsuccessful in documenting microscopic or molecular data to suggest that any of the traditional locations within the GALT or the spleen are strong sources for IgA CSR. Compared with WT mice, the CD40−/− mice exhibited dramatically reduced levels of IgA CSR in PP and MLN, whereas the ILF and LP were poor sites for IgA class switching in both strains. B cells in the PerC demonstrated significant levels of AID mRNA expression in both strains, and an equal but low frequency of postswitch circular DNA transcripts were detected. These findings are consistent with the notion that IgA in CD40−/− mice is produced against TI Ags and that B1 cells are potentially the main source of this IgA (10, 60). The question of whether it is the B1 PerC population or the strongly impaired IgA CSR in PP or MLN (in B1 or B2 cells) that contributes to most of the LP IgA plasma cells in CD40−/− mice, and to what extent, cannot be answered by the present study. However, we can conclude that the gut LP is unlikely to be a primary site for IgA CSR in either CD40−/− or WT mice because of a complete lack of AID mRNA expression and no evidence of postswitch circular DNA transcripts.

We made some remarkable observations regarding expression of AID mRNA, germline α transcripts, and postswitch circular DNA transcripts was clearly found in WT PP, denoting this site a true place for IgA CSR. This finding was also supported by strong labeling with specific Abs for GC and IgA in tissue sections of PP from WT mice. By contrast, CD40−/− mice had PP that failed to label for GC or IgA and exhibited substantial levels of germline α transcripts and AID mRNA but substantially reduced postswitch circle DNA transcripts. Therefore, we believe that PP may not be an important site for IgA CSR in CD40−/− mice unless activated IgA CSR-committed B cells migrate out of the PP to complete the CSR. Alternatively, only 25% of normal CSR-activity in PP, as in the CD40−/− mice, suffices to provide WT levels of gut LP IgA plasma cells. Interestingly, despite the lack of GC in CD40−/− mice, the AID mRNA expression in PP B cells was prominent. AID is normally thought of in the context of GL7high B cells in the GC and is critically required for CSR and SHM (33, 61). Recent studies have also documented AID-expression in B cell tumors and extrafollicular large proliferating normal B cells (62–64). However, contrary to our finding, these latter cells showed evidence of CD40 signaling, were Ki67+, and resulted from TD Ag activation (62). Of note, CD40 signaling is one pathway that can induce AID expression as can IL-4 and TLR4 signaling or pathways that affect intracellular levels of protein kinase A (65–68).

The B cell population that transcribed AID in the PP of CD40−/− animals expressed an intermediate level of GL7 undetectable by histology, whereas the GL7-negative B cells were also AID mRNA-negative. To our knowledge, no previous study has reported AID mRNA transcription in relation to GL7 membrane expression. We found that microscopically GL7-negative tissues can, indeed, host substantial AID mRNA expression. Both in PP and PerC B cells AID mRNA expression was found in GL7-intermediate cells, indicating that these cells have the potential to undergo CSR. It is also possible that these cells undergo IgA CSR, although this process would have to be nondeletional, as seen, e.g., in hairy leukemic cells, which also fail to produce circle DNA transcripts (63). Rather, we believe that IgA CSR in the activated B cells in PP or PerC is completed at some other not yet defined location before seeding the gut LP. This location does not appear to be the spleen or MLN. However, the significance of extrafollicular AID mRNA expression for CSR and SHM is still controversial (62). Therefore, it remains to be seen to what extent GL7-intermediate B cells are responsible for IgA CSR in response to TI Ags in normal individuals. Nevertheless, in this study we provide compelling evidence that CD40−/− mice have near normal total gut IgA plasma cell levels and yet exhibit only extrafollicular sites of AID mRNA expressing B cells. In addition, preliminary studies of SHM in IgA V_H genes from gut LP cells from CD40−/− mice surprisingly revealed many mutated clones even compared with those observed in WT gut IgA cells, suggesting that even this normally GC-restricted quality could be provided by extrafollicular sites (P. Bergqvist, unpublished observation).

Evidence that LP would be a primary site for IgA CSR was first reported by Fagarasan et al. (24). The background to the study was that AID−/− mice had increased levels of B220+IgM+ cells in the LP, and the authors hypothesized that that these cells could undergo IgA CSR in the LP itself. However, their data have recently been challenged by at least two studies that cannot confirm the notion that gut LP would be a primary site for IgA CSR (25, 26). The ILF compartment is grossly enlarged in AID−/− mice, and it is probable that the nonorganized LP is diminished in these mice in keeping with the interpretation that the purified gut cells were likely to contain cells from ILF and not exclusively nonorganized LP in AID−/− mice (24). In the present study we found that AID mRNA expression was detected in ILF-containing biopsies but not in the biopsies of normal nonorganized small intestinal LP. Shikina et al. (26) also used careful dissection of LP tissue to exclude ILF and, in agreement with our study, failed to detect AID mRNA and postswitch circle DNA transcripts. In human LP tissue, Boursier et al. (25) failed to detect the molecular markers for IgA CSR, including a lack of AID mRNA. Furthermore, clonal analysis of somatic mutations in the rearranged IgV_H5-gene segment in IgA+ cells isolated from distant sites in the nonorganized LP showed greater similarity than when compared with mutations in neighboring sites, favoring a model of dissemination of precursor cells derived from a common source within the organized GALT (25). At variance with our study and that of Boursier et al., Shikina et al. did not detect germline α transcripts in LP (25, 26). The reason for this is unknown. Nevertheless, because expression of these germline α transcripts is necessary and precedes CSR but cannot initiate CSR in the absence of AID expression, these transcripts merely indicate the potential of B cells in the gut LP to undergo CSR (69, 70). We believe that the lack of AID mRNA and postswitch circle DNA transcripts as well as failure to demonstrate B cell proliferation, i.e., Ki67-labeling cells (P. Bergqvist, unpublished observation), disqualify gut LP as a primary site for IgA CSR. For these reasons we cannot ascribe IgA CSR to the PP or, indeed, all of the GALT, including MLN, in CD40−/− mice, as these sites fail to exhibit the signifying factors required for an active IgA CSR. If CSR is initiated in the GALT of these mice at all, cells must leave, e.g., the
PP, before undergoing full deletional CSR to IgA. We believe that this site or sites are to be found.

In agreement with previous studies we found that CD40 deficiency mice lack GC completely (56). A common notion is that the presence of GC in the PP of WT mice constitute a source of gut LP IgA plasma cell precursors (6, 71). These GC B cells are predominantly B2 cells that appear to respond not only to TD Ags, but to a significant degree also to TI Ags associated with the commensal flora (13). Hence, the PP GC formations seen in CD19−/− mice, which largely lack B1 cells, could be B2 cells reacting to the commensal flora, whereas these mice are unable to respond to oral immunizations with TD Ags (31). In fact, GC formations in PP may not even be dependent on BCR recognition but result from the presence of luminal bacteria and T cells (16). Because genetically deficient mice such as CD19−/− or TNFR1−/− mice differ with regard to the presence of GC in their PP and still make good levels of IgA, it must be concluded that there is no direct relationship between GC in GALT and IgA CSR in either B1 or B2 cells (7, 31).

Two tissues were surprisingly poor sites for IgA CSR also in WT mice. Considering the paucity of detectable molecular markers for IgA CSR in both MLN and the ILF, the relative to that in PP, we must conclude that they appear to be subordinate for the total gut IgA formation. Because previous reports have demonstrated active CSR in LP IgA plasma cells (8). However, we found almost no IgA in young (3-wk-old) mice, but substantial IgA SFC were detected in gut LP in both CD40−/− and WT mice. Although the ILF were formed independent of CD40 and appeared to develop in response to the luminal flora of bacteria, they appear to have little to do with populating the gut LP with IgA plasma cells. The ILF in CD40−/− mice were comparable in numbers and distribution to those in WT mice and hosted naive IgM− cells. In both WT and CD40−/− mice IgA CSR in ILF were not prominent, as determined by the lack of postswitch circle DNA transcripts and poor correlation to gut IgA SFC (P. Bergqvist, unpublished observation). Furthermore, MLN has been found to be critical for processing luminal bacteria carried to the node by DC migrating from PP and the gut LP (72). These and other findings have focused attention on MLN as a possible site for IgA CSR in the GALT. Our study does not support this idea. Rather, it is the phagocytic and eliminating activity against the transported bacteria that is critically located at the MLN, and the MLN is not a site for IgA CSR of the gut LP IgA plasma cells.

Previous reports have suggested that PerC B-1 cells could contribute to as much as half of the total IgA production in the mouse small intestine (11, 55). The PerC B-1 cells in both WT and CD40−/− did not express IgA (P. Bergqvist, unpublished results) but, interestingly, were strong in AID mRNA transcription. This report is, to our knowledge, the first to document AID mRNA and postswitch circle DNA transcripts in PerC B cells. However, the frequency of postswitch circle DNA transcripts was very low, and relatively few cells were Ki67-positive as compared with PP, suggesting little ongoing proliferation. At variance with de Waard et al. (20), we failed to detect germline α transcripts in the PerC B cells. Taken together, this raises the question of whether these cells were already committed or were on their way to initiate or complete IgA CSR at some other location. AID mRNA expression by itself is not sufficient for CSR; additional cofactors are required (73). Of note, compared with PP B2 cells in the PP of WT mice, the frequency of postswitch circle DNA expression was very low in PerC B1 cells. Moreover, none of the earlier reports have established where, indeed, PerC B-1 cells are exposed to Ag or where the cells undergo IgA CSR. It has been suggested that PerC B1 cells can undergo IgA CSR in MLN, but our results in CD40−/− mice largely fail to support this theory because post-switch circular DNA transcripts were hardly found (72, 74). Future studies using germfree and recently colonized mice will explore these possibilities.

Disclosures
The authors have no financial conflict of interest.

References


