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AID-Expressing Germinal Center B Cells Cluster Normally within Lymph Node Follicles in the Absence of FDC-M1+ CD35+ Follicular Dendritic Cells but Dissipate Prematurely

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Upon activation with T-dependent Ag, B cells enter germinal centers (GC) and upregulate activation-induced deaminase (AID). AID+ GC B cells then undergo class-switch recombination and somatic hypermutation. Follicular dendritic cells (FDC) are stromal cells that underpin GC and require constitutive signaling through the lymphotoxin (LT) β receptor to be maintained in a fully mature, differentiated state. Although it was shown that FDC can be dispensable for the generation of affinity-matured Ab, in the absence of FDC it is unclear where AID expression occurs. In a mouse model that lacks mature FDC, as well as other LT-sensitive cells, we show that clusters of AID+PNA+GL7+ Ag-specific GC B cells form within the B cell follicles of draining lymph nodes, suggesting that FDC are not strictly required for GC formation. However, later in the primary response, FDC-less GC dissipated prematurely, correlating with impaired affinity maturation. We examined whether GC dissipation was due to a lack of FDC or other LTβ receptor–dependent accessory cells and found that, in response to nonreplicating protein Ag, FDC proved to be more critical for long-term GC maintenance. Our study provides a spatial-temporal analysis of Ag-specific B cell activation and AID expression in the context of a peripheral lymph node that lacks FDC-M1+ CD35+ FDC and other LT-sensitive cell types, and reveals that FDC are not strictly required for the induction of AID within an organized GC-like environment. The Journal of Immunology, 2013, 191: 4521–4530.

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Abbreviations used in this article: AID, activation-induced deaminase; BM, bone marrow; CSR, class-switch recombination; DC, dendritic cell; FDC, follicular dendritic cell; FRC, fibroblastic reticular cell; GC, germinal center; iLN, inguinal lymph node; LN, lymph node; LT, lymphotoxin; LTβR, lymphotoxin receptor; NP, 4-hydroxy-3-nitrophénylacetil hapten conjugated to chicken gamma globulin; p.i., postimmunization; PNA, peanut agglutinin; R-PE, R-phycoerythrin; SCS M, subcapsular sinus macrophage; SHM, somatic hypermutation; WT, wild-type.

Although small soluble Ag can enter lymph node (LN) follicles through conduits (4), large Ag can be trapped and transported into follicles by subcapsular sinus macrophages (SCS M) in a complement receptor–dependent manner (5, 6). The nature of an Ag affects the kinetics and magnitude of the response that it elicits: smaller protein Ag accumulates poorly in follicles until the appearance of Ag-specific Ig enables the formation of larger immune complexes, whereas larger multimeric Ag, such as virus-like particles, can be bound early on by natural IgM, enabling them to form complement-containing immune complexes within follicles (7).

Once Ag is transported into the follicle, it is deposited on a type of nonhematopoietic stromal cell known as the follicular dendritic cell (FDC). FDC secrete chemokines and survival factors important for GC structure and function (8). The maintenance of FDC maturation status (phenotype and function) requires constitutive signaling through the lymphotoxin (LT) β receptor (LTβR); FDC are induced to mature in situ from a perivascular precursor through engagement of LTβR by membrane-bound LTαβ expressed on lymphocytes (9, 10). Within the GC, B cells express even higher levels of LTαβ, which is likely important for orchestrating a tight FDC network to support maturation of the Ab response (11, 12). Because LTβR signaling is required to maintain identifiable FDC within lymphoid tissues (13), mice rendered deficient in LTβR signaling by deletion of LTα, LTβ, or LTβR have been studied to query the role of FDC in the GC response. Notably, inhibition of LTβR signaling results in the impaired formation of splenic GC (14–16). Supporting the concept that FDC are required for GC structures, selective ablation of FDC using diphtheria toxin terminates the GC response in peripheral LN (17). However, GC formation in the mesenteric LN was shown to occur normally in LTβ−/− mice (18), and mice deficient in LTβR only exhibit a defect in affinity maturation at low doses of Ag (19). Variable dependency on FDC for a GC response may depend on whether Ag is particulate.
versus adjuvanted, as demonstrated in TNFR1−/− mice, which also lack FDC (20). Indeed, in the context of viral infection, although immunization of TNFR1−/− mice with viral protein results in a defective Ab response, immunization with live virus induces strong titers of neutralizing IgG (21). Thus, the mode of Ag delivery and the site of Ag encounter (spleen versus peripheral LN versus mucosal LN) are variables that likely affect the dependency on FDC for an effective humoral immune response.

Once formed, GC are the primary location of AID expression (3, 22). Although the mutagenic activity of AID is required for the generation of a high-affinity Ab response, this mutagenic activity carries the collateral risk of the potential generation of autoreactive B cells. For this reason, it would be expected that the expression of AID is restricted to B cells in microenvironments where they are regulated and subjected to tolerance checks. GC, which contain FDC and CD4+ T follicular helper cells, could be such an environment, explaining why AID activity is primarily observed in GC (3, 22). However, somatically mutated autoreactive B cells in MRL/lpr mice have been found outside of GC (23), and clusters of AID+ GC B cells in the absence of FDC in the periphery are observed in LT3−/− mice (20). The location and persistence of AID expression in the context of an FDC-less LN is not known. Understanding the minimal spatio-temporal requirements for AID expression has important clinical implications because inflamed nonlymphoid tissues can also support B cell–rich follicle-like structures (24).

We sought to initiate a robust, class-switched humoral response that would generate GC B cells whose AID expression and Ag specificity could be tracked by flow cytometry and immunofluorescent histology in FDC-less LN of LT3−/− chimeric mice. We observed clusters of AID+ GC B cells in the absence of FDC in the follicles of LN of LT3−/− chimeric mice. These AID+ GC B cells appeared with surprisingly normal kinetics, and their phenotype was very similar to AID+ GC B cells generated within FDC-sufficient GC. However, following the peak of the response, in the absence of LTβ and FDC, GC disappeared abruptly, and such abortive GC correlated with impaired affinity maturation. During this later phase of the GC, we used a combination of genetic and pharmacological approaches to ablate SCS Mφ and/or FDC and observed a weak correlation between the number of SCS Mφ and the number of GC B cells, whereas a significant decrease in the number of GC B cells late in the primary response occurred only when FDC were ablated. Our findings show that FDC-M1+ CD35+ FDC are not strictly required for the generation or clustering of AID+ GC B cells. In addition, our results suggest that FDC are required for the long-term maintenance of GC and the resulting production of high-affinity Ab.

Materials and Methods

Mice and immunizations

C57BL/6 mice were purchased from Charles River Laboratories. AID-GFP–transgenic mice were obtained from Rafael Casellas (National Institutes of Health, Bethesda, MD). LTβ−/− mice were purchased from B&K Universal and crossed with AID-GFP mice to produce LTβ−/− × AID-GFP mice. LTβ−/− mice with the CD45.1 congenic allele were obtained from Rodney Newberry (Washington University School of Medicine, St. Louis, MO). Bone marrow (BM) chimeras were generated as previously described (25). Chimeric mice were immunized s.c. in both hind flanks with 50 μl of a 1:1 PBS/CFA mixture (CFA from Difco Laboratories) containing 0.4 mg/ml (high-dose) or 0.035 mg/ml (low-dose) R-phycocerythrin (R-PE, Anaspec) or an irrelevant Ag (4-hydroxy-3-nitrophenylacetyl hapten conjugated to chicken gamma globulin [NP-CCG]; Biosearch Technologies). In some experiments, mice were treated by injecting 100 μg LTβR-Ig or control MOPC21 mAb (26) (Biogen-Idec) i.p. at days −2 and 5 postimmunization (p.i.). All mice (both chimeric and nonchimeric) were housed in specific pathogen–free conditions. All animal experiments were performed in accordance with end points and standards of animal care approved by the University of Toronto, Faculty of Medicine Animal Ethics Committee (Protocol # 20008480).

Cell isolation and flow cytometry

Inguinal LN (iLN) from immunized mice were ground between glass slides and suspended in HBSS with 1 mg/ml collagenase D and 60 μg/ml DNase I (Roche Diagnostics) for 30 min at 37˚C. Cell suspensions were then filtered through a 70-μm strainer and resuspended in PBS. Cells were labeled with Live/Dead Aqua (Life Technologies) prior to staining or with 7-aminoactinomycin D (BD), and dead cells were gated out. Prior to staining, cells were blocked with mouse serum and 2.4G2 Ab (Fc block). Cells were stained with Abs against B220 (eFluor 450; mAb RA3-6B2), CD1d (PE; mAb B3B4), CD11c (allophycocyanin; mAb N418), F4/80 (biotin; mAb BM8) (all from eBioscience); with Abs against CD16/32 (FITC; mAb MOMA-1) (GenTex); or with Abs against IgM (PerCP-Cy5.5; mAb R5-60.2); CD21 (FITC; mAb 7G6), GL7 (Alexa Fluor 647), and Fas (PECy7; mAb Jo2) (all from BD). Biotin-conjugated Abs were stained with streptavidin–allophycocyanin or streptavidin–allophycocyanin–eFluor 780 (eBioscience). Ag-specific B cells were labeled with R-PE. Fluorescent-labeled cells were analyzed on a BD FACSCanto II flow cytometer.

Histology

iLN from immunized mice were processed for histology, as previously described (12). Tissue sections were blocked with mouse serum prior to staining. Serial sections were stained with allophycocyanin-conjugated Abs against B220 (mAb RA3-6B2; eBioscience), FDC-M1, CD35 (mAb SC12), or CD138 (mAb 281-2) (BD) or biotin-conjugated peanut agglutinin (PNA, Vector Labs). Serial sections were also stained with anti-IgD (mAb 11-26; eBioscience) or anti-fibronectin (Sigma). Biotin-conjugated Abs were then stained with streptavidin–allophycocyanin (eBioscience). Unlabeled rat anti-mouse ER-TR7 (BMA Biomedicals) was labeled with anti-rat–FITC (Southern Biotech). Ag-specific Ig was detected by applying R-PE directly to the tissue (Anaspec). The AID-GFP reporter transgene was too dim for direct histological visualization and so was amplified by staining with chicken anti-GFP polyclonal Ab (Aves Labs), followed by anti-chicken–Alexa Fluor 488 (Life Technologies). Stained sections were mounted with Dako Fluorescent Mounting Medium and visualized on a Leica DMRA2 microscope at room temperature. Photographs were taken with 10× and 20× objectives using a QImaging Retiga EXi camera with an EXPO X-Cite 120 lamp using Openlab software.

ELISA

Blood was collected from immunized mice and centrifuged to isolate sera. To measure Ag-specific Ab, wells in NUNC MaxiSorp plates (Thermo Scientific) were coated with 10 μg/ml R-PE (Anaspec) overnight at 4˚C and then blocked with 2% BSA/PBS for 1 h at 37˚C. To measure relative Ab titters, R-PE–coated wells were incubated with a dilution series of serum for 2 h at 37˚C and then probed with biotinylated Abs against IgG (polyclonal Ab Poly4053) or IgM (mAb RMM-1) (BioLegend). Wells were then incubated with streptavidin-HRP enzyme, developed with liquid tetramethylbenzidine substrate (BioShop), stopped with 1 M H2SO4, and read at 450 nm. Relative titters were calculated as the serum dilution at 1 OD relative to a serum control. To measure relative Ag affinity, we used a protocol adapted from the literature (27). Briefly, R-PE–coated wells were incubated with a quantity of serum, such that each well for each sample contained the same amount of anti-R-PE Ab (as calculated by relative titer ELISA). Wells were then incubated for 15 min with 0.4 M ammonium thiocyanate in 0.1 M phosphate buffer at pH 7.15. Wells were then probed and developed as described above. The resulting dilution curve was fitted with a third-order polynomial regression line. The affinity index of each sample was calculated from the regression line as the molar concentration of ammonium thiocyanate required to decrease 50% of bound anti-R-PE serum Ab.

Measuring SHM in LN GC B cells

AID-GFP (wild-type [WT]) and AID-GFP × LTβ−/− BM chimeras were immunized with a high dose of R-PE, as described above, and LN were collected at day 9 p.i. LN B cells from three mice of each genotype were pooled together for sorting. B220+ AID-GFP–R-PE+ GL7+ GC B cells were isolated at FACS using a BD FACSAria flow cytometer. Sorted cells were suspended in TRIZol (Life Technologies), and the JH2–JH4 region of
extracted DNA was amplified, as previously described (28), with the following modifications: PCR products were amplified by Q5 High-Fidelity DNA polymerase (New England BioLabs) at 68°C annealing temperature. Amplified DNA was gel purified with QIAquick gel extraction kit (QIAGEN) and cloned into Zero Blunt TOPO plasmid vector (Life Technologies) before transformation into DH5α bacteria. Plasmid DNA was prepared using an E-Z 96 FastFilter plasmid kit (Omega Bio-Tek) and sequenced by an AB13730XL automatic sequencer (Macrogen).

Results

Phenotypically normal GC B cells are observed in the absence of LTβ

In this study, we developed a system for examining the humoral immune response within LN using tools that allow us to track Ag-specific B cells that express AID. Specifically, we immunized mice with the 240-kDa fluorochrome R-PE in CFA to follow an Ag-specific response within the endogenous polyclonal repertoire because B cells activated by R-PE in vivo can later be stained with R-PE ex vivo (29, 30). For detecting AID expression in Ag-specific B cells, we used mice harboring a transgene with an AID-GFP fusion protein under the control of AID cis-elements. The AID-GFP reporter induces fluorescence in AID-expressing GC B cells, which is subsequently quenched upon downregulation of AID (3). LTβ−/− mice lack peripheral LN. Therefore, we crossed LTβ−/− mice with AID-GFP reporter mice to track AID expression in the absence of LTβ and used these mice (as well as WT AID-GFP control mice) as the source of donor BM to transfer into C57BL/6 hosts. This allowed us to induce GC within iLN in the presence or absence of LTβ, creating AID-GFP (WT) BM chimeras and AID-GFP × LTβ−/− BM chimeras (Supplemental Fig. 1A).

We confirmed the ablation of LtβR-induced LTβR signaling in AID-GFP × LTβ−/− BM chimeras based on the lower cellularity of the peripheral LN (Supplemental Fig. 1B), consistent with a role for LTβR signaling in regulating the maturation status of high endothelial venules (31). AID-GFP × LTβ−/− BM chimeras also had severely reduced populations of splenic marginal zone B cells, consistent with observations that the marginal zone of the spleen collapses in the absence of LTβR signaling (Supplemental Fig. 1C) (32).

We next tested the GC response to R-PE in the draining iLN of AID-GFP (WT) and AID-GFP × LTβ−/− BM chimeras. Validating the specificity of R-PE staining, we observed that mice immunized with an irrelevant Ag (NP-CGG) generated AID-GFP+ B cells that are not stained by R-PE, and B cells from mice immunized with R-PE do not fluoresce in the PE channel when R-PE is not added to the flow cytometry panel (Fig. 1A). We then immunized AID-GFP (WT) and AID-GFP × LTβ−/− BM chimeras s.c. with R-PE in each hind flank to generate humoral responses in both iLN. At day 7 of the response, we observed a population of AID-GFP×R-PE− B cells in AID-GFP (WT) and AID-GFP × LTβ−/− BM chimeras by flow cytometry (Fig. 1B). Surprisingly, the percentage of AID-GFP×R-PE− B cells in the iLN of AID-GFP × LTβ−/− BM chimeras was equal to or greater than in the iLN of AID-GFP (WT) chimeric mice (Fig. 1C). We analyzed the expression of other GC B cell markers on AID-GFP×R-PE− B cells from both types of BM chimeric mice and found that they displayed the same levels of Fas, GL7, and AID-GFP (Fig. 1D). A population of AID−R-PElow cells was also observed (Fig. 1B). It is unclear whether these are B cells with low affinity for R-PE or represent cells staining for FcR-bound anti-R-PE Ab; nevertheless, the frequency and number of these cells were similar for both groups (data not shown). Taken together, these observations indicate that significant numbers of phenotypically normal GC B cells are produced in the draining iLN in the absence of LTβ.

![FIGURE 1. Flow cytometry of AID-GFP×R-PE− GC B cells. (A) AID-GFP (WT) BM chimeric mice were immunized s.c. with R-PE/CFA or NP-CGG/CFA (left to right), and B cells (B220+) from the draining iLN were analyzed by flow cytometry on day 7 p.i. Data are mean ± SEM. (B) Representative graphs of the levels of Fas, GL7, and AID-GFP on B220+ AID-GFP×R-PE− GC B cells from AID-GFP (WT) and AID-GFP × LTβ−/− BM chimeras on day 7 p.i. Data are mean ± SEM. (C) The percentage of B220+AID-GFP×R-PE− GC B cells from seven AID-GFP (WT) and seven AID-GFP × LTβ−/− BM chimeras on day 7 p.i. Representative data from three independent experiments are shown.](http://www.jimmunol.org/)

AID-GFP+ GC B cell clusters form in the absence of LTβ and FDC

Given that AID-GFP+ GC phenotype B cells are readily detected in the draining iLN of R-PE–immunized AID-GFP × LTβ−/− BM chimeric mice, we sought to determine the location of AID expression by immunofluorescence microscopy. At day 7 p.i., serial sections of iLN from AID-GFP (WT) BM chimeric mice were generated, and clusters of AID-GFP+ B cells were observed within B cell follicles (Fig. 2A). These clusters of AID-GFP+ cells stained with PNA, indicating that they contained activated GC B cells (Fig. 2A). These GC were populated with FDC-M1+ CD35+ FDC (Fig. 2A). Staining serial sections with R-PE revealed R-PE–specific Ab secreted by CD138+ plasma cells in clusters adjacent to GC and in the medullary cords (Fig. 2A). Surprisingly, iLN from AID-GFP × LTβ−/− BM chimeras also contained clusters of AID-GFP×PNA+ B cells within B cell follicles (Fig. 2B), as well as R-PE×CD138+ cells in clusters adjacent to GC and in the medullary cords (Fig. 2B). However, as expected, these clusters did not contain any FDC, as verified by the absence of FDC-M1+ and CD35+ staining (Fig. 2B, Supplemental Fig. 2A, 2B).
Interestingly, although FDC networks were disrupted in LTβ^{−/−}\textsuperscript{BM} chimeras, ERTR7/fibronectin conduits produced by fibroblastic reticular cells (FRC) (4, 33) remained intact and indeed infiltrated FDC-less GC (Fig. 3).

**FIGURE 2.** In situ examination of the anti–R-PE response in iLN. Representative serial cross-sections of iLN GC are shown. All serial sections were stained with anti-GFP (green) and with R-PE (red), which stains secreted and membrane-bound anti–R-PE Ig. Serial sections were then stained with one of five allophycocyanin-conjugated Abs (B220, PNA, FDC-M1, CD35, or CD138). (A) Representative cross-section of an iLN from an AID-GFP (WT) BM chimera on day 7 p.i. with R-PE/CFA. The large center panel is stained for B220 (blue) and depicts the entire LN as a composite of images taken at original magnification \(\times 100\). Yellow boxes denote areas displayed to either side at original magnification \(\times 200\). The upper yellow box denotes a GC that is shown in serial sections at original magnification \(\times 200\) stained for each PNA, FDC-M1, CD35, and CD138 (all depicted here as blue color). The lower yellow box denotes the LN medullary cords and is stained for CD138 (blue) in a serial section. (B) Representative cross-section of an iLN from an AID-GFP \(\times\) LTβ^{−/−} BM chimera on day 7 p.i. with R-PE/CFA. Serial stains and magnifications are the same as in (A). The left yellow box highlights a GC, and the right yellow box highlights the medullary cords. Scale bars, 50 \(\mu\)m for images at original magnification \(\times 200\), 100 \(\mu\)m for images at original magnification \(\times 100\). The images are derived from an experiment in which three AID-GFP (WT) and five AID-GFP \(\times\) LTβ^{−/−} BM chimeric mice were examined and are representative of three separate experiments.
Taken together, these observations show that clusters of AID⁺ PNA⁺ GC B cells form in the iLN of AID-GFP /LTb₂/₂ BM chimeras in the absence of FDC. Because these B cells form clusters situated in the follicle and because they express numerous markers of the GC B cell phenotype (AID, GL7, Fas, PNA), we elected to call these clusters GC, despite the lack of an FDC-M1⁺ CD35⁺ FDC network.

LTb is required for the maintenance, but not the initiation, of GC

We next investigated the kinetics of the GC response in FDC-less BM chimeras to examine whether the absence of LTb could result in any kinetic or qualitative differences. We immunized AID-GFP (WT) and AID-GFP × LTb⁻/⁻ BM chimeric mice as before and quantified AID-GFP⁺R-PE⁺ B cells over time. GC B cells were not present in iLN at day 5 p.i., but they appeared at day 6 (Fig. 4A). The initial response in AID-GFP × LTb⁻/⁻ BM chimeras appeared delayed; there were slightly fewer AID-GFP⁺R-PE⁺ B cells in AID-GFP × LTb⁻/⁻ BM chimeras at day 6; however, that number had caught up to or exceeded the number found in AID-GFP (WT) BM chimeras by day 7 (Fig. 4A). GC B cells were still present by day 12 in AID-GFP (WT) BM chimeras (Fig. 4B). In contrast, the response in AID-GFP × LTb⁻/⁻ BM chimeras had vanished by day 12 (Fig. 4B). Similar results were observed using immunofluorescence microscopy (Fig. 5).

Because FDC are thought to trap Ag through complement and FcRs for protracted presentation to Ag-specific B cells (8), we considered that high doses of Ag may circumvent the requirement for FDC-mediated Ag capture. To test this, we repeated the kinetic analysis by immunizing with a low dose of Ag (1.75 mg R-PE/injection, as opposed to 20 μg for the high dose). The low-dose immunization resulted in much weaker GC responses that were sustained until day 12 in AID-GFP (WT) BM chimeras, whereas they had completely dissipated by day 12 in the AID-GFP × LTb⁻/⁻ BM chimeric mice (Fig. 4C, 4D). Therefore, LTb is dispensable for the initiation of Ag-specific GC B cell expansion in the draining iLN but is critical for postpeak maintenance of GC B cell numbers both at low and high doses of Ag.
Each time point by 3–12 individual mice. Data are mean ± SEM.

To determine whether the impairment in affinity maturation was due to reduced SHM, we isolated B220+AID-GFP+R-PE+GL7+ GC B cells from the iLN of AID-GFP (WT) and AID-GFP × LTβ−/− BM chimeras to systematically evaluate the contribution of each cell type to GC maintenance.

Using this approach, we immunized LTβ−/− and control WT (CD45.1) BM chimeras with R-PE as before and treated them with LTβR-Ig or control mAb. iLN from control-treated LTβR−/− BM chimeras had significantly fewer SCS MΦ than did WT BM chimeras (Fig. 7A) but still contained mature FDC (Supplemental Fig. 3). On average, 97% of SCS MΦ were CD45.1+ in LTβR−/− BM chimeras, indicating that the vast majority of SCS MΦ were donor derived (data not shown). Consistent with previous reports (6), treatment with LTβR-Ig resulted in a near complete loss of iLN SCS MΦ (Fig. 7A), as well as ablation of FDC in both LTβR−/− and WT BM chimeras. In LTβR−/− BM chimeras treated with control Ab, the number of GC B cells at day 12 p.i. was slightly lower than in WT BM chimeras, although this reduction was not statistically significant (Fig. 7B). A significant decrease in the number of GC B cells at day 12 p.i. was observed only when LTβR−/− BM chimeras were treated with LTβR-Ig (Fig. 7B), and we only observed a weak correlation between the number of SCS MΦ and the number of GC B cells in the iLN at day 12 p.i. (Fig. 7C). Together, these observations suggest that SCS MΦ play a partial role in the prolonged maintenance of GC in response to protein Ag, but that FDC, or the cooperation between FDC and SCS MΦ, are required for GC to persist beyond the peak of the primary response.

**Discussion**

Studies examining the spleens of LT-deficient mice and non-human primates treated with LT pathway inhibitors have consistently

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**FIGURE 4.** Kinetics of the R-PE–specific B cell response. Kinetics of appearance of B220+AID-GFP+R-PE+ GC B cells in the iLN of AID-GFP (WT) and AID-GFP × LTβ−/− BM chimeras after s.c. immunization with a high dose (20 µg) (A, B) or a low dose (1.75 µg) (C, D) of R-PE in CFA, measured by flow cytometry. Each type of BM chimera is represented at each time point by 3–12 individual mice. Data are mean ± SEM. Representative flow cytometry of B220+AID-GFP+R-PE+ GC B cells on day 12 p.i. with a high dose (B) or a low dose (D) of R-PE in CFA. Data from five (A) and four (C) independent experiments are shown. *p < 0.05, **p < 0.01, ***p < 0.001, Student unpaired t test.

**LTβ is required for optimal affinity maturation**

Given that GC B cells were not sustained in the draining iLN of AID-GFP × LTβ−/− BM chimeras following immunization, we assessed the impact of GC collapse on the affinity of the anti-R-PE humoral response after high-dose immunization. Using ELISA, we found that the production of serum anti-R-PE IgM and IgG was roughly equal in AID-GFP (WT) and AID-GFP × LTβ−/− BM chimeras (Fig. 6A, 6B). However, AID-GFP × LTβ−/− BM chimeras produced serum IgG with significantly lower affinity for R-PE, indicating a defect in affinity maturation (Fig. 6C). To determine whether the impairment in affinity maturation was due to reduced SHM, we isolated B220+AID-GFP+R-PE+GL7+ GC B cells from the iLN of AID-GFP (WT) and AID-GFP × LTβ−/− BM chimeras by FACS and sequenced their JH2–JH4 regions to look for AID-induced mutations. Because Ag-specific AID+ GC B cells are not detectable at day 12 p.i., we elected to look for evidence of SHM at day 9 p.i. At this time point we found that the mutation frequency was ∼30% lower among GC B cells in LTβ−/− versus WT chimeric mice, although the difference was not statistically significant (Table I, Supplemental Fig. 3). Therefore, we conclude that, during the first 9 d of the immune response, SHM occurs in AID-GFP × LTβ−/− BM chimeras. Together, these results indicate that the inability to sustain the GC response in AID-GFP × LTβ−/− BM chimeric mice corresponds with poor affinity maturation.

**Premature GC dissipation is primarily due to the absence of FDC**

Although the defects of early GC dissipation and poor affinity maturation in AID-GFP × LTβ−/− BM chimeras correlate with the absence of FDC, we could not conclude from those experiments that these defects were caused by the absence of FDC. This is because abrogation of LTβR signaling by removing LTβR has effects other than ablating FDC. Most relevant to the LN GC response is that the abrogation of LTβR signaling results in a significant reduction in the number of SCS MΦ (6). SCS MΦ trap immune complexes in the SCS and then pass them on to non-cognate B cells, which, in turn, deliver the immune complexes to FDC for long-term retention (5, 6). Thus, interactions between FDC and SCS MΦ may be important for the generation of Ag deposits within GC that are required to sustain a prolonged GC response.

To determine whether the absence of FDC, SCS MΦ, or both was responsible for the collapse of GC that we observe at day 12 p.i., we generated BM chimeras in which C57BL/6 mice were reconstituted with BM from LTβ−/− congenic allele. In these LTβ−/− BM chimeras, the lack of LTβR on hematopoietic cells results in a significant deficiency in SCS MΦ (6), whereas FDC, which are radioresistant, are unaffected. Treatment of LTβR−/− BM chimeras with LTβR-Ig, a soluble decoy receptor that effectively blocks LTβR interactions in vivo (26, 34), allowed us to ablate FDC and/or SCS MΦ to systematically evaluate the contribution of each cell type to GC maintenance.

Using this approach, we immunized LTβR−/− and control WT (CD45.1) BM chimeras with R-PE as before and treated them with LTβR-Ig or control mAb. iLN from control-treated LTβR−/− BM chimeras had significantly fewer SCS MΦ than did WT BM chimeras (Fig. 7A) but still contained mature FDC (Supplemental Fig. 2C, 2D). On average, 97% of SCS MΦ were CD45.1+ in LTβR−/− and WT BM chimeras, indicating that the vast majority of SCS MΦ were donor derived (data not shown). Consistent with previous reports (6), treatment with LTβR-Ig resulted in a near complete loss of iLN SCS MΦ (Fig. 7A), as well as ablation of FDC in both LTβR−/− and WT BM chimeras. In LTβR−/− BM chimeras treated with control Ab, the number of GC B cells at day 12 p.i. was slightly lower than in WT BM chimeras, although this reduction was not statistically significant (Fig. 7B). A significant decrease in the number of GC B cells at day 12 p.i. was observed only when LTβR−/− BM chimeras were treated with LTβR-Ig (Fig. 7B), and we only observed a weak correlation between the number of SCS MΦ and the number of GC B cells in the iLN at day 12 p.i. (Fig. 7C). Together, these observations suggest that SCS MΦ play a partial role in the prolonged maintenance of GC in response to protein Ag, but that FDC, or the cooperation between FDC and SCS MΦ, are required for GC to persist beyond the peak of the primary response.
demonstrated poor or absent GC formation correlating with a lack of FDC (14–16, 35). Furthermore, FDC within Peyer’s patches play an important role in regulating GC responses to gut-derived Ag (36), and FDC networks are strongly associated with AID expression in ectopic lymphoid structures in the salivary glands of patients with Sjögren’s syndrome (24) and in the synovium of patients with rheumatoid arthritis (37). These previous studies suggest that FDC are critical for the formation of GC and for the concomitant expression of AID by GC B cells. However, the AID-dependent processes of SHM and CSR still occur in immunized LTα−/− mice, which lack FDC and display absent or abortive splenic GC (38). Therefore, we sought to determine where AID expression can occur if not in canonical GC.

Although we initially expected to observe abnormal locations for AID-expressing B cells, we were surprised to see clusters of AID+ GC B cells in the follicles of iLN that resembled GC, despite their lack of FDC. This suggests that FDC-M1+CD35+ FDC are not required to initiate GC or to foster AID expression in Ag-specific B cells within iLN in response to protein Ag. Our results suggest that peripheral LN may have different types of stromal cells that could substitute for some of the functions attributed to mature FDC. Indeed, we observed the presence of an ERTR7+Fibronectin+ network within FDC-less GC. Such matrix elements are produced by FRC and were shown to facilitate the movement of follicular chemokines, such as CXCL13, which could conceivably draw Ag-specific B cells together into a GC niche (4). Both FRC and marginal reticular cells can produce CXCL13 and are a putative source of this chemokine in the absence of FDC (39, 40). Indeed, it was reported that ablation of FDC does not result in a significant decrease in CXCL13 transcripts in peripheral LN (17), suggesting that FDC are not an essential source of CXCL13 in this location and that FRC or marginal reticular cells could potentially organize B cell follicles and GC. Therefore, our findings suggest a role for other stromal cell types in the formation of GC in peripheral LN. Future studies that perform proteomic analysis of putative compensatory non-FDC stromal cells in the LTβ-deficient environment potentially would provide a more complete understanding of LN stroma. Alternatively, noncognate B cells, which also can capture and transport Ag in the follicle, could potentially display Ag to GC B cells in the absence of FDC.

**FIGURE 5.** Kinetics of GC appearance in the LN of immunized BM chimeric mice. All tissues were stained with anti-GFP (green), R-PE (red), which stains secreted and membrane-bound anti–R-PE Ig, and anti-B220 (blue). Representative cross-sections of iLN from AID-GFP (WT) mice (A) and AID-GFP x LTβ−/− BM chimeric mice (B) at days 5, 7, and 12 p.i. with R-PE/CFA. Images are composites of pictures taken at original magnification ×100. Scale bars, 100 μm. Three experiments were performed with similar results on three to six mice/group/experiment.
providing a partial redundancy for the Ag-trapping role of FDC. The collapse of the GC in FDC-less mice at day 12 may reflect turnover of these particular B cells (5, 41, 42). Previous studies demonstrating the correlation between a lack of FDC and a lack of GC focused on the splenic lymphoid environment. In this study, we investigated peripheral LN and observed GC forming in the absence of FDC. Our findings highlight the fact that the lymphoid environments in the spleen and in LN are likely structured differently. A previous study reported GC without FDC in mesenteric LN (18). However, mesenteric LN are unique in a few respects. Although peripheral LN in SPF mice experience periods of quiescence during which the follicles are devoid of GC, mesenteric LN are sites of constitutive GC activity as the result of stimulation by gut Ag (43). As well, the ontogeny of mesenteric LN differs from other LN; although peripheral LN require signaling through LTβR for their development, mesenteric LN are able to form in the absence of LTβ (44). For these reasons, mesenteric LN were perhaps thought of as a special case, and the prevailing view that FDC were crucial to GC formation remained. Our findings support these previous observations in the GALT and extend them to peripheral LN where we show that organized GC-like clusters of

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** ELISA measurements of R-PE–specific Ab. Kinetics of appearance of serum anti-R-PE IgM (A) and IgG (B), measured by ELISA. Each type of BM chimera is represented at each time point by four to nine individual mice. (C) Measurement of the affinity of serum IgG for R-PE by denaturing ELISA. Higher values for affinity index denote higher affinity for Ag. Each type of BM chimera is represented at each time point by six to nine individual mice. All data are mean ± SEM. Data from five (A, B) and two (C) independent experiments are shown. *p < 0.05, ***p < 0.001, Student unpaired t test. ns, Not significant.

### Table I. SHM in GC B cells from WT and LTβ<sup>−/−</sup> chimeras

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>LTβ&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>No. sequences analyzed</td>
<td>47</td>
<td>39</td>
</tr>
<tr>
<td>No. mutations</td>
<td>19</td>
<td>11</td>
</tr>
<tr>
<td>Mutation frequency&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.68 x 10&lt;sup&gt;−4&lt;/sup&gt;</td>
<td>1.87 x 10&lt;sup&gt;−4&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mutations at WRC (%)&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>18.2</td>
</tr>
<tr>
<td>Mutations at WA (%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>47.4</td>
<td>81.8</td>
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<sup>a</sup>Frequency is defined as mutations/bp sequenced. WT: 19/70,782; LTβ<sup>−/−</sup>: 11/58,734.

<sup>b</sup>Percentage of mutations calculated over total number of mutations. W, A/T nucleotides; R, A/G nucleotides.

![Figure 7](http://www.jimmunol.org/)

**FIGURE 7.** iLN GC response in LTβR<sup>−/−</sup> BM chimeras. WT→WT and LTβR<sup>−/−</sup>→WT BM chimeras immunized with R-PE/CFA and treated at days −2 and 5 p.i. with LTβR-Ig or MOPC21 control mAb. Cells from iLN were analyzed by flow cytometry at day 12 p.i. (A) Number of CD11b<sup>−</sup>CD11c<sup>−</sup>CD169<sup>+</sup>F4/80<sup>+</sup> SCS MΦ in the draining iLN. (B) Number of B220<sup>+</sup>GL7<sup>+</sup>Fas<sup>+</sup>R-PE<sup>+</sup> GC B cells in the draining iLN. All data are mean ± SEM. (C) Number of SCS MΦ versus the number of GC B cells in the draining iLN at day 12 p.i. Each symbol represents one iLN from one BM chimera. Plot includes LTβR<sup>−/−</sup> and WT BM chimeras from both LTβR-Ig– and MOPC21–treated groups. Plot is fitted with a second-order polynomial regression line. Each chimeric/treatment group is represented by 8–13 individual mice. Data from two independent experiments are shown. *p < 0.05, **p < 0.01, ***p < 0.001, Student unpaired t test. ns, Not significant.
AID-expressing B cells are effectively induced within the LN B cell follicle at the peak of the response to protein Ag, despite a lack of FDC-M1\(^+\) CD35\(^+\) FDC.

A more recent study by Wang et al. (17) used a different experimental approach to investigate the role of FDC in supporting GC by eliminating FDC with diphtheria toxin. They found that their model of FDC ablation resulted in the abrupt disappearance of LN GC B cells, demonstrating that FDC are required to maintain normal GC at the peak of the immune response. We found that FDC were dispensable for the formation of GC that support peak numbers of AID\(^+\)Fas\(^+\)PNA\(^+\)GL7\(^+\)Ag-specific B cells and that it was not until late in the response that GC were negatively affected by a lack of FDC. One difference between these two studies is that, in our study, FDC-M1\(^+\) CD35\(^+\) FDC were absent for the entire duration of the primary response, whereas in the study by Wang et al. FDC were ablated only after GC had already formed. Nevertheless, both studies demonstrate that an absence of FDC results in the disappearance of GC later in the primary response.

Although we found that FDC-M1\(^+\) CD35\(^+\) FDC are dispensable for the initial formation of GC in LN, we determined that the LT pathway is crucial for the long-term maintenance of GC. By using a combination of LTBR\(^--\)/BM chimeras and treatment with LTBR–Ig, our study was able to determine the specific contribution of FDC to the formation of GC and to contrast this with the role of other LTBR-dependent cells, such as SCS MΦ. Other effects of abrogating LTBR signaling include loss of a mature high endothelial venule phenotype (31), impaired dendritic cell (DC) function and/or homeostasis (45–47), and loss of SCS MΦ (6). SCS MΦ are important for the optimal transport of Ag into the follicle (5, 6). We observed a weak correlation between the number of SCS MΦ and the number of GC B cells late in the primary response. However, significantly reducing the number of SCS MΦ did not result in a significant decrease in GC B cells at day 12 p.i. Therefore, we concluded that SCS MΦ play a secondary role (or cooperate with FDC) in sustaining GC that form in response to nonreplicating protein Ag, although in the context of a viral infection it is likely that SCS MΦ are more critical (48, 49).

With respect to DC function, our BM chimera experiments also suggest that DC-intrinsic LTBR signaling does not play a significant role in the maintenance of the GC microenvironment. Indeed, we showed that the role of LTBR signaling in DC is more important in the context of cross-presentation of protein Ag to CD8\(^+\) T cells (45). In contrast, only the treatment of LTBR\(^--\)/BM chimeras with soluble LTBR–Ig resulted in a significant decrease in GC B cells late in the response. This evidence suggests that FDC play a primary role in sustaining GC.

Given that we observed a reduction in affinity maturation at days 20 and 48 p.i. in the AID-GFP \(\times\) LTBR\(^--\) BM chimeras, it is possible that this could be due to impaired survival of Ag-specific GC B cells; indeed, the number of Ag-specific GC B cells rapidly dwindles at day 12 (Fig. 4), concomitant with the collapse of GC structures at this time point (Fig. 5). However, if this were the case, the fittest GC B cells would need to be selectively lost in the later stages of the GC response to account for the reduced Ab affinity observed at days 20 and 48. Moreover, if GC B cell death were to account for the reduction in Ab affinity, we would note an overall reduction in anti-P E iterates (irrespective of affinity), which is something that, for the most part, we do not observe (Fig. 6A, 6B). We favor the alternative interpretation that, in the presence of FDC, GC are sustained for a longer period of time. This extra time would allow for additional cycles of maturation and selection that are conducive to the emergence of higher-affinity B cell clones.

In summary, we conclude that FDC-M1\(^+\) CD35\(^+\) FDC are not required for the formation of GC (i.e., clusters of Ag-specific PNA\(^+\)GL7\(^+\)Fas\(^+\)AID\(^+\) B cells) in LNs. This finding highlights differences in the structure and function of follicular stroma in the spleen versus peripheral LN. However, we suggest that FDC are required for the prolonged maintenance of GC, allowing for robust affinity maturation and the production of high-affinity Ab.

**Note added in proof.** A recent study using Cc19-cre conditional ablation of LTBR found that ERTR7\(^+\) networks are still formed in LNs in the absence of LTBR on FRC (50).

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**Disclosures** The authors have no financial conflicts of interest.

**References**


Figure S1. Confirmation of ablation of LTβR signaling in BM chimeric mice. (A) A diagram illustrating how we generated AID-GFP (WT) and AID-GFP x LTβ<sup>−/−</sup>BM chimeric mice. (B) The number of total LN cells and B cells in the iLNs of BM chimeras day 5 p.i. with R-PE/CFA, prior to the appearance of GC. AID-GFP→WT n=8; AID-GFP x LTβ<sup>−/−</sup>→WT n=7. Student’s unpaired t-test; *, P<0.05. Columns depict mean ± SEM. Data from two independent experiments is shown. (C) Flow cytometry of splenocytes from BM chimeras. The left column depicts total B220<sup>+</sup> cells. The right column depicts cells from the IgM<sup>+</sup>CD23<sup>+</sup> gate. In the right column a gate is drawn around CD1d<sup>hi</sup>CD21<sup>+</sup> marginal zone (MZ) B cells. Note the reduction in MZ B cells in AID-GFP x LTβ<sup>−/−</sup>→WT mice.

Figure S2. AID-GFP<sup>+</sup> GC B cell clusters in the absence of FDC in AID-GFP x LTβ<sup>−/−</sup>BM chimeras. (A,B) Representative serial cross sections of two GC from two AID-GFP x LTβ<sup>−/−</sup>→WT mice at day 7 p.i. stained with anti-GFP (green), R-PE (red), which stains secreted and membrane-bound anti-R-PE Ig; and with PNA, FDC-M1, or CD35 (blue). Representative serial cross sections of GC from a (C) WT→WT BM chimera and a (D) LTβR<sup>−/−</sup>→WT BM chimera treated with MOPC21 control mAb and stained with anti-B220 (Green) and PNA, anti-CD35, or FDC-M1 (blue). Columns depict serial sections of iLN GC. Pictures are taken at 20x objective magnification. Scale bars represent 50µm.

Figure S3. Mutation frequency in JH sequences from WT and LTβ<sup>−/−</sup> GC B cells.
B220<sup>+</sup>AID-GFP<sup>-</sup>R-PE<sup>-</sup>GL7<sup>-</sup> GC B cells from AID-GFP (WT) and AID-GFP x LTβ<sup>-/-</sup>BM chimeric mice were isolated by FACS sorting at day 9 p.i. Pie charts depict the number of mutations found per sequence. The total number of sequences analyzed is indicated in the center. Pie chart segments depict the proportion of these segments that carry 0, 1, 2, 3, or 4 mutations.