AID and Caspase 8 Shape the Germinal Center Response through Apoptosis

Bryant Boulianne, Olga L. Rojas, Dania Haddad, Ahmad Zaheen, Anat Kapelnikov, Thanh Nguyen, Conglei Li, Razq Hakem, Jennifer L. Gommerman and Alberto Martin

*J Immunol* published online 15 November 2013
http://www.jimmunol.org/content/early/2013/11/15/jimmunol.1301776
AID and Caspase 8 Shape the Germinal Center Response through Apoptosis

Bryant Boulianne,1 Olga L. Rojas,1 Dania Haddad,1 Ahmad Zaheen, Anat Kapelnikov, Thanh Nguyen, Conglei Li, Razq Hakem, Jennifer L. Gommerman,2 and Alberto Martin2

Germinal centers (GCs) are clusters of activated B cells that form in secondary lymphoid organs during a T-dependent immune response. B cells enter GCs and become rapidly proliferating centroblasts that express the enzyme activation-induced deaminase (AID) to undergo somatic hypermutation and class-switch recombination. Centroblasts then mature into centrocytes to undergo clonal selection. Within the GC, the highest affinity B cell clones are selected to mature into memory or plasma cells while lower affinity clones undergo apoptosis. We reported previously that murine Aicda+/− GC B cells have enhanced viability and accumulate in GCs. We now show that murine Aicda+/− GC B cells accumulate as centrocytes and inefficiently generate plasma cells. The reduced rate of plasma cell formation was not due to an absence of AID-induced DNA lesions. In addition, we show that the deletion of caspase 8 specifically in murine GC-B cells results in larger GCs and a delay in affinity maturation, demonstrating the importance of apoptosis in GC homeostasis and clonal selection.  The Journal of Immunology, 2013, 191: 000–000.

During a T-dependent immune response, germinal centers (GCs) containing a dark zone and a light zone form in secondary lymphoid organs (1, 2). B cells first enter the dark zone as proliferating, centroblasts, undergo class-switch recombination (CSR) and somatic hypermutation (SHM) through the action of activation-induced deaminase (AID) (3, 4), and then migrate to the light zone to become centrocytes. Centrocytes with the highest affinity for Ag are selected to recycle to the centroblast stage to expand a successful clone or to exit the GC and mature into memory or plasma cells (5). GC B cells, which are out-competed during clonal selection, die by apoptosis.

Apoptosis in GC B cells can be triggered by the extrinsic pathway through Fas receptor ligation and subsequent downstream caspase 8 activation. GC B cells express elevated levels of Fas, and cultured GC B cells are highly susceptible to Fas-mediated apoptosis, which can be blocked by inhibiting caspase 8 (6–8). Fas deficiency results in impaired selection of high-affinity clones (9) and the emergence of self-reactive GC B cell clones (10). Alternatively, the intrinsic apoptotic pathway can be triggered by DNA damage that leads to the activation of caspase 9 (11, 12).

During the affinity maturation process, there is a risk that GC B cells can become autoreactive or cancerous. Culling low-affinity and potentially harmful B cell clones is therefore crucial to the health of the organism, and apoptosis is central to this process (9, 13, 14). We previously investigated Aicda+/− GC B cells, which do not express AID and do not undergo SHM or CSR, and we found that they overpopulate GCs, have lower rates of apoptosis, and have higher rates of proliferation (15); however, the underlying reason for this unconstrained GC response was not determined.

In this study, we evaluated the stage of GC development at which AID-deficient B cells were accumulating. We show that Aicda+/− B cells accumulate as CXCR4low centrocytes and fail to mature efficiently into plasma cells. The reduced rate of plasma cell maturation in Aicda+/− GC B cells, combined with a previously reported reduction in apoptosis in Aicda+/− GC B cells (15), could explain the accumulation of these cells in GCs in AID-deficient mice. Interestingly, the observed defect in plasma cell formation was not due to the absence of DNA double-strand breaks (DSBs). Furthermore, we show that deficiency of caspase 8 within GC B cells leads to enlarged GCs and a delay in the appearance of high-affinity Ab.

Materials and Methods

Mice

AID−/−, YFPfl/fl mice were provided by Dr. R. Casellas (National Institutes of Health, Bethesda, MD) (5). Caspase 8−/− mice were crossed with AID−/−, YFPfl/fl mice to produce AID−/−YFPfl/fl Caspase8−/− and AID−/−YFPfl/fl Caspase8+/− mice. CD45.1- and IgH+ congenic mice on the C57BL/6 background were obtained from the Jackson Laboratory (stocks 002014 and 001317, respectively). Aicda−/− mice were obtained from Tasuku Honjo (Kyoto University, Japan). Aicda−/− and CD45.1 mice were crossed to produce Aicda−/−CD45.2/0.1 bone marrow (BM) hosts. OTII and MD4 mice were obtained from the Jackson Laboratory (stocks 004194 and strain 002595, respectively). MD4 mice were crossed with Aicda−/− and CD45.1 mice to obtain Aicda−/−/MD4 and CD45.1/MD4 mice. Msh2−/− mice were obtained from Tak Mak (University of Toronto). UNG−/− mice were obtained from Hans Krokan (Norwegian University of Science and Technology, Trondheim, Norway). UNG−/− and Msh2−/− mice were crossed to

Received for publication July 5, 2013. Accepted for publication October 15, 2013.

This work was supported by a grant from the Canadian Institutes of Health Research (89783 to J.L.G. and A.M.). A.M. is supported by a Canada Research Chair award.

B.B. planned and performed experiments and wrote the manuscript; O.L.R., D.H., A.Z., C.L., and A.K. planned and performed experiments; T.N. and R.H. helped with experiments and contributed input into experimental design; and J.L.G. and A.M. conceived the experimental approach and wrote the manuscript.

Address correspondence and reprint requests to Dr. Jennifer L. Gommerman and Dr. Alberto Martin, University of Toronto, 1 King’s College Circle 7302, Toronto, ON M5S 1A8, Canada. E-mail addresses: jen.gommerman@utoronto.ca (J.L.G.) and alberto.martin@utoronto.ca (A.M.)

Abbreviations used in this article: AID, activation-induced deaminase; BM, bone marrow; CSR, class-switch recombination; DSB, double strand break; GC, germinal center; HSL, hen egg lysozyme; NP-CGG, nitrophenyl-chicken gamma globulin; p.i., postimmunization; SHM, somatic hypermutation; WT, wild type.

Copyright © 2013 by The American Association of Immunologists, Inc. 0022-1767/13/$16.00

The Journal of Immunology
obtain Moh2−/− UNG−/− mice. All mice were housed in specific pathogen-free conditions. All animal experiments were performed in accordance with endpoints and standards of animal care approved by the University of Toronto Faculty of Medicine Animal Ethics Committee.

Bone marrow chimeras, adoptive transfers, and immunizations

BM chimeras were generated as described previously (15). Eight weeks after transfer, chimeric mice were immunized i.p. with 100 μg nitrophenyl-chicken gamma globulin (NP-CCG) (Biosearch Technologies) in alum adjuvant (Thermo Scientific). Some BM chimeras received a secondary radiation dose of 500 cGy to induce DNA DSBs. For adoptive transfer experiments, B cells from Aicda−/−/MD4 and CD45.1/MD4 mice and T cells from OTII mice were isolated by negative selection with EasySep kits (Stemcell Technologies). Both genotypes of B cell and 3.5 × 106 T cells from OTII mice were injected i.v. into host Aicda−/− CD45.2/0.1 mice. Twenty-four hours after transfer, host mice were immunized s.c. in each hind flank and i.p. with 50 μg hen egg lysozyme (HEL)-OVA and 0.07 μg LPS in alum adjuvant. HEL-OVA protein was produced by conjugating Hen Egg Lysozyme with Chicken OVA (Sigma-Aldrich) using a conjugation kit (SoluLink).

Cell isolation and flow cytometry

Single-cell suspension of spleen and BM (femur, tibia) cells were suspended in 1 ml RBC lysis buffer for 3 min on ice and then resuspended in PBS. Single-cell suspensions from inguinal lymph nodes were 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 strained and resuspended in PBS, followed by incubation with normal mouse serum and 2.4G2 mAb before staining with Abs against B220 (mAb A3809); B220 (mAb RA3-6B2), CD45.1 (mAb A20), Fas (mAb N418), and CD45.2 (mAb 104) (eBioscience); GL7 Ag, Fas (mAb Jo2), CXCR4 (mAb 2B11/CXCR4), IgM (mAb A85-1), and CD138 (mAb 281-2) (BD Biosciences); B220 (mAb RA3-6B2), IgM (mAb RMM-1), and IgD (mAb 11-26c.2a; Biologend). Biotinylated Abs were labeled with streptavidin conjugated to PE-TexasRed (BD) or APC-eFluor780 (eBioscience). Cells were labeled for Ag specificity with NP-PE (Biosearch Technologies). Intracellular stains were performed using Cytotox/Cytoperm (BD). Cells were stained for active caspase 8 using IETD-FMK from the CaspGLOW Caspase 8 Kit (BioVision).

V-region sequencing

RNA was isolated from NP-immunized AcreC8fl/+ and AcreC8fl/fl mice according to manufacturer’s instructions (TRIzol Reagent, Invitrogen). cDNA was synthesized using oligo(dT) primed SuperScript III reverse transcriptase (Invitrogen), and VH186.2 joined to IgG1 C region was sequenced as described previously (16).

ELISA

Sera from nonimmunized or NP-immunized AcreC8fl/+ or AcreC8fl/fl mice were taken after immunization and added to ELISA plates were coated with 0.2 μg/ml NP4-BSA or NP32-BSA (Biosearch Technologies) at 4°C overnight. Later steps were performed as described previously (17).

ELISPOT

MultiScreen-HTS-HA filter plates (Millipore) were coated with 50 μg/ml NP30-BSA (Biosearch Technologies) in 1%BSA/PBS overnight at 4°C and blocked with 2%BSA/PBS for 4 h at 37°C. RBC-lysed splenic or BM cells were suspended in RPMI 1640 with 50 μM 2-ME, 10% FBS, and 1× Pen/Strep (Sigma-Aldrich), plated in wells at 5 × 103 to 5 × 105 cells/well, and incubated overnight at 37°C. Plates were washed with 0.05% Tween20/PBS and probed with FITC-conjugated or biotinylated mAbs against IgM, IgG, and IgG (Biolegend). Anti-FITC (pAb; Roche Diagnostics) or streptavidin (ProZyme) conjugated to alkaline phosphatase were used as a secondary probe. Wells were then developed with 0.2 mg/ml SigmaFast BCIP/NBT substrate (Sigma-Aldrich) for 20 min and washed.

Results

Aicda−/− B cells accumulate as CXCR4low centocytes in the GCs of mixed BM chimeras

We previously showed that Aicda−/− mice have enlarged GCs and that Aicda−/− GC B cells out-compete wild type (WT) B cells in a GC reaction. We also observed significantly less apoptosis of Aicda−/− GC B cells compared with WT B cells (15). Although these results suggested that reduced apoptosis in Aicda−/− GC B cells results in their accumulation within GCs, it is unclear that this is due to increased numbers of centroblasts or centrocytes. To address this unanswered question, we generated mixed BM chimeras in which donor BM cells from Aicda−/− (CD45.2) and WT (CD45.1) mice are used to reconstitute Aicda−/− CD45.1/CD45.2 hosts (15). We immunized BM chimeras with NP-CCG in alum and measured the ratio of Aicda−/− to WT GC B cells (Fig. 1A). This ratio was then normalized to the background ratio of Aicda−/− to WT naive B220+ Fas+ GL7+ follicular B cells by dividing the ratio among GC B cells by the ratio among follicular B cells. This generated a ratio normalized for variation in chimerism between

![Image](http://www.jimmunol.org/DownloadedFrom)
individual BM chimeras that could be compared with the expected null value of 1. We also generated control BM chimeras that contained two types of congenically labeled WT BM cells (CD45.1 and CD45.2). In these control BM chimeras, neither of the donor B cell types are expected to have an advantage over the other, and we found that, as expected, the normalized ratio was ~1.0 (Fig. 1D, 1E). Using this approach, we evaluated the mean fluorescence intensity of CXCR4, using CXCR4low/high as a marker to investigate centrocyte–centroblast phenotype (2, 18). Validating the CXCR4low/high gating strategy, we found that the CXCR4high cells were radiosensitive and the CXCR4low cells were radioresistant (data not shown), which is consistent with the highly proliferative nature of centroblasts (2, 18). We observed that Aicda−/− GC B cells expressed lower levels of CXCR4 than WT GC B cells did at all time points (Fig. 1B). We gated on all GC B cells, divided this population into CXCR4low and CXCR4high gates (Fig. 1C), and measured the ratio of Aicda−/− to WT cells among CXCR4high and CXCR4low populations. Among CXCR4high centroblast GC B cells, there was a small but significant skew toward Aicda−/− cells at day 7 postimmunization (p.i.), which was not detectable at any other time point (Fig. 1D). However, there was a significant skew toward Aicda−/− GC B cells among CXCR4low cells at all time points (Fig. 1E). This finding indicates that, for the most part, Aicda−/− GC B cells are accumulating as CXCR4low centrocytes.

Accumulation of Aicda−/− GC B cells is not due to affinity of the BCR for Ag
Because GC B cells undergo clonal selection at the centrocyte stage, we assessed whether the accumulation of Aicda−/− GC B cells was due to the lack of affinity maturation of the BCR for Ag. We crossed Aicda−/− (CD45.2) and WT (CD45.1) mice with MD4 mice, which contain transgenic, rearranged Ig heavy and light chains that encode a high-affinity Ab for the hen HEL protein (19, 20), thereby fixing the Ag affinity (and specificity) of the BCR in both cell populations. We transferred 2 × 10^6 of each Aicda−/− MD4 (IgMbCD45.2) and WT MD4 (IgMaCD45.1) B cell along with 3.5 × 10^5 OTII T cells (as a source of T cell help), into C57BL/6 (IgMbCD45.2) hosts and immunized them s.c. with HEL-OVA in alum. At 7 d p.i., we examined the normalized ratio of GC B cells within the draining lymph node and found that there was still a significant skew toward Aicda−/− GC B cells at day 7 postimmunization (p.i.), which was not detectable at any other time point (Fig. 1D). However, there was a significant skew toward Aicda−/− GC B cells among CXCR4low cells at all time points (Fig. 1E). This finding indicates that the affinity of the BCR for Ag cannot solely explain the observed accumulation of Aicda−/− B cells in the GC.

Aicda−/− GC B cells do not efficiently form plasma cells
Aicda−/− GC B cells accumulated as centrocytes, yet were present in normal numbers as centroblasts (thus were not simply moving out of the dark zone into the light zone en masse); therefore, we hypothesized that Aicda−/− GC B cells were experiencing a block in fate decisions following their tenure in the light zone. We needed to determine whether Aicda−/− GC B cells were experiencing a block in fate decisions following their tenure in the light zone. We generated mixed BM chimeras in which the WT B cells possess the IgH allotype to differentiate them from IgH expressing Aicda−/− B cells. We then measured the number of BM plasma cells 21 d p.i. that were producing IgM or IgG (WT) versus the number of BM plasma cells producing IgM (Aicda−/−) by Ag-specific ELISPOT (Fig. 2C). Flow cytometry revealed significantly more Aicda−/− than WT B cells in splenic GCs, but ELISPOT counts showed approximately equivalent numbers of Aicda−/− and WT BM plasma cells (i.e., a 1:1 ratio; see Fig. 2C). This result suggests that although Aicda−/− GC B cells develop into long-lived BM plasma cells, they do so inefficiently.

DNA double-strand breaks do not rescue plasma cell maturation in Aicda−/− GC B cells
A recent report shows that the formation of DNA DSBs during CSR in cultured human B cells initiates a signal that promotes B cell development into plasma cells (21). Because Aicda−/− B cells cannot generate DSBs in the IgH locus, we hypothesized that Aicda−/− B cells might be missing a signal required for normal plasma cell maturation. To test this notion, we immunized BM chimeras with NP-CCG in alum and dosed them with 500 cGy ionizing irradiation at day 10 p.i. to induce DNA DSBs in GC B cells and then analyzed the ratio of NP-specific Aicda−/− BM plasma cells to NP-specific WT BM plasma cells using flow cytometry. We first analyzed BM cells from unimmunized and HEL-immunized control mice (Fig. 3) and observed that B220low
CD138+ cells stained nonspecifically for intracellular NP-PE Ag (Fig. 3D), while B220<sub>medium</sub>CD138+ BM cells labeled specifically for intracellular NP-PE (Fig. 3B). Hence, at day 21 p.i., we analyzed NP-specific B220<sub>medium</sub>CD138+ BM cells and measured the normalized ratio of Aicda<sup>−/−</sup> (CD45.2<sup>+</sup>) to WT (CD45.1<sup>+</sup>) plasma cells (Fig. 3B, 3C).

Irradiation-induced genomic DSBs were found to occur equally in Aicda<sup>−/−</sup> and WT B cells, as verified by staining for γH2AX, which is a phosphorylated histone component present at sites of DNA DSBs (Fig. 4A) (22). The ratio of Aicda<sup>−/−</sup> to WT BM plasma cells in irradiated BM chimeras did not increase compared with control mice, indicating that radiation-induced DNA DSBs did not promote the maturation of Aicda<sup>−/−</sup> GC B cells into plasma cells (Fig. 4B).

Because our irradiation strategy might not have induced optimal DNA breaks within the IgH locus, we further investigated the role of DNA DSBs in plasma cell development using UNG<sup>−/−</sup>Msh2<sup>−/−</sup> mice. UNG and Msh2 are DNA repair factors that convert AID-induced cytosine deaminations at the Ig locus into DSBs (23) and UNG<sup>−/−</sup>Msh2<sup>−/−</sup> are unable to produce DSBs in response to AID (24). If DSBs are required for plasma cell maturation, UNG<sup>−/−</sup>Msh2<sup>−/−</sup> GC B cells should be incapable of maturing into plasma cells. We generated mixed BM chimeras with WT (CD45.1) and UNG<sup>−/−</sup>Msh2<sup>−/−</sup> BM and immunized as done previously. UNG<sup>−/−</sup>Msh2<sup>−/−</sup> B cells undergo high rates of apoptosis, evident by TUNEL staining (Fig. 4C). This apoptosis resulted in a low number of UNG<sup>−/−</sup>Msh2<sup>−/−</sup> B cells in the GC and a low

---

**FIGURE 3.** Gating on Ag-specific BM plasma cells. Representative flow cytometry of BM cells from mixed BM chimeras immunized i.p. with NP-CGG in alum at day 21 p.i. We gated on (A) B220<sub>medium</sub>CD138<sup>+</sup> BM cells and then analyzed this cell population for (B) Ag-specific plasma cells by intracellular staining with NP-PE. NP-PE staining labeled intracellular Ig in Ag-specific plasma cells. (B) A large fraction of B220<sub>medium</sub>CD138<sup>+</sup> BM cells stained positive for NP-PE in NP immunized mice. Few of these cells stained positive for NP-PE in unimmunized mice or in mice immunized with an irrelevant Ag (HEL). No autofluorescence was detected in cells not stained with NP-PE (FMO). NP-PE specific cells were then analyzed for (C) CD45.1 (WT) or CD45.2 (Aicda<sup>−/−</sup>) expression. (D) B220<sub>medium</sub>CD138<sup>+</sup> cells stained nonspecifically for NP-PE. These controls demonstrated that cells outside of the gate (B) stained nonspecifically for NP-PE.

**FIGURE 4.** Irradiation-induced DNA DSBs do not rescue Aicda<sup>−/−</sup> plasma cell formation. (A) Mixed BM chimeras were immunized and then irradiated at day 10 p.i. to induce DSBs. Staining splenic B220<sup>+</sup> B cells 3 h after irradiation shows elevated γH2AX in both Aicda<sup>−/−</sup> (CD45.2<sup>+</sup>) and WT (CD45.1<sup>+</sup>) B cells by flow cytometry. (B) The ratio of gated Aicda<sup>−/−</sup> to WT B220<sub>medium</sub>CD138<sup>+</sup>NP<sup>+</sup> BM plasma cells measured by flow cytometry. Irradiation-induced DSBs has not resulted in an increase of the ratio of Aicda<sup>−/−</sup> to WT BM plasma cells. Data from two independent experiments are shown. (C) and (D) Mixed BM chimeras of UNG<sup>−/−</sup>Msh2<sup>−/−</sup> (double knockout [DKO]) and WT BM donor cells were immunized, and their splenic GCs and BM plasma was analyzed with flow cytometry. Data from two independent experiments are shown. (C and D) Mixed BM chimeras of UNG<sup>−/−</sup>Msh2<sup>−/−</sup> (DKO) and WT BM donor cells were immunized, and their splenic GCs and BM plasma was analyzed with flow cytometry. Data from two independent experiments are shown. (C) and (D) Mixed BM chimeras of UNG<sup>−/−</sup>Msh2<sup>−/−</sup> (double knockout [DKO]) and WT BM donor cells were immunized, and their splenic GCs and BM plasma was analyzed with flow cytometry. Data from two independent experiments are shown. (C and D) Mixed BM chimeras of UNG<sup>−/−</sup>Msh2<sup>−/−</sup> (DKO) and WT BM donor cells were immunized, and their splenic GCs and BM plasma was analyzed with flow cytometry. Data from two independent experiments are shown. (C and D) Mixed BM chimeras of UNG<sup>−/−</sup>Msh2<sup>−/−</sup> (DKO) and WT BM donor cells were immunized, and their splenic GCs and BM plasma was analyzed with flow cytometry. Data from two independent experiments are shown. (C and D) Mixed BM chimeras of UNG<sup>−/−</sup>Msh2<sup>−/−</sup> (DKO) and WT BM donor cells were immunized, and their splenic GCs and BM plasma was analyzed with flow cytometry. Data from two independent experiments are shown.
ratio of UNG*/Msh2* to WT GC B cells (Fig. 4D). Nevertheless, although low, the ratio of UNG*/Msh2*/ to WT GC B cells was the same as the ratio of UNG*/Msh2*/ to WT BM plasma cells. This finding indicates that although the UNG*/Msh2*/ B cells fare poorly within the GC, they formed plasma cells efficiently despite their lack of DNA DSBs (Fig. 4D). These results suggest that DNA DSBs, though potential contributors, are neither necessary nor sufficient for the in vivo maturation of GC B cells into plasma cells.

Deletion of caspase 8 in GC B cells results in larger GCs and delayed affinity maturation

GC B cells lacking AID have lower rates of apoptosis than WT GC B cells do and are less likely to activate caspases 3, 8, and 9 (15), suggesting that the enhanced viability of Aicda*/GC B cells might be responsible for the enlarged GCs observed in Aicda*/mice. A previous study conditionally ablated Fas in GC B cells and noted hyperlymphoproliferation and disrupted homeostasis (14). These observations suggest that AID and Fas can promote apoptosis in GC B cells and that this promotion of apoptosis is important in regulating GC responses. To test more precisely the hypothesis that reduced apoptosis in GC B cells leads to enhanced GC B cell numbers, we conditionally deleted caspase 8, which is downstream of Fas signaling, within AID-expressing GC B cells. Mice expressing cre recombinase under the control of the AID promoter along with loxP sites flanking the caspase 8 gene (AcreC8fl/fl) were crossed with mice containing a YFP fluorescent reporter transgene containing a loxP-flanked stop codon. Heterozygous AcreC8fl/+ mice were used as controls. AcreC8fl/fl B cells conditionally delete caspase 8 and produce YFP upon expressing AID. Consistent with AID not being expressed in naive B cells, YFP was not observed in B220*GL7 Fas* follicular B cells (Fig. 5A). We found that caspase 8 expression was negligible in GC B cells from AcreC8fl/fl mice at days 7 and 10 p.i. compared with controls (Fig. 5B). Caspase 8–positive GC B cells were observed in AcreC8fl/fl mice at day 15 p.i., although in lower numbers than in AcreC8fl/+ mice (Fig. 5C). Because caspase 8 can promote proliferation in certain conditions (25, 26), this might reflect the selection of B cell clones that escaped deletion of caspase 8 earlier in the response. Deletion of caspase 8 did not affect the proportions of total splenic B cells (Fig. 5D).

We immunized AcreC8fl/+ and AcreC8fl/fl mice with NP-CGG in alum and measured the percentage of splenic B220*GL7 Fas* GC B cells by flow cytometry at various time points p.i. (Fig. 6A). At days 7 and 15 p.i., AcreC8fl/fl mice had a significantly higher percentage of GC B cells than AcreC8fl/+ mice (Fig. 6B), although in lower numbers than in AcreC8fl/+ mice (Fig. 5C). Because caspase 8 can promote proliferation in certain conditions (25, 26), this might reflect the selection of B cell clones that escaped deletion of caspase 8 earlier in the response. Deletion of caspase 8 did not affect the proportions of total splenic B cells (Fig. 5D).

We immunized AcreC8fl/+ and AcreC8fl/fl mice with NP-CGG in alum and measured the percentage of splenic B220*GL7 Fas* GC B cells at day 15 p.i. (Fig. 6A). At days 7 and 15 p.i., AcreC8fl/fl mice had a significantly higher percentage of GC B cells than AcreC8fl/+ mice (Fig. 6B), although in lower numbers than in AcreC8fl/+ mice (Fig. 5C). Because caspase 8 can promote proliferation in certain conditions (25, 26), this might reflect the selection of B cell clones that escaped deletion of caspase 8 earlier in the response. Deletion of caspase 8 did not affect the proportions of total splenic B cells (Fig. 5D).

To confirm the delay in affinity maturation at the molecular level, we sequenced the V186.2 subfamily of V-regions that is selected during anti-NP responses, and we examined V186.2 canonical Trp to Leu mutations (W→L) (27, 28). At day 10 p.i., 35.3% of V186.2 sequences from AcreC8fl/+ GC B cells had codon 33 mutations and 11.8% had W→L mutations (Table I). In contrast, we did not detect any codon 33 mutations in V186.2 sequences from AcreC8fl/fl GC B cells at day 10 despite a similar
overall mutation frequency. This result suggests that although SHM functions normally, caspase 8 deficiency delays the selection of high-affinity clones. By day 15 p.i., W→L mutations at codon 33 appeared in AcreC8fl/fl GC B cells, although at a slightly reduced frequency compared with controls (Table I). Although this method of sequencing V regions from RNA might result in a bias toward plasma cell sequences, it should still provide a measure of mutations induced during the GC response because extrafollicular Ab-secreting cells begin to wane by day 8 p.i., and are mostly absent by day 15 p.i. of the NP response (29, 30). The V region sequences we analyzed are therefore likely to have been derived from GC-derived plasma cells, and as such reflect SHM induced during the GC response. This notion is supported by the fact that many V-regions harbored the W→L mutations at codon 33 in WT mice at day 10 p.i. In conclusion, these results suggest that apoptosis mediated by caspase 8 constrains the size of GCs and is important in ensuring efficient affinity maturation.

**Discussion**

GC B cells express elevated levels of Fas and endure genotoxic stress, and are therefore prone to apoptosis. Although Fas-mediated apoptosis is crucial for clonal selection of B cells with high affinity for Ag (9), it is increasingly evident that apoptosis is also important in regulating the GC response (10, 14). We have shown previously that Aicda<sup>−/−</sup> mice exhibit large, constitutive GCs, correlating with reduced apoptosis and enhanced proliferation of Aicda<sup>−/−</sup> GC B cells (15). These findings suggest that apoptosis through genotoxicity is important in GC B cell regulation by promoting the activation of caspases 3, 8, and 9. Apoptosis through the Fas receptor is also important in curtailing the GC response, as Fas-deficiency in GC B cells results in hyperproliferation (14). Although we suggested previously that the reduced apoptosis in Aicda<sup>−/−</sup> GC B cells might account for the enlarged GC B cell population in these mice, we wanted to test this question directly by conditionally deleting a known downstream effector of apoptosis (caspase 8) in GC B cells and assessing its effect on GC B cell homeostasis. We show that caspase-8 deficiency in AID-expressing GC B cells resulted in enlarged splenic GC populations, similar to Aicda<sup>−/−</sup> GCs, confirming a role for apoptosis in constraining GCs.

Moreover, we observed that caspase-8 deficiency in GC B cells was associated with a delay in the appearance of high-affinity mutated IgG. However, the defect in the Aicda<sup>−/−</sup> mice is stronger and more persistent than the defect in the AcreC8fl/fl mice. This suggests that AID lies upstream of C8 activation, and perhaps other death mechanisms can replace the loss of C8 at later time points. Nevertheless, we postulate that inappropriate survival of low-affinity B cell clones could result in reduced competition within GCs and
relaxed negative selection of low-affinity B cell clones presumably affects the quality (affinity) of the Ab responses.

We have demonstrated that Aicda<sup>−/−</sup> B cells preferentially accumulate within GCs as CXCR4<sup>low</sup> centrocytes, the stage at which B cells undergo clonal selection and commit to a maturation fate (1). In addition, a recent study has analyzed the transcriptional profiles of GC B cells from Aicda<sup>−/−</sup> mice and found that they express a centrocyte signature (31) supporting our observations in competitive BM chimeras. It is likely that Aicda<sup>−/−</sup> GC B cells undergo lower rates of attrition and higher rates of proliferation (15) as centroblasts and then accumulate as they mature into centrocytes. The fact that Aicda<sup>−/−</sup> GC B cells accumulated as centrocytes suggests that they are not succumbing to negative clonal selection at a normal rate. Similarly, A<sup>−/−</sup>C<sup>−/−</sup> GC B cells expressed, on average, lower levels of surface CXCR4 than WT GC B cells did, suggesting that caspase 8-deficient GC B cells also accumulate as centrocytes.

Our data suggest that both AID-induced DNA damage and Fas-signaling are important in inducing apoptosis in centrocytes. Specifically, cells that are not appropriately mutated to succeed during clonal selection in the light zone can perish by Fas signaling, which is executed by caspase 8 downstream of Fas ligation (7, 32). Fas-deficiency results in enlarged GCs (14), and we have now shown that even partial deletion of caspase 8 likewise results in enlarged GCs. On the other hand, our experiments with MD4 Aicda<sup>−/−</sup> GC B cells demonstrate that even with a fixed BCR, Aicda<sup>−/−</sup> GC B cells accumulate in GCs. We hypothesize that DNA damage-induced apoptosis is responding to maladaptive AID-mediated mutations and is responsible for the accumulation of MD4 Aicda<sup>−/−</sup> GC B cells. Accordingly, we previously showed that Aicda<sup>−/−</sup> GC B cells exhibit lower activation of caspases 3, 8, and 9 (15). Collectively, these data highlight that there are likely redundancies in the use of different apoptotic pathways to constrain the GC response effectively. Future experiments using mice that are double deficient in AID and caspase 8 could determine whether the contributions of these pathways are additive or synergistic in regulating GCs.

We also demonstrate that Aicda<sup>−/−</sup> GC B cells mature into plasma cells at a lower frequency, and this could contribute to the accumulation of Aicda<sup>−/−</sup> GC B cells. The observation that Aicda<sup>−/−</sup> GC B cells accumulated as CXCR4<sup>high</sup> centrocytes suggests that they were experiencing a reduced commitment to fate decisions (maturation, recycling, apoptosis). Because Aicda<sup>−/−</sup> B cells were present in expected numbers in the CXCR4<sup>high</sup> centroblast population, it seemed that they were not being induced to recycle at a normal frequency (33)—nor were Aicda<sup>−/−</sup> GC B cells committing to the plasma cell fate at a normal frequency. In addition, we have shown previously that Aicda<sup>−/−</sup> GC B cells undergo lower rates of apoptosis (15). These observations suggest that the accumulation of Aicda<sup>−/−</sup> GC B cells could also be due in part to reduced commitment to fate decisions.

The signals that determine specific GC B cell fates are still a matter of intense investigation (34). Because AID-induced DNA DSBs provide a signal that promotes plasma cell maturation in vitro (21), we evaluated whether this could explain the observed inefficiency in generation of Aicda<sup>−/−</sup> plasma cells in vivo. Whole-body irradiation induces DNA breaks in dividing B cells and has been shown to alleviate the defect partially in V(D)J recombination in SCID mice (35, 36). Although there is good evidence in vitro that DSBs play a role in plasma cell maturation, our experiments suggest that DSBs alone are not sufficient in vivo. Although it is possible that our irradiation procedure did not induce DSBs at specific regions of the Ig locus necessary to induce plasma cell maturation, because UNG<sup>−/−</sup>Msh2<sup>−/−</sup> GC B cells still form plasma cells efficiently, the possibility is unlikely (Fig. 3F). Alternatively, the plasma cell maturation defect we observed could be due to the Ig isotype expressed on the surface of the GC cell; the intracellular region of IgG contains an extended tail not present in IgM that enhances BCR signaling (37, 38). Indeed, IgG<sup>+</sup> memory B cells are more likely to differentiate into plasma cells upon BCR stimulation, whereas IgM<sup>+</sup> memory B cells are more likely to become GC B cells (39). In addition, BCR stimulation generates a stronger proliferative response in class-switched IgG<sup>+</sup> plasmablasts than in unswitched plasmablasts (40). Thus, Aicda<sup>−/−</sup> GC B cells, which possess only IgM<sup>+</sup> BCRs, could be lacking a BCR signal that promotes efficient plasma cell differentiation or expansion. Another possibility is that increasing the Ag affinity of the BCR promotes the generation of plasma cells and that Aicda<sup>−/−</sup> GC B cells, which cannot undergo SHM, simply lack the high-affinity BCR interactions necessary to promote robust plasma cell formation. Indeed, GC B cells with higher affinity for Ag are preferentially selected early to populate the post-GC plasma cell population (41). Alternatively, Aicda<sup>−/−</sup> GC B cells may simply be unable to acquire specific signals from CD4<sup>+</sup> T follicular helper cells, such as IL-21 (42), to promote survival or to induce maturation into plasma cells. Normally, GC B cells that cannot obtain positive interactions with CD4<sup>+</sup> T follicular helper cells should undergo apoptosis. However, the intrinsically lower rate of apoptosis in Aicda<sup>−/−</sup> GC B cells could

### Table I. Analysis of NP-Specific Sequences

<table>
<thead>
<tr>
<th></th>
<th>Day 10</th>
<th>Day 15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of sequences analyzed</td>
<td>34</td>
<td>21</td>
</tr>
<tr>
<td>Codon 33 mutations</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>% Mutation&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35.3</td>
<td>0</td>
</tr>
<tr>
<td>% W→L&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.8</td>
<td>0</td>
</tr>
<tr>
<td>CDR2 mutations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutation frequency&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.3 × 10&lt;sup&gt;−2&lt;/sup&gt;</td>
<td>9 × 10&lt;sup&gt;−2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total mutations</td>
<td>109</td>
<td>96</td>
</tr>
<tr>
<td>% GC mutations</td>
<td>51.3</td>
<td>63.5</td>
</tr>
<tr>
<td>% Transition mutations</td>
<td>63.3</td>
<td>55.2</td>
</tr>
<tr>
<td>% Transversion mutations</td>
<td>36.7</td>
<td>44.8</td>
</tr>
<tr>
<td>Replacement/silent ratio&lt;sup&gt;d&lt;/sup&gt;</td>
<td>10</td>
<td>21.67</td>
</tr>
</tbody>
</table>

<sup>a</sup>Percent mutation of codon 33 calculated by dividing the total codon 33 mutations by the number of sequences analyzed.

<sup>b</sup>The percent of affinity enhancing mutations (tryptophan to leucine) at codon 33 calculated by dividing the number of these mutations by the total number of sequences analyzed.

<sup>c</sup>Mutation frequency in the CDR2 region encompassing 51 bp.

<sup>d</sup>Replacement-silent mutations ratio calculated in the CDR2 region.
allow them to survive the absence of T cell help and to simply accumulate at the centrocyte checkpoint rather than undergo apoptosis (15).

In conclusion, using both AID- and caspase 8-deficient mice, we have established a molecular framework that limits the size of the GC response. Specifically, we attribute the accumulation of Aicda

- deficient GC B cells to a combination of enhanced viability owing to AID deficiency and a reduced emigration of cells from GCs during plasma cell maturation. Because caspase 8 deficient GC B cells were also shown to accumulate within the GCs, we believe that caspase 8 is involved in constraining the size of GCs. Our findings contribute to our understanding of how apoptosis influences the GC response and suggests that AID expression is important for plasma cell maturation.

Acknowledgments

We thank Dr. Marc Shulman for comments on this manuscript and the Martin and Gommerman laboratories for helpful discussions.

Disclosures

The authors have no financial conflicts of interest.

References


In conclusion, using both AID- and caspase 8-deficient mice, we have established a molecular framework that limits the size of the GC response. Specifically, we attribute the accumulation of Aicda

- deficient GC B cells to a combination of enhanced viability owing to AID deficiency and a reduced emigration of cells from GCs during plasma cell maturation. Because caspase 8 deficient GC B cells were also shown to accumulate within the GCs, we believe that caspase 8 is involved in constraining the size of GCs. Our findings contribute to our understanding of how apoptosis influences the GC response and suggests that AID expression is important for plasma cell maturation.

Acknowledgments

We thank Dr. Marc Shulman for comments on this manuscript and the Martin and Gommerman laboratories for helpful discussions.

Disclosures

The authors have no financial conflicts of interest.

References