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*J Immunol* 2007; 178:2192-2203; doi: 10.4049/jimmunol.178.4.2192
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AID−/− μs−/− Mice Are Agammaglobulinemic and Fail to Maintain B220−CD138+ Plasma Cells

Kaori Kumazaki,* Boaz Tirosh,* René Maehr,† Marianne Boes,‡ Tasuku Honjo,§ and Hidde L. Ploegh*†

The terminal stage of B cell differentiation culminates in the formation of plasma cells (PC), which secrete large quantities of Igs. Despite recent progress in understanding the molecular aspect of PC differentiation and maintenance, the requirement for the synthesis of secretory Igs as a contributing factor has not been explored. To address this issue, we generated activation-induced cytidine deaminase (AID)/secretory μ-chain (μs) double-knockout mice, in which a normally diverse repertoire of B cell receptors is retained, yet B cells are unable to synthesize secretory Igs. These mice possess polyclonal B cells but have no serum Igs. Following immunization in vivo, PCs, identified by CD138 expression and loss of the B220 marker, were starkly reduced in number in spleen and bone marrow of AID−/− μs−/− agammaglobulinemic mice compared with wild-type mice. Upon mitogenic stimulation in vitro, AID−/− μs−/− B cells differentiated into plasmablasts to some extent, but showed reduced survival compared with wild-type B cells. We found no evidence that this reduced survival was attributable to accumulation of membrane IgM. Our results indicate that the synthesis of secretory Igs is a requirement for maintenance of B220−CD138+ PCs. The Journal of Immunology, 2007, 178: 2192–2203.

Plasma cells (PC)3 are terminally differentiated secretory cells that play a critical role in humoral immunity by producing copious amounts of soluble Abs. Upon encounter with Ags, B cells either differentiate into short-lived PCs that secrete low-affinity IgM, or participate in a germinai center (GC) reaction accompanied by class switch recombination (CSR) and somatic hypermutation (SHM), to yield PCs that secrete high-affinity IgG or IgA (1, 2). Terminal differentiation into PCs requires excessive remodeling of the endoplasmic reticulum (ER) to accommodate the large quantities of newly synthesized Igs, and to control the quality of assembled multimeric Igs in preparation for secretion (3–5). The molecular mechanisms involved in the regulation of terminal PC differentiation are being deciphered at the level of the transcription factors responsible, but little is known about the significance of the secretory Igs themselves as a factor that contributes to terminal differentiation and maintenance of PCs.

Several transcriptional regulators have been implicated in the control of PC differentiation (1), of which XBP-1 was the first shown to be essential (6). There is a close correlation between high rates of Ig secretion and the activation of XBP-1 (7). Expression of the active form of XBP-1 requires engagement of the IRE-1 protein, whose activation is believed to be triggered by the accumulation of misfolded polypeptides, although the agents commonly used to activate IRE-1 are rather toxic compounds such as tunicamycin, DTT, or thapsigargin (8). In the absence of XBP-1, B cells fail to differentiate into PCs (6). The inability to deal with an onslaught of newly ER-inserted proteins and the misfolded proteins that are the inevitable byproduct of protein synthesis might propel such XBP-1 deficient B cells toward programmed cell death. However, we have shown that XBP-1 deficient B cells contain comparable levels of μ-chain mRNA, yet are far less efficient at translating this mRNA than their wild-type counterparts (7). These observations suggest that the relationship between PC differentiation and high rates of Ig secretion is more complex than envisioned originally.

Even though the transcripts that encode the membrane and secreted forms of Igs are part of the same transcription unit, polyclonal activation of B cells incapable of IgM secretion does not result in a measurable increase in the synthesis of membrane IgM (7). The decision between the synthesis of membrane vs secreted Igs is made by choice of polyadenylation site (9), but exactly what regulates the levels of these different mRNAs is not known. Clearly, the inability to generate an mRNA that encodes the secreted μ-chain is not automatically linked to an increase in the synthesis of the membrane bound product, notwithstanding seemingly adequate secretory capacity (7). These observations, too, suggest a hitherto unappreciated relationship between Ig secretion by B cells and their ability to differentiate into PCs.

To explore the relationship between secretory Ig and PC differentiation more directly, we generated mice that are unable to synthesize any secretory Ig in significant quantities, yet retain a normally diverse repertoire of BCRs. We used μs−/− μc−/− mice, unable to secrete IgM because of the elimination of elements that enable synthesis of the secretory form of IgM (10). These mice retain the capacity to engage other Ig constant regions by CSR, and so possess normal serum levels of all Ig isotypes except IgM. Crossing the μs−/−...
animals onto an AID-deficient background eliminates both the possibility of SHM and CSR (11) and consequently such animals should lack circulating serum Igs, as is indeed the case (see Results). Although agammaglobulinemic animals have been produced previously through introduction of a membrane IgM-encoding transgene on a genetic background incapable of executing VDJ recombination of the H chain locus (12, 13), the B cells in such animals lack the normally diverse repertoire of BCRs. The rearranged Ig transgene is not embedded in the usual chromosomal environment that may contribute to its regulated expression, and so might impinge on the terminal stages of B cell differentiation. Moreover, the issue of PC generation has not been previously examined in the context of the B cell’s ability to secrete Igs. Upon stimulation of secretory Ig-deficient, yet otherwise normal B cells, can PC equivalents be generated and maintained?

In the present study, we compared in detail the B cell differentiation pathway of agammaglobulinemic AID−/− mice with wild type and each of the single knockout strains. We show that Ag-independent B cell development/differentiation is close to normal in AID−/− mice. However, following T-dependent Ag immunization in vivo, B220−CD138+ stage PCs were starkly reduced in number in the spleen and bone marrow of AID−/− and agammaglobulinemic mice. Upon mitogenic stimulation in vitro, AID−/− mice proliferated and differentiated into CD138+ plasmablasts to some extent, but showed reduced survival compared with B cells from wild-type mice. Our results suggest that the synthesis of secretory Igs is required to maintain PCs.

Materials and Methods

Mice

AID−/− (11) and each of the single knockout strains. We show that Cam- bridge, MA) were provided by Dr. J. Chen (Massachusetts Institute for Technology, Boston, MA). The AID−/− mice were backcrossed to C57BL/6J for six generations in Dr. Alt’s laboratory. The AID−/− mice (10) were provided by Dr. J. Chen (Massachusetts Institute for Technology, Cambridge, MA) and Dr. M. Carroll (Harvard Medical School). The AID−/− mice used had been backcrossed to C57BL/6J for 10 generations in Dr. Chen’s laboratory. AID−/− mice were generated by crossing AID−/− mice to AID−/− mice. C57BL/6J mice were used as wild-type controls, and were originally purchased from The Jackson Laboratory. All mice were bred and maintained under pathogen-free environment.

Age- (8–11 wk) and sex- matched mice from each group, which were raised at the same time in the same environment at the Harvard Medical School animal facility, were used for serum analyses and B cell characterization by flow cytometry. Two to four mice from each group were analyzed per experiment, and each experiment was repeated four times. Flow cytometry analyses were performed using the same reagents and instruments for each experiment, and the results were pooled for statistical analysis. For in vitro experiments, spleens from age- (12–14 wk) and sex- matched mice from each group that were raised in the same environment at the Whitehead Institute for Biomedical Research animal facility (Cam- bridge, MA) were used. All the studies were performed according to institutional guidelines for animal use and care.

Immunization

For each group, age- (9–11 wk) and sex- matched mice were immunized with nitrophenyl-conjugated chicken gammaglobulin (NP-CG; Biosearch Technologies) or OVA (grade V, Sigma-Aldrich) to elicit a response to TD-Ags. For NP-CG immunization, 100 μg of NP-CG emulsified in alum was injected i.p., and experiments were performed 9 days and 2 wk after the immunization. For immunization with OVA, mice were injected i.p. with 100 μg of OVA emulsified in CFA (Sigma-Aldrich) on day 0, then boosted with the same dose of OVA emulsified in IFA (Sigma-Aldrich) on days 7, 14, and 21 by i.p. injection. At day 24, blood was taken from each group of mice for assessment of specific Ab production by ELISA. A significant increase in anti-OVA Abs was detected in the sera of wild-type (both IgG1 and IgM), AID−/− mice (IgM), and μs−/− mice (IgG1) (data not shown), as evidence of successful immunization. Mice were sacrificed on day 28 for analysis of bone marrow and spleen.

Serum analysis

Serum samples were assayed for Ig isotype levels by ELISA using goat anti-mouse Ig, HRP-conjugated goat Abs specific for each mouse Ig isotype (Southern Biotechnology Associates), and a 3′, 5′-tetramethylbenzidine-based developing reagent (Sigma-Aldrich). Ig concentrations were calculated using ELISA software (Softmax Pro; Molecular Devices) from a standard curve produced from serial 2-fold dilutions of mouse Ig standards (Southern Biotechnology Associates).

Immunoblotting of serum Ig was conducted using HRP-conjugated goat Abs specific for IgM (μ), IgG1 (γ1), IgA (α), κ-chain, and λ-chain (Southern Biotechnology Associates). The level of total protein in each serum sample was measured before SDS-PAGE, and the same amount of total serum protein was loaded in each lane in each immunoblotting.

Cell preparation and flow cytometry

Spleens, lymph nodes, and Peyers’ patches were dissociated into single-cell suspensions by mechanical disruption in ice-cold RPMI 1640 medium (Invitrogen Life Technologies) containing 10% FCS. Peritoneal cells were obtained by lavage with 10 ml of the same medium. Bone marrow cells were obtained by flushing femurs and tibias of the mice. Erythrocytes in (plasma/fix) and snap-frozen, then cryostat sections (6-μm thick) were thawed, air-dried and fixed in ice-cold acetone for 10 min. For detection of GC B cells, sections were incubated with biotinylated PNA (Vector Laboratories) and rat anti-mouse CD19 (BD Biosciences), followed by incubation with Alexa 568-conjugated streptavidin and Alexa 488-conjugated goat anti-rat Ab ( Molecular Probes). For detection of PCs or plasmablasts, sections were incubated with PE-conjugated anti-mouse CD138 and FITC-conjugated anti-mouse CD45RB220 (BD Biosciences). For proliferation assays, cells were labeled with CFSE (Molecular Probes) according to the manufacturer’s instructions.

Cells were stimulated with 100 nM CpG (1826-CPG; TIB Molbiol) followed by 20 μg/ml LPS (Sigma-Aldrich) added after 24 h of culture. For conditions that promote in vitro class switching, cells were incubated with 1 μg/ml anti-mouse CD40 (HM40-3; BD Biosciences) and 25 ng/ml recombinant murine IL-4 (Sakura Fintek) and snap-frozen, then cryostat sections (6-μm thick) were loaded in each lane in each immunoblotting.

Histological and immunofluorescence analyses

For histological examination, organs were fixed with 4% paraformalde- hyde, embedded in paraffin, and stained with H&E. For immunofluo- rescence staining, tissues were embedded in Tissue Tek OCT compound (Sakura Fintek) and snap-frozen, then cryostat sections (6-μm thick) were thawed, air-dried and fixed in ice-cold acetone for 10 min. For detection of GC B cells, sections were incubated with biotinylated PNA (Vector Laboratories) and rat anti-mouse CD19 (BD Biosciences), followed by incubation with Alexa 568-conjugated streptavidin and Alexa 488-conjugated goat anti-rat Ab ( Molecular Probes). For detection of PCs or plasmablasts, sections were incubated with PE-conjugated anti-mouse CD138 and FITC- conjugated anti-mouse CD45RB220 (BD Biosciences).

Cell culture and in vitro stimulation

The conditions for in vitro B cell stimulation were as described (7). In brief, B cells were purified from mouse spleenocytes by magnetic depletion with anti-CD43 beads (Miltenyi Biotec), then cells were plated at 106 cells/ml in complete medium containing RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, 50 μg/ml streptomycin, 50 μg/ml mic- nonoessential amino acids, and 1 mM sodium pyruvate. For proliferation assays, cells were labeled with CFSE (Molecular Probes) according to the manufacturer’s instructions.

RT-PCR analysis

Total RNA was isolated using TRIzol (Invitrogen Life Technologies). RNAs were used for first-strand synthesis with the Superscript reverse transcriptase (Invitrogen Life Technologies). PCR primers 5′-ACACGCT TGGGAAATGCGACAC-3′ and 5′-CCATGGAAGATTTATTCGGG-3′ encompassing the missing sequences in XBP-1 were used for the PCR amplification with Platinum PCR Supermix (Invitrogen Life Technologies).

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Early B cell development in the bone marrow in AID\(^{-/-}\) μs\(^{-/-}\) mice

To characterize B cell development and differentiation in AID\(^{-/-}\) μs\(^{-/-}\) mice, we performed flow cytometric analyses using the cells collected from bone marrow, spleen, Peyer’s patches, and peritoneal cavity of each of the four groups of mice. There was no difference in total number of bone marrow cells among the four groups (Table I). The different populations of bone marrow B cells were classified according to the scheme proposed by Hardy et al. (15, 16), by the surface expression of S7 (leukosialin, CD43), BP-1, 30F1 (heat stable Ag, CD24), B220, and IgM. There was no significant difference in the proportions of pre-pro-B, early pro-B, late pro-B, and pre-B cells (fraction (Fr.) A, B, C, and D, respectively) among the four groups.

Results

Lack of serum IgM, IgG, and IgA in AID\(^{-/-}\) μs\(^{-/-}\) mice

To determine whether AID\(^{-/-}\) μs\(^{-/-}\) mice are deficient in circulating IgGs, we performed immunoblotting and ELISA analyses using the sera from AID\(^{-/-}\) μs\(^{-/-}\) mice as well as the sera from wild-type, AID\(^{-/-}\), and μs\(^{-/-}\) mice. As expected from the previous reports (10, 11), IgG1 (γ1 chain) and IgA (α-chain) were not detected in the sera of AID\(^{-/-}\) mice, and IgM (μ chain) was not detected in the sera of μs\(^{-/-}\) mice by immunoblotting (Fig. 1A). AID\(^{-/-}\) mice had more IgM than wild-type mice, consistent with the “hyperIgM” syndrome that also characterizes AID-deficient humans (14). B cells of neither the AID\(^{-/-}\) nor the μs\(^{-/-}\) mice have an intrinsic defect in Ig secretion.

No μ, γ1, α, κ, or λ polypeptides were detected in the sera of AID\(^{-/-}\) μs\(^{-/-}\) mice by immunoblotting (Fig. 1A). ELISA analysis confirmed the absence of IgG1 (γ1 chain), IgG2a (γ2α chain), IgG2b (γ2β chain), IgG3 (γ3 chain), and IgA (α-chain) in the sera of AID\(^{-/-}\) μs\(^{-/-}\) mice (Fig. 1B). In the sera of AID\(^{-/-}\) μs\(^{-/-}\) and μs\(^{-/-}\) mice, very low levels of IgM (μ chain) were detected by ELISA (Fig. 1B). The ELISA does not distinguish between intact IgM and shed IgM-derived fragments that retain immunoreactivity. The absence of intact μ-chain by immunoblotting, even for overdeveloped blots, suggests that the levels of intact μ-chain are negligible, if present at all. Because L chains are synthesized in molar excess over H chains, even B cells that produce only membrane IgM will still release some L chain. This accounts for the low, but detectable level of κ and λ chain in AID\(^{-/-}\) μs\(^{-/-}\) mice as measured by ELISA (data not shown).

Flow cytometric analyses demonstrated the presence of membrane IgM (Fig. 2C) and the absence of membrane IgG (data not shown) in the B cells freshly harvested from AID\(^{-/-}\) μs\(^{-/-}\) mice.
In this level of analysis, early development of B cells in AID mice appear to be affecting early B cell development. We conclude that at and percentage of B cells between AID mice, whereas the total number of splenocytes was smaller in AID mice, which might reflect the absence of follicular B cells on the basis of CD21 and CD23 expression. The percentage of follicular B cells (Fr. C) was higher in AID mice than in wild-type mice (Table II, Fig. 2). The percentage of MZ (B220−/H11001) B cells was much higher in AID mice (Fig. 2). The percentage of MZ (B220−/H11001) B cells was much higher in AID mice (Fig. 2).

The percentage of newly generated immature B cells (Fr. E, B220+/Hi/S7/IgM+) was higher in AID−/− μs−/− and μs−/− mice than in wild-type mice (Fig. 2A). The percentage of recirculating mature B cells (Fr. F, B220+/Hi/Hi/S7/IgM+) was lower in μs−/− but not in AID−/− or AID−/− μs−/− mice, which might reflect the decreased percentage of B cells in the spleen of μs−/− mice (Table II, differences that are significant at p < 0.05 are indicated in bold).

Thus, we observed only minor changes in the B cell compartment in the bone marrow of AID−/− μs−/− mice, and these changes did not appear to be affecting early B cell development. We conclude that at this level of analysis, early development of B cells in AID−/− μs−/− is close to normal.

**Splenic B cell and T cell compartment in AID−/− μs−/− mice**

There was no significant difference in total number of splenocytes and percentage of B cells between AID−/− μs−/− and wild-type mice, whereas the total number of splenocytes was smaller in AID−/− mice, and the percentage of B cells was lower in μs−/− mice than in wild type (Table II). The ratio of surface κ+ B cells/λ+ B cells was somewhat lower in AID−/− μs−/− and μs−/− mice than in wild type (Table II), suggesting that the absence of μs may affect the type of B cell (κ+ or λ+) that survive.

The percentages of CD4+ T cells were equivalent for the four groups. We observed decreases in the percentage of CD8+ T cells in AID−/− μs−/− and AID−/− μs−/− mice compared with wild type, and an increase in the percentage of CD8+ T cells in μs−/− mice. Although statistically significant, these differences were not radical and therefore considered unlikely to have a major effect on development and homeostasis of the T cell compartment.

**Marginal zone (MZ) and follicular B cells in the spleen of AID−/− μs−/− mice**

Mature B cells in the spleen can be classified into MZ and follicular B cells on the basis of CD21 and CD23 expression. The percentage of MZ (B220+/AA4.1−CD21hiCD23−) B cells was 4- to 5-fold higher in AID−/− μs−/− mice compared with wild-type mice (Table II, Fig. 2B). We also examined the expression of CD1d, which is more highly expressed on MZ than on follicular B cells (17, 19, 20). The percentage of CD1dhiCD23− MZ B cells in AID−/− μs−/− showed a 4-fold increase compared with wild-type mice (data not shown). The percentages of follicular (B220+/AA4.1−CD21hiCD23−) B cells in AID−/− μs−/− did not differ compared with wild-type mice. MZ B cells express high levels of surface IgM and low levels of surface IgD, while follicular B cells express low levels of surface IgM and high levels of surface IgD (21). The percentage of AA4.1− IgMhiIgDlow mature B cells was much higher in AID−/− μs−/− than in wild-type mice (Fig. 2C), again indicative of an increased percentage of MZ B cells in AID−/− μs−/− mice. Because PCs are very few in the spleen of AID−/− μs−/− mice, as will be described below, the

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**FIGURE 2.** B cell development and differentiation in AID−/− μs−/− mice by flow cytometric analyses. Representative results from at least four separate experiments are shown. A, Bone marrow cells from each group of mice were stained with B220, CD43 (S7), and IgM. Dot plots are gated on B220+/H11001 B cells. Fraction (Fr.) D (B220+/Hi/S7/IgM+) corresponds to newly generated immature B cells, and Fr. E (B220+/Hi/S7/IgM+) corresponds to recirculating mature B cells. The numbers indicate the percentages of gated cells in total bone marrow cells. B and C, Splenocytes from four groups of mice were stained with B220, AA4.1, CD21, and CD23 (B) or with B220, AA4.1, IgM, and IgD (C). Dot plots are gated on B220+/AA4.1− mature B cells. CD21hiCD23− cells were defined as MZ B cells, and CD21hiCD23− cells were defined as follicular (FO) B cells (B). Numbers indicate the percentages of gated cells in total splenocytes. D, PNAhiFashi GC B cells were analyzed in the splenocytes of four groups of mice 2 wk after immunization with NP-CG. Dot plots are gated on B220+ B cells. Numbers indicate the percentages of gated cells in total splenocytes.
increase in MZ B cell number could be related to the reduction in number of PCs. If correct, this could be evidence for a role for PCs in limiting the number of MZ B cells.

**Germinal center reaction in AID−/− μs−/− mice**

The total number and percentage of PNA^hi^Fas^hi^ GC B cells was significantly higher in the spleen of AID−/− μs−/− mice than in wild-type mice, both before and after immunization (Table II, Fig. 2D). This observation fits the phenotype of AID−/− mice (11).

### Table I. Number and frequency of cells in different organs

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<th>Wild type</th>
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<th>μs−/−</th>
<th>AID−/− μs−/−</th>
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<td>(n = 8–14)</td>
<td>(n = 8–12)</td>
<td>(n = 8–14)</td>
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<td><strong>Bone marrow</strong></td>
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<td>Total cell number</td>
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<td>2.7 ± 0.6</td>
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<td>% Fr.A: HSA− BP1</td>
<td>2.6 ± 1.2</td>
<td>2.7 ± 1.2</td>
<td>3.3 ± 1.6</td>
<td>2.4 ± 0.9</td>
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<tr>
<td>% Fr.H: HSA^+ BP1−</td>
<td>2.8 ± 1.6</td>
<td>2.9 ± 1.6</td>
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<td>3.5 ± 1.8</td>
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<td>% Fr.C: HSA^+ BP1^+</td>
<td>0.8 ± 0.5</td>
<td>1.0 ± 0.5</td>
<td>0.9 ± 0.5</td>
<td>1.0 ± 0.6</td>
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<tr>
<td>% Fr.D: B220^+ IgM− IgM^+</td>
<td>15.1 ± 4.2</td>
<td>12.3 ± 6.1</td>
<td>17.5 ± 5.1</td>
<td>13.9 ± 4.5</td>
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<td>% Fr.E: B220^+ IgM− IgM^+</td>
<td>6.6 ± 2.1</td>
<td>7.0 ± 3.0</td>
<td>11.5 ± 2.1</td>
<td>10.2 ± 2.4</td>
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<td>% Plasma cells</td>
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<td>1.3 ± 0.4</td>
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<td>% Plasma cells (TD-IM)^f−</td>
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<td>1.0 ± 0.1</td>
<td>0.5 ± 0.3</td>
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<td>Total plasma cells</td>
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<td>Total cell number</td>
<td>5.5 ± 2.7</td>
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<td>21.5 ± 6.4</td>
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<td>% Gerinal center B cells</td>
<td>6.7 ± 0.6</td>
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<td>19.9 ± 7.6</td>
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<td>Gerinal center B cells (×10^5)</td>
<td>3.9 ± 3.3</td>
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<td>% B-1 cells (B220^+ Mac-1^−)</td>
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<td>% B-2 cells (B220^+ Mac-1^+)</td>
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<td>% B-2 cell number (×10^5)</td>
<td>7.0 ± 4.5</td>
<td>11.5 ± 7.7</td>
<td>12.8 ± 6.2</td>
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### Table II. Numbers and frequencies of splenic B and T cell populations

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<td>(n = 8–14)</td>
<td>(n = 8–12)</td>
<td>(n = 8–14)</td>
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<tr>
<td><strong>Spleen</strong></td>
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<td></td>
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<td></td>
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<tr>
<td>Total cell number</td>
<td>8.7 ± 1.7</td>
<td>5.7 ± 1.9</td>
<td>7.9 ± 1.9</td>
<td>9.9 ± 4.1</td>
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<tr>
<td>Total B220^+ B cells (×10^5)</td>
<td>5.2 ± 1.4</td>
<td>3.2 ± 1.1</td>
<td>3.6 ± 0.9</td>
<td>6.5 ± 2.9</td>
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<tr>
<td>% B220^+ B cells</td>
<td>58.1 ± 7.8</td>
<td>597.7 ± 75</td>
<td>447.4 ± 47</td>
<td>59.4 ± 9.8</td>
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<tr>
<td>κ/λ ratio (κ^−^ B cells/κ^+^ B cells)</td>
<td>18.3 ± 2.3</td>
<td>19.4 ± 1.5</td>
<td>15.9 ± 1.8</td>
<td>13.2 ± 2.1</td>
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<tr>
<td>% CD4 T cells</td>
<td>23.7 ± 5.7</td>
<td>24.8 ± 5.1</td>
<td>29.3 ± 5.9</td>
<td>27.4 ± 7.7</td>
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<tr>
<td>% CD8 T cells</td>
<td>14.0 ± 2.2</td>
<td>10.8 ± 1.7</td>
<td>17.4 ± 2.3</td>
<td>10.1 ± 2.5</td>
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<td>% Marginal zone B cells</td>
<td>2.1 ± 1.2</td>
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<td>10.0 ± 3.0</td>
</tr>
<tr>
<td>% Follicular B cells</td>
<td>26.2 ± 6.9</td>
<td>29.4 ± 7.3</td>
<td>13.1 ± 4.5</td>
<td>22.7 ± 7.4</td>
</tr>
<tr>
<td>% AA4.1-IgM^hi^IgD^hi^ B cells</td>
<td>7.3 ± 2.3</td>
<td>10.2 ± 2.5</td>
<td>12.3 ± 3.8</td>
<td>18.4 ± 5.2</td>
</tr>
<tr>
<td>% AA4.1-IgM^hi^IgD^hi^ B cells</td>
<td>7.5 ± 2.5</td>
<td>7.6 ± 3.2</td>
<td>5.0 ± 0.1</td>
<td>7.4 ± 0.9</td>
</tr>
<tr>
<td>% GC B cells</td>
<td>0.5 ± 0.3</td>
<td>2.2 ± 0.6</td>
<td>0.5 ± 0.4</td>
<td>1.9 ± 0.5</td>
</tr>
<tr>
<td>% GC B cells/total SPC (TD-IM)^f−</td>
<td>1.9 ± 0.9</td>
<td>5.7 ± 0.2</td>
<td>0.9 ± 0.2</td>
<td>3.8 ± 1.4</td>
</tr>
<tr>
<td>Total plasmablasts (×10^5) (TD-IM)^f−</td>
<td>2.5 ± 1.7</td>
<td>2.7 ± 0.7</td>
<td>4.7 ± 1.2</td>
<td>6.0 ± 2.4</td>
</tr>
<tr>
<td>% Plasma cells</td>
<td>1.4 ± 0.8</td>
<td>1.4 ± 0.4</td>
<td>1.3 ± 0.8</td>
<td>1.0 ± 0.5</td>
</tr>
<tr>
<td>% Plasma cells (TD-IM)^f−</td>
<td>1.6 ± 0.9</td>
<td>1.8 ± 0.5</td>
<td>1.4 ± 0.7</td>
<td>1.1 ± 0.6</td>
</tr>
<tr>
<td>% Plasma cells</td>
<td>3.4 ± 2.6</td>
<td>3.9 ± 3.0</td>
<td>3.0 ± 3.4</td>
<td>2.8 ± 2.3</td>
</tr>
<tr>
<td>Total plasma cells</td>
<td>9.1 ± 5.8</td>
<td>10.1 ± 9.4</td>
<td>6.9 ± 5.1</td>
<td>3.5 ± 4.7</td>
</tr>
<tr>
<td>% Plasma cells (TD-IM)^f−</td>
<td>9.8 ± 6.2</td>
<td>12.4 ± 12.2</td>
<td>6.5 ± 4.9</td>
<td>3.7 ± 3.3</td>
</tr>
</tbody>
</table>

f Data are presented by per million (%) for the frequency of plasma cells.

* Analyses were performed after immunization with T-dependent Ags (TD-IM).
mice (Fig. 3C). Thus, the features of enhanced GC reaction in AID<sup>−/−</sup>μs<sup>−/−</sup> mice were fully consistent with those seen in AID<sup>−/−</sup> mice.

**B-1B cell population in AID<sup>−/−</sup>μs<sup>−/−</sup> mice**

Because μ<sup>−/−</sup> mice show an increase in the number of B-1 cells (10), we also examined the population of B-1a cells in the spleen and peritoneal cavity in each group of mice. The percentages of splenic CD5<sup>+</sup>IgM<sup>+</sup> B-1a B cells were much higher in both AID<sup>−/−</sup>μ<sup>−/−</sup> and μ<sup>−/−</sup> mice than in wild-type mice (Table II). In peritoneal lavage cells, the total numbers of both B220<sup>+</sup>Mac-1<sup>−</sup>CD5<sup>+</sup>B-1a B cells and B220<sup>+</sup>Mac-1<sup>−</sup>CD5<sup>−</sup>B-1b B cells were much higher in both AID<sup>−/−</sup>μ<sup>−/−</sup> mice and μ<sup>−/−</sup> mice than in wild-type mice (Table I). The total number of B220<sup>+</sup>Mac-1<sup>−</sup>B-2 B cells was also higher in AID<sup>−/−</sup>μ<sup>−/−</sup> mice than in wild-type mice, although the percentage of B-2 B cells was similar (Table I). Thus, the feature of increased B-1 B cells in the spleen and peritoneal cavity in AID<sup>−/−</sup>μ<sup>−/−</sup> mice was consistent with that observed for μ<sup>−/−</sup> mice.

We conclude that the changes in B cell differentiation in naive AID<sup>−/−</sup>μ<sup>−/−</sup> mice, as assessed by cytfluorometry, are modest, and the perturbations in the B cell compartment have minimal effects on the CD4 and CD8 T cell populations. Therefore Ag-independent B cell development/differentiation and T cell homeostasis are close to normal in AID<sup>−/−</sup>μ<sup>−/−</sup> mice.

**Morphological features of the spleens and Peyer’s patches of AID<sup>−/−</sup>μ<sup>−/−</sup> mice**

We conducted histological examinations of spleen, thymus, peripheral lymph nodes, and GALT. The spleens of AID<sup>−/−</sup>μ<sup>−/−</sup> mice were enlarged compared with the other three groups by macroscopic observation (data not shown). H&E-stained sections of the spleens of AID<sup>−/−</sup>μ<sup>−/−</sup> mice revealed less-structured white pulp in comparison with the other groups of mice (Fig. 3A). Peyer’s patches of AID<sup>−/−</sup>μ<sup>−/−</sup> and AID<sup>−/−</sup> mice were obviously enlarged compared with wild-type mice, by both macroscopic inspection and microscopic examination (Fig. 3B). Mesenteric lymph nodes of AID<sup>−/−</sup>μ<sup>−/−</sup> and AID<sup>−/−</sup> mice were also enlarged compared with wild-type mice (data not shown). Because increased GC formation was evident in both AID<sup>−/−</sup>μ<sup>−/−</sup> and AID<sup>−/−</sup> mice by flow cytometry (Tables I and II, Fig. 2D) and immunofluorescence microscopy (Fig. 3C), the enlargement of GALT in these mice may be due to the enhanced GC reactions in response to intestinal microbial Ags. There were no obvious histological differences in H&E-stained sections of thymus and peripheral lymph nodes among the four groups of mice (data not shown).

The enlargement of GALT in AID<sup>−/−</sup>μ<sup>−/−</sup> mice as well as in AID<sup>−/−</sup> mice can be caused by hyperreaction of GC. However, the disorganization of white pulp that characterizes the spleen of the AID<sup>−/−</sup>μ<sup>−/−</sup> mice, is likely caused by the unique features of this strain, i.e., impaired secretion of any class of Ig, or impaired PC maintenance, as will be described below.

**Decreased PCs in the spleen and bone marrow of AID<sup>−/−</sup>μ<sup>−/−</sup> mice**

To determine whether PCs are maintained normally in AID<sup>−/−</sup>μ<sup>−/−</sup> mice, we examined the population of B220<sup>−/−</sup>CD138<sup>+</sup> PCs in the spleen and bone marrow. To focus on terminally differentiated PCs, we excluded from our analysis the B220<sup>+</sup>CD138<sup>+</sup> population, which includes B220<sup>int-low</sup>CD138<sup>+</sup> plasmablasts (22–24). There was no significant difference in the percentage of B220<sup>int-low</sup>CD138<sup>+</sup> plasmablasts among the four groups of mice (Table II). Following immunization with OVA (Fig. 4A) and NP-CG (Fig. 4B), both the percentage and total number of B220<sup>−/−</sup>CD138<sup>+</sup> PCs were significantly lower in the spleen of AID<sup>−/−</sup>μ<sup>−/−</sup> mice compared with wild-type mice (Table II, Fig. 4). In the bone marrow of AID<sup>−/−</sup>μ<sup>−/−</sup> mice, the percentages of B220<sup>−/−</sup>CD138<sup>+</sup> PCs were significantly lower, both before and after immunization (Table I, Fig. 4A). The total number of PCs in the bone marrow was also much lower in AID<sup>−/−</sup>μ<sup>−/−</sup> mice than in wild-type mice after immunization.

Although PCs are generally considered to express little or no surface Ig (2, 25), there are some recent reports that PCs can express Ig on their surface (26–28). We could divide B220<sup>−/−</sup>CD138<sup>+</sup> PCs of wild type and each of the single knockout mice into two groups, according to a clear distinction in surface IgM expression.
XBP-1 splicing is not inhibited in AID<sup>−/−</sup> μ<sup>−/−</sup> B cells upon mitogenic stimulation in vitro

Splicing of XBP-1 is imperative for differentiation of B cells into PCs (4, 6). Because PCs were not detected in AID<sup>−/−</sup> μ<sup>−/−</sup> mice in vivo, a plausible explanation might be the failure to evoke splicing of XBP-1. Such failure might derive from the absence of secretory Ig and misfolded byproducts, usually held responsible for induction of an unfolded protein response (UPR), which starts with activation of IRE-1 and is followed by IRE-1 degradation and

FIGURE 4. PCs are nearly absent in AID<sup>−/−</sup> μ<sup>−/−</sup> mice. A, After repeated immunization with OVA, bone marrow cells, and splenocytes from each group of mice were stained with B220, CD5, CD138, and IgM for flow cytometric analyses. Dot plots are gated on B220<sup>−/−</sup> cells. Numbers indicate the percentages of gated cells in total bone marrow cells or in total splenocytes. The percentage of B220<sup>−/−</sup>CD138<sup>−/−</sup> PCs was markedly decreased in AID<sup>−/−</sup> μ<sup>−/−</sup> mice. In the analyses of wild-type, AID<sup>−/−</sup>, and μ<sup>−/−</sup> mice, there was a clear distinction between IgM<sup>−/−</sup> and IgM<sup>+</sup> PCs. B, Nine days after immunization with NP-CG, splenocytes from each group of mice were stained with B220, CD138, IgM, and IgG1. Dot plots are gated on B220<sup>−/−</sup> cells, and numbers indicate the percentages of gated cells in total splenocytes. Pink dots represent IgM<sup>−/−</sup> B220<sup>−/−</sup>CD138<sup>−/−</sup> PCs. Majority of IgM<sup>−/−</sup> B220<sup>−/−</sup>CD138<sup>−/−</sup> PCs express detectable levels of IgG1, whereas most of IgM<sup>−/−</sup> B220<sup>−/−</sup>CD138<sup>−/−</sup> PCs are negative for IgG1. Representative results from at least three separate experiments are shown.

FIGURE 5. A, XBP-1 splicing in splenic B cells from AID<sup>−/−</sup> μ<sup>−/−</sup> mice under in vitro stimulation. B cells were purified from the spleens of each group of mice, and stimulated with CpG and LPS. RNA was extracted at the indicated times, and splicing of XBP-1 mRNA was analyzed by RT-PCR. Spliced XBP-1 mRNA was observed for AID<sup>−/−</sup> μ<sup>−/−</sup> mice similar to wild type. B, semiquantitative RT-PCR analysis of cDNA prepared from RNA of CpG/LPS-treated B cells. Three-fold dilution series of cDNA were used as input material for the PCR primers specific for μm or GAPDH as reference. There was no significant difference in the levels of μm mRNA in wild-type and AID<sup>−/−</sup> μ<sup>−/−</sup> B cells.
splicing of XBP-1 mRNA. To address this possibility, we examined by RT-PCR the splicing of XBP-1 mRNA in response to mitogenic stimulation of isolated splenic B cells with CpG/LPS. Similar to wild-type controls, spliced XBP-1 mRNA was readily seen for AID−/−μS−/− mice at day 1, and was continuously generated until day 3 (Fig. 5A). Spliced XBP-1 mRNA was also observed for AID−/−μS−/− mice from days to day 3 (data not shown). The absence of secretory Ig does not prevent the activation of IRE1 and splicing of XBP-1 mRNA in the course of polyclonal B cell activation, nor do we observe an excessive level of

FIGURE 6. In vitro stimulation of splenic B cells from AID−/−μS−/− mice. B cells were purified from the spleens of each group of mice and stimulated with CpG followed by stimulation with LPS after 24 h. For the proliferation assay (D), cells were stained with CFSE before the stimulation. Dead cells were identified by TOPRO-3 staining. A and B, CD138+ plasmablasts were detectable in the culture of all four groups of mice, however, the number and percentage of live plasmablasts (TOPRO3− CD138+ cells) were lower in the culture of AID−/−μS−/− and μS−/− mice compared with wild type (⁎, p < 0.05 compared with wild). Data represent mean ± SD from three separate experiments. C, Annexin V/TOPRO3 costaining of stimulated B cells on day 2. Dot plots are gated on CD138+ plasmablasts. Annexin V+, TOPRO3− cells are early apoptotic cells, and Annexin V+, TOPRO3+ cells are dead cells that include both late apoptotic cells and necrotic cells. There was no significant difference in the ratio of early apoptotic plasmablasts to dead plasmablasts between AID−/−μS−/− and wild type. D, Flow cytometry on day 3 showed B cell proliferation in all four groups, although distribution of CFSE fluorescence was heterodisperse in the analyses for AID−/−μS−/− and μS−/− mice. Histograms (left and middle panels) are shown for TOPRO3− live cells, and dot plots (right panels) are shown for all the cells in each culture.
spliced XBP-1 mRNA in AID−/−μs−/− mice compared with wild type.

**μμ mRNA is not up-regulated in AID−/−μs−/− B cells upon mitogenic stimulation in vitro**

We considered a possibility that overproduction of membrane IgM due to the increase in membrane μ (μμ) mRNA, which would normally be channeled into μs mRNA in the course of PC differentiation, may exert toxicity and result in failure of PC maintenance in AID−/−μs−/− mice. To address this possibility, we compared the levels of μμ mRNA by semiquantitative RT-PCR in CpG/LPS treated B cells. We found similar levels of μμ mRNA in wild-type and AID−/−μs−/− mice on days 2 and 3 (Fig. 5B). Therefore, we consider the presence of toxic level of μμ unlikely. However, we cannot formally exclude the possibility that AID−/−μs−/− B cells/plasmablasts that express elevated μμ transcript levels rapidly die and are lost to analysis, and therefore μμ mRNA levels were similar among the groups. This explanation would require selective death of those cells that express enhanced levels of μμ mRNA, such that the residual population of survivors would express the identical mRNA levels as seen in wild type, not withstanding the fact that these survivors are still AID−/−μs−/−.

Alternatively, because the μs−/− mouse strain retains a neomycin phosphotransferase (neo)-gene insertion, the failure of generating PCs in AID−/−μs−/− mice might be attributed to increased synthesis of the Neo cassette in the course of PC differentiation. We consider this possibility unlikely, because normal differentiation into GFP+ PCs is seen in IgH<sup>CF136</sup> mice, in which GFP is inserted into IgH locus with a Neo cassette (29).

**Differentiation and survival of AID−/−μs−/− plasmablasts in vitro**

Many aspects of B cell differentiation into Ig-secreting plasmablasts can be recapitulated in vitro by exposing splenic B cells to polyclonal B cell activators such as CpG and LPS. We purified B cells from spleens of each group of mice and stimulated them with CpG and LPS to induce differentiation of B cells into B220<sup>+</sup> CD138<sup>+</sup> plasmablasts. We plated identical numbers of B cells from each group, and performed flow cytometry every 24 h. Dead cells were identified by labeling with TOPRO-3.

The formation of CD138<sup>+</sup> plasmablasts was observed for all four groups of mice. We assessed TOPRO3−CD138<sup>+</sup> cells, i.e., live plasmablasts, by the following criteria: 1) number of live CD138<sup>+</sup> plasmablasts relative to total cell number (Fig. 6A), 2) total number of live CD138<sup>+</sup> plasmablasts in each culture (Fig. 6B), 3) number of live CD138<sup>+</sup> plasmablasts relative to total (live and dead) CD138<sup>+</sup> plasmablasts (data not shown). By all of these criteria, the numbers of live CD138<sup>+</sup> plasmablasts were lower for the AID−/−μs−/− and μs−/− mice compared with wild type. For the 48–72 h interval, we observed significantly lower numbers of live plasmablasts for the AID−/−μs−/− and μs−/− mice compared with wild type (Fig. 6B). Our results indicate that B cells from AID−/−μs−/− mice can differentiate in vitro at least to the stage of CD138<sup>+</sup> plasmablasts; but survival of these cells appears to be compromised.

To see whether impaired survival of AID−/−μs−/− plasmablasts is due to apoptotic censoring, we performed annexin V/TOPRO3 costaining using cultured B cells stimulated by CpG/LPS. Although the percentage of total Annexin V<sup>+</sup> plasmablasts was much higher in AID−/−μs−/− culture with wild type, the proportion of early apoptotic plasmablasts (annexin V<sup>+</sup>, TOPRO3<sup>+</sup>) relative to total dead plasmablasts (annexin V<sup>+</sup>, TOPRO3<sup>+</sup>) was similar between AID−/−μs−/− and wild type (Fig. 6C). Therefore, we cannot conclude that impaired survival of AID−/−μs−/− plasmablasts is simply due to apoptosis.

Because only IgM<sup>+</sup> switched PCs were detected in the spleen of μs−/− mice in vivo (Fig. 4), we assumed that only PCs that undergo CSR and acquire the capacity to secrete isotype switched Igs are maintained in μs−/− mice. Therefore, to examine whether signals that trigger in vitro CSR improve the survival of μs−/− plasmablasts, we induced class switching by inclusion of anti-CD40 and IL4. We observed 25–30% of IgG<sup>+</sup> cells in cultures from wild-type and μs−/− mice (data not shown). For the 72–96 h interval, the ratio of CD138<sup>+</sup> live plasmablasts tended to be higher in the culture from μs−/− mice compared with AID−/−μs−/− mice (data not shown), although this difference was not statistically significant.

To determine whether AID−/−μs−/− B cells proliferate normally upon stimulation in vitro, we labeled purified B cells with CFSE and then incubated them with CpG and LPS. Flow cytometric analysis on day3 revealed B cell proliferation in all four groups (Fig. 6D). The distribution of CFSE fluorescence was heterodisperse with less discrete peaks for μs−/− mice compared with AID−/−μs−/− mice (Fig. 6G). Therefore, we cannot conclude that impaired survival of AID−/−μs−/− plasmablasts is simply due to apoptosis.

**Absence of PCs in the spleen of AID−/−μs−/− mice by immunohistochemistry after immunization with TD-Ag**

After four sequential immunizations with OVA, we performed immunohistochemical analysis on spleens from each group of mice. We noted the presence of B220 CD138<sup>+</sup> PCs in wild-type, AID−/− and μs−/− mice, but not in AID−/−μs−/− mice (Fig. 7). Immunohistochemistry also confirmed the presence of B220<sup>+</sup> CD138<sup>+</sup> plasmablasts in the spleen of AID−/−μs−/− mice.

This result is consistent with, and extends the in vitro data, and strongly suggests that in AID−/−μs−/− mice plasmablasts can arise, but PCs, if they arise, do not persist.
FIGURE 8. Schematic summary of B cell and PC differentiation in AID−/−μ−/− agammaglobulinemic mice. Switched PCs are nearly absent because of the defects in SHM and CSR. Enhanced GC reaction may be caused by impaired feedback regulation due to the lack of SHM and CSR. B cells develop and differentiate into unswitched PC to some extent. However, unswitched PCs are not maintained normally; failure in production of secretory IgM (sIgM) may cause early death of unswitched PCs. Accumulation of MZ B cells may be caused by impaired feedback regulation due to the lack of unswitched PCs and/or sIgM. FoB, follicular B cell; PB, plasmablasts; κ, κ-chain; mIgM, membrane IgM.

Discussion
We observed a near complete absence of PCs in the AID−/−μ−/− agammaglobulinemic mice. Some of the other features in B cell differentiation in AID−/−μ−/− mice were compatible with the observations in either of single knockout mice (10, 11). An increase of the B-1 B cell population was reported in μ−/− mice, and a physiologic role of secretory IgM was inferred as a component of a feedback loop that contributes to B-1 cell differentiation and/or maintenance (10). AID−/−μ−/− mice show an enhanced GC reaction, observed also in AID−/− mice. Upon encounter with Ag and Th cells, some follicular B cells migrate into the primary follicle and expand within the GC, where B cells undergo proliferation accompanied by SHM and CSR (1, 30). The inhibition of this proliferation may be provoked by SHM or CSR, so that activated B cells are given the opportunity to differentiate (11). Because IgM+ PCs were few in both AID−/− and AID−/−μ−/− mice, IgM+ switched splenic PCs that should have undergone SHM and CSR may also be required under normal circumstances for feedback regulation of B cell proliferation in the GC (Fig. 8). The increase in numbers of MZ B cells in AID−/−μ−/− mice can also be explained by impaired negative feedback regulation due to a lack of IgM− unswitched splenic PCs (Fig. 8). The disorganized structure of white and red pulp in H&E staining of AID−/−μ−/− spleen may be due to the accumulation of MZ B cells. These changes in B the cell compartment are not radical, and therefore we conclude that in AID−/−μ−/− mice, B cell development and differentiation are close to normal, at least before the terminal stage of PC formation.

Although PC differentiation is characterized by complex pathways and heterogeneity in marker expression (31–33), we could classify B220+ CD138+ PCs into two groups, according to surface IgM expression. In AID−/− mice, the absence of IgM+ switched PCs is consistent with a lack of SHM and CSR. IgM− unswitched PCs were observed not only in the spleen but also in the bone marrow of AID−/− mice, and of wild-type mice at lower frequency. Class-switched PCs that find survival niches become long-lived and migrate into bone marrow, which is their preferred location (1, 34). The IgM+ PCs detected in the bone marrow of AID−/− mice cannot have undergone CSR or SHM (i.e., unswitched and “short-lived” in general), and therefore certain populations of “short-lived” unswitched PCs may also recirculate through the bone marrow, although the lifespan of these cells is not clear.

In the spleen and bone marrow of μ−/− mice, we observed only IgM− switched PCs, whereas both types of PCs were very few in AID−/−μ−/− mice. The absence of switched PCs in AID−/−μ−/− mice, as well as in AID−/− mice, must be due to the defect in CSR. However, in AID−/−μ−/− and μ−/− mice, it was unclear whether B cells fail to differentiate into unswitched PCs, or whether unswitched PCs survive less well than in wild-type mice. To address this question, we performed in vitro stimulation using CpG and LPS, agents that rapidly induce differentiation of MZ B cells into plasmablasts, the precursors of unswitched PCs (17, 35). Because we detected CD138+ cells in cultures from both AID−/−μ−/− and μ−/− mice, MZ B cells in these strains can differentiate at least to the stage CD138+ plasmablasts by T-independent Ag stimulation in vitro. The detection of B220+ CD138+ cells in both AID−/−μ−/− and μ−/− mice after TD-Ag immunization in vivo also demonstrates the potential of follicular B cells to differentiate into plasmablasts. The decrease in number of “live” CD138+ cells in AID−/−μ−/− and μ−/− cultures in the course of stimulation with CpG/LPS indicates impaired survival of plasmablasts/PCs. The tendency toward improved survival of μ−/−CD138+ cells by induction in vitro of CSR suggests that in μ−/− mice, death of plasmablasts/PCs can in part be rescued by CSR.

The shortened survival of unswitched PCs in AID−/−μ−/− and μ−/− mice is likely attributable to the shared feature of these two strains, i.e., abolished secretion of IgM. We might consider the possibility that plasmablasts/PCs of AID−/−μ−/− and μ−/− mice are unable to accommodate excess L chain, not all of which is necessarily secreted if it failed to assemble with μ chain. Plasmablasts/PCs might therefore die as they ramp up L chain synthesis. Under this assumption, plasmablasts/PCs in μ−/− mice can survive only by CSR, as L chain can be secreted with γ-, α-, or κ-chains. Although we observed κ-chain secretion in AID−/−μ−/− cells as well as in wild type upon in vitro stimulation (data not shown), our qualitative analysis cannot directly exclude this possibility.

XBP-1 is required for PC differentiation (6), and is a key element of the UPR, activated during ER stress imposed by treatment with drugs such as tunicamycin, thapsigargin, or with DTT (8). The UPR is required a properly functioning ER (36, 37). Iwakoshi et al. (4, 38) established the relationship between PC differentiation, the UPR, and XBP-1. Treatment with tunicamycin, thapsigargin, and DTT usually result in robust splicing of XBP-1 mRNA. However, the physiological factors that trigger the UPR in the course of PC differentiation are not at all well defined. Mature naïve B cells synthesize equal amounts of μm and μs (39). In the course of PC differentiation, B cells shift from synthesis of the μm to predominantly μs (3). The steep increase in μs synthesis, perhaps to a level that exceeds the folding capacity of...
the ER of the developing PCs, might indeed entail ER stress, as evident from splicing of XBP-1 mRNA. Treatment of B cells with low concentrations of cycloheximide abrogates XBP-1 splicing, suggesting that at least de novo protein synthesis is required for induction of the UPR (4). Additionally, Iwakoshi and colleagues (7) demonstrated that Cre-mediated removal of a floxed μH chain locus dramatically reduces the levels of XBP-1s generated in LPS-treated B cells. Finally, we have shown that XBP-1 is required to maintain high levels of synthesis of μs chains, even though μ mRNA levels hardly differ for wild-type and XBP-1−/− B cells. Taken together, these data suggest a feed-forward loop that connects the synthesis of μs with the generation of XBP-1s. Against expectations, we observed that in the absence of μs, XBP-1 mRNA is spliced in response to CpG/LPS treatment. This suggests that factors other than induction of Ig synthesis contribute to the activation of IRE1 upon stimulation. Indeed, careful analysis of the kinetics of μs chain synthesis and the appearance of XBP-1s in CH12 B cells subjected to LPS treatment showed that splicing of XBP-1 preceded the up-regulation of μ chain synthesis (40). We therefore propose that the initial activation of IRE1 does not necessarily rely on de novo synthesis of μs chains.

The intrinsic survival of B cells ex vivo is limited to 3–4 days. This confounds a quantitative assessment of the contribution of individual factors to the viability of B cells in culture. Nonetheless, we observed reduced survival of AID−/− μs−/− and μs−/− in comparison to wild-type or AID−/− B cells. This indicates that synthesis of μs protects B cells from cell death. The μs glycoprotein contains 5 N-linked glycosylation sites and many disulfide bonds. In the course of its folding, μs interacts directly and indirectly with a multitude of chaperones, including Bip, PDI, Ero1, ERp44 and membrane IgM. Their mouse lacks the JH segments required for genic mouse that lacks circulating Abs but with B cells that retain the absence of CSR (AID

VDJ recombination and B cell development, and monoclonal B cells facilitate further studies of the roles of serum Igs in immune re-

References

1. Shapiro-Shelef, M., and K. Calame. 2005. Regulation of plasma-cell develop-


3. van Anken, E., E. P. Romijn, C. Maggioni, A. Mezghrani, R. Sitia, I. Braakman, and L. M. Hendershot. 2003. Pro-apoptotic function of the UPR is dispensable for degrada-


6. Zhang, K., and R. J. Kaufman. 2006. Protein folding in the endoplasmic reticu-


7. Tirosh, B., N. N. Iwakoshi, L. H. Glimer, and H. L. Ploegh. 2005. XBP-1 specifically promotes IgM synthesis and secretion, but is dispensable for degra-

dation of glycoproteins in primary B cells.


Disclosures

The authors have no financial conflict of interest.

Acknowledgments

We thank Amy McQuay for her excellent technical assistance. We also thank Dr. Klaus Rajewsky for discussion of the results.
frequency of surface IgA-positive plasma cells in the intestinal lamina propria and decreased IgA excretion in hyper IgA (HIGA) mice, a murine model of IgA nephropathy with hyperserum IgA. *J. Immunol.* 165: 1387–1394.


