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T-Independent Activation-Induced Cytidine Deaminase Expression, Class-Switch Recombination, and Antibody Production by Immature/Transitional 1 B Cells

Yoshihiro Ueda, Dongmei Liao, Kaiyong Yang, Anjali Patel, and Garnett Kelsoe

Inflammation elicits a splenic lymphopoiesis of unknown physiologic significance but one that juxtaposes developing B cells and exogenous Ag. We show that immature and transitional 1 (immature/T1) B cells constitutively express activation-induced cytidine deaminase and B lymphocyte-induced maturation protein 1 in amounts that support accelerated plasmacytic differentiation and limited class-switch recombination. In vivo, activation of immature/T1 B cells by TLR ligands or bacterial vaccine rapidly induces T1 cells to divide, proliferate, and secrete IgM, IgG, or IgA Ab; in vitro, proliferation and differentiation are substantially enhanced by B cell-activating factor. We propose that inflammation-induced extramedullary lymphopoiesis represents a specialized mechanism for innate Ab responses to microbial pathogens. The Journal of Immunology, 2007, 178: 3593–3601.

Inflammation mobilizes developing lymphocytes from the bone marrow (BM) by suppressing CXCCL12 and stem cell factor levels and initiates extramedullary lymphopoiesis in the spleen (1). Concurrently, immature (im) granulocyte numbers increase in the BM and expand into developmental niches vacated by emigrant lymphocytes. We have proposed that this redirection of leukopoiesis represents an innate immune response to microbial pathogens (1).

If expanded BM granulopoiesis is an adaptive response, for what purpose and benefit are lymphocyte progenitors mobilized to the periphery? Unlike granulocytes, mature B cells are capable of mitotic expansion and have half-lives measured in weeks or months, rather than hours or days. Thus, mobilization of B cell progenitors and the establishment of extramedullary lymphopoiesis could be inconsequential. On the other hand, the signals that retain developing myeloid and lymphoid cells in the BM appear well regulated as do the localization and persistence of lymphoid progenitors in the periphery (1). This regulation suggests that inflammation-induced extramedullary B lymphopoiesis may have a significant physiologic role.

We demonstrate that the splenic lymphopoiesis that follows inflammatory stimuli generates large numbers of im and transitional 1 (im/T1) B cells that express low, but significant, levels of activation-induced cytidine deaminase (AID). In these cells, AID expression is independent of T cells, CD154, or the IL-1R-associated kinase 4 (IRAK4). This intrinsic AID expression is developmentally regulated; AID message is greatly diminished or undetectable in pro/pre-B, T2, or mature B cells. Splenic im/T1 B cells from CD154−/− mice contain germline γ3 transcripts (γ3 GLT) and the molecular intermediates of IgM→IgG3, IgG2a, IgG2b, and IgA class-switch recombination (CSR). Splenic im/T1 B cells from CD154−/− mice also carry low levels of message for B lymphocyte-induced maturation protein 1 (BLIMP-1) and respond to TLR ligands by rapid entry into cell cycle and production of IgM and IgG Ab; immunization with a bacterial vaccine efficiently differentiates im/T1 B cells into CD138+ plasmacytes. Taken together, these unique properties suggest that the peripheral im/T1 B cell compartment elicited by inflammation is specialized for T cell-independent (T1) humoral responses to microbial infection in extravascular tissues.

Materials and Methods

Mice

Female C57BL/6 (BL/6), nude, and CD154-deficient congenic (CD154−/−; Ref. 3) mice were purchased from The Jackson Laboratory and maintained in our colony. Mice deficient for linker of activated T cells (LAT−/−) bred onto the BL/6 genetic background (4) were the gift of Dr. W. Zhang (Duke University, Durham, NC). BM samples from BL/6 mice deficient in IRAK4−/− (5) or AID−/− (6) were provided by Drs. J. Aliberti (Duke University) and T. Imanishi-Kari (Tufts University, Boston, MA), respectively. Mice constitutively expressing GFP in all tissues (BL/6 × BL/6-Tg(Actb-CAG-GFP)1Osb/J) F1 animals, GFP transgenic (Tg) (7) were obtained from Dr. M. Kondo (Duke University); mice carrying an IgH transgene (H50GTg) were bred and maintained locally (8). Mice were housed under specific pathogen-free conditions at the Duke University Animal Care Facility with sterile bedding, water, and food. Mice used in these experiments were 6–16 wk old. All experiments involving animals were reviewed and approved by the Duke University Institutional Animal Care and Use Committee.
Mice were immunized by single, i.p. injections of 25 µg of (4-hydroxy-3-nitrophenyl)acetyl (NP) chicken gammaglobulin (CGG) in IFA (Sigma-Aldrich) as described previously (1). NP-CGG contained 10–15 mol NP/mol CGG. A bacterial vaccine was prepared by boiling small volumes of washed Escherichia coli (DH5α) in HBSS; this vaccine (5 × 10^10 bacteria; 200 µl) was given i.v.

Flow cytometry

FITC-, PE-, PE-Cy5-, PE-Cy7-, biotin-, or allophycocyanin-conjugated mAb specific for mouse B220, CD4, CD8, CD21, CD23, IgM, GL7, TER-119, Gr-1, CD11b, and CD180 were purchased (BD Bioscience or eBioscience). PE-, biotin-, and Texas Red-conjugated Ab for mouse IgD, IgM, and λ L chain were purchased from Southern Biotechnology Associates. Streptavidin-allophycocyanin-Cy7 (eBioscience) identified biotinylated mAb.

Mice were killed at various times after injection/immunization, and cells were harvested from spleen, BM, and/or blood. RBC were lysed in ammonium chloride buffer before immunolabeling. Typically, 10^6 nucleated cells were suspended in 50–100 µl of labeling buffer (HBSS with 2% FCS and labeled mAb) and incubated on ice for 20 min. 7-Aminoactinomycin D (Molecular Probes) or propidium iodide (Sigma-Aldrich) was included to identify dead cells. Labeled cells were analyzed/sorted in a FACSVantage with DIVA option or FACScan (BD Biosciences). Flow cytometric data were analyzed with FlowJo software (Tree Star).

Specific B cell populations from the BM and spleen cells were identified with fluorochrome mAb specific for CD21, CD23, IgM, or IgD; pro/pre-B, im/T1, T2, mature follicular (MF), marginal zone (MZ),...
and germinal center (GC) B cells were identified/isolated based on distinctive expression phenotypes (9–12). Dead cells and cells expressing the Gr1, CD11b, CD4, CD8, or Ter119 Ags (Lin+) were excluded in a dump channel. In some experiments, populations of CD93+ GL7 or CD93-GL7 mice were determined by flow cytometry.

Adaptive transfer of B cells

Immunized and naive recipients were determined by flow cytometry. Growth factor genes were calculated by the comparative threshold cycle (CT) method. Supernatants were analyzed by RT-PCR, ELISA, and ELISPOT assays. Cells were labeled with CFSE (1) before culture. After culture for 1–3 days, cells and supernatants were harvested, and cell and supernatant frequencies were determined by flow cytometry.

Cell culture

Sorted B cells (~3 × 10^6) were cultured with 5 µg/ml LPS (Sigma-Aldrich). 5 µg/ml CpG (InvivoGen), or 50 µg/ml anti-IgM Ab Fab (1:2) (Jackson ImmunoResearch Laboratories) in the presence or absence of B cell-activating factor (BAFF; 500 ng/ml; R&D Systems) at 37°C in humidified air supplemented with 5% CO_2. In some experiments, cells were labeled with CFSE (1) before culture. After culture for 1–3 days, cells and supernatants were harvested, and cell and supernatant frequencies were determined by flow cytometry.

RT-PCR

Total RNA was extracted from 0.5–2 × 10^6 cells in TRizol reagent (Invitrogen Life Technologies); mRNA was reverse transcribed (Superscript III; Invitrogen Life Technologies) with oligo(dT) primer for 1 h at 42°C. PCR were performed on serial dilutions (4-fold) of cDNA using TaqDNA polymerase (Denville Scientific). The following PCR primers were used: β-actin, forward, 5'-AGCCATGTACGTCATCCT-3' and reverse, 5'-CTCACTGACGGTACACACC-3'; IgG3, forward, 5'-TGTGGAATCTGCAAATGGA-3' and reverse, 5'-CATAGCAC-3'; IgM, forward, 5'-TGGGAGTTCCATCTCAG-3' and reverse, 5'-CATAGCAC-3'; GL7, PCR primers for IgM, forward, 5'-TGTGGGAATCTGCAAATGGA-3' and reverse, 5'-CATAGCAC-3'; IgG3, forward, 5'-TGTGGAATCTGCAAATGGA-3' and reverse, 5'-CATAGCAC-3'; β-actin, forward, 5'-AGGAGGATGGGCTGTAG-3' and reverse, 5'-GCCTACGC-3'; and TLR4, forward, 5'-GGCCCTTC-3' and reverse, 5'-CAGACGTCAGCGCTTCAGATG-3' (RAG1-2); BCL-6, forward, 5'-GAAGATGTCCTGACACGCG-3'; and reverse, 5'-GGAAATGATGACGTCATCC-3'; BLIMP-1, forward, 5'-CTGGTGAACGGGATGAAC-3' and reverse, 5'-TGGGAGACCTTCTGAGT-3'; TLR2, forward, 5'-TGCTTCCTGCTGGAGATTT-3' and reverse, 5'-TGATACCAAGTCTGCCCAG-3'; TLR4, forward, 5'-ACCTGCGCTGTGTTAAGCT-3' and reverse, 5'-AGAAATCCTGGCAGAACTAATG-3'; and TLR9, forward, 5'-ACCTGCGCCCTTTCGCCAGACATC-3' and reverse, 5'-GCCTCAGCTCAAGGTTAGG-3'.

To determine the frequencies of AID expression in im/T1, MF, and GC B cells, single cells were sorted into 96-well plates containing 10^5/g of yeast tRNA (Ambion). Sorted B cell populations (5–10^6) were transferred into congenic BL/6 recipients (1). Fifteen minutes after transfer, selected recipients were given PBS or bacterial vaccine i.v. Five days after immunization, B220 and CD138 expression by GFP+ splenocytes from immunized and naive recipients were determined by flow cytometry.

FIGURE 2. Immunization elicits increase of developmentally immature B cells independent of T cell signaling. Splenocytes from naive (n = 4) and immunized (d14; n = 5) BL/6 mice were harvested and labeled with Abs for B220, CD21, CD23, CD93, IgM, and IgD to enumerate developing pro/pre-B (im/T1, im/T2), MF B cells (im/T1 and MF B cells were enriched from the spleens of naive GFP-Tg (8) and immunized (d14; n = 5) BL/6 mice were transferred into congenic BL/6 recipients (1). Fifteen minutes after transfer, selected recipients were given PBS or bacterial vaccine i.v. Five days after immunization, B220 and CD138 expression by GFP+ splenocytes from immunized and naive recipients were determined by flow cytometry.

ELISA and ELISPOT assay

Ig concentrations in culture supernatants were determined by a standard ELISA method. Briefly, 96-well plates (BD Biosciences) were coated overnight with goat anti-mouse Ig (H+L) mAb (Southern Biotechnology Associates) or anti-mouse Fcy (Sigma-Aldrich) (both 10 µg/ml in PBS) at 4°C and blocked for 1 h with 3% BSA. After washing (PBS with 0.1% Tween 20), 25-µl aliquots of serially diluted (4-fold) culture supernatant were loaded, incubated for 3 h at room temperature, and extensively washed. Bound IgM and IgG3 were detected by HRP conjugates of isotype-specific goat Ab (Southern Biotechnology Associates). Bound HRP activity was visualized using tetramethylbenzidine peroxidase (Bio-Rad). Ab-secretting cells in cultures were identified by ELISPOT assay. Cultured B cells were harvested, washed, and resuspended in fresh medium before being plated onto nitrocellulose filters coated with Ab specific for mouse IgG (Southern Biotechnology Associates). Plated cells were incubated for 3 h at 37°C in humidified air supplemented with 5% CO_2. Subsequently, filters were washed and flooded with alkaline phosphatase (AP)-conjugated mAb specific for mouse IgM, IgG3, IgG2a, or HRP-conjugated anti-IgG3 (Southern Biotechnology Associates). Bound AP and HRP activity was determined for AP- and HRP-conjugated mAbs.
visualized using Naphthol AS-MX (Sigma-Aldrich) and 3-amino-9-ethyl carbazole (Sigma-Aldrich), respectively.

**Statistics**
Statistical significance of data was determined by Student’s t test.

**Results**

**Identification and isolation of specific B cell compartments**

Pro/pre-B, im/T1 B, T2, MF, and MZ B cells were isolated from the BM (Fig. 1A) and spleen (Fig. 1B) of naive or immunized mice by established methods. Following precedent (9, 11, 12), we define pro/pre-B cells as Lin+/H11002 B220lowCD93+/H11001 CD21+/H11002 CD23+/H11002 IgM+/H11002 IgD+/H11002; im/T1 B cells as Lin+/H11002 B220lowCD93+/H11001 IgM+/H11001 IgD+/H11002 CD21+/H11002 CD23+/H11002; and T2 B cells as Lin+/H11002 B220lowCD93+/H11001 IgD+/H11002 CD21lowCD23+/H11001 IgM+/H11001 IgDlow.Mature B2 (MF) cells are Lin+/H11002 B220highCD93+/H11002 IgM+/H11001 IgD+/H11002 CD21highCD23+/H11001 CD23lowIgMhiIgDlow, whereas MZ B cells are Lin+/H11002 B220highCD93+/H11002 IgM+/H11001 IgD+/H11002 CD21+/H11002 CD23lowIgMhiIgDlow (Fig. 1, A and B).

Representative postsort FACS analyses of these B cell subsets reveal typical purities of ∼95% and diagnostic phenotypes, including modulation of B220, CD21, and CD93 expression during B cell maturation (Fig. 1C). We note that IgM expression by BM im/T1 B cells is lower than the im/T1 splenic compartment, suggesting increased maturity of the peripheral pool. IgD and CD23 first appear on T2 B cells and are increased in the MF compartment and reduced on MZ B cells (Fig. 1C).

**Inflammation expands splenic im/T1 B cell numbers**

Inflammation increases the number of developmentally im B cells (Lin−CD93+ GL7intB220lowIgM+ and Lin−CD93+ GL7intB220low IgM+) in the spleen (1, 13, 15). To determine whether all stages of B cell development expand equivalently, we enumerated splenic pro/pre-B and im/T1 B cell numbers in naive and adjuvant-immunized mice. Fourteen days after immunization, splenic pro/pre-B and im/T1 B cell numbers were 2- to 8-fold higher than in naive controls, constituting 3–6% (1.5–2.0 × 10⁶) of splenic B cells (Fig. 2A) (1, 13). In contrast, adjuvant did not change splenic T2, MF, and MZ B cell numbers (Fig. 2, A and B). Inflammation-induced increases in splenic pro/pre-B and im/T1 B cells were comparable in both BL/6 and congenic nude mice (16) (Fig. 2C).

**TLR ligands activate im/T1 B cells and induce rapid Ab secretion**

Expression of the RP105 TLR (Fig. 3A) (17) and TLR9 message...
(Fig. 3B) is substantial in im/T1 cells, suggesting the possibility of responses to bacterial components. Therefore, we cultured splenic im/T1, MF, and MZ B cells (3 × 10^6) from BL/6 and CD154^−/− mice with LPS or CpG (5 µg/ml) for 24, 48, or 72 h (Fig. 3, C–F). In some experiments, cells were labeled with CFSE (1) before culture. At 72 h, secreted IgM and IgG were quantified by ELISA and Ab-forming cells (AFC) enumerated by ELISPOT. TLR expression and the responses of BL/6- and CD154-deficient B cells to TLR ligands are comparable (data not shown).

Activation (Fig. 3C) and CFSE dilution (Fig. 3, D and E) were comparable for im/T1, MF, and MZ B cells cultured with LPS or CpG. In contrast to MF and MZ B cells, however, im/T1 B cell numbers did not increase, indicating concurrent proliferation and death (Fig. 3F).

Despite the increased rates of cell death, LPS induced rapid Ab secretion by im/T1 B cells. After 24 h with LPS, im/T1 (~20 AFC/10^6 cells, p = 0.02) and MZ (~100 AFC/10^6 cells; p = 0.001) B cells produced higher frequencies of IgM AFC than MF B cells (<1 AFC/10^6 cells) (Fig. 4A); by 48 h, frequencies of IgM AFC in all cultures were comparable, and by 72 h, LPS-stimulated MF and MZ cultures produced 300–450 AFC/10^6 cells compared with ~230 AFC/10^6 cells in im/T1 cultures (Fig. 4A).

LPS and CpG also elicited IgG3 and IgG2a AFC in im/T1, MF, and MZ B cells from BL/6 and CD154^−/− mice (Fig. 4B). Rapid Ab production by im/T1 and MZ B cells was also evident in the higher amounts of Ab in culture supernatants; at 72 h, im/T1 and MZ B cell supernatants contained twice as much IgM and 4- to 6-fold more IgG3 than MF B cell cultures (data not shown).

**BAFF promotes survival and CSR in im/T1 B cells**

BAFF expression is increased by inflammation (18). To determine whether BAFF might promote survival and CSR in activated im/T1 B cells (19), we cultured im/T1, MF, and MF B cells for 72 h with LPS (with or without BAFF) and counted the viable cells and AFC produced (Fig. 5). BAFF increased im/T1 B cell survival ≥4-fold (p = 0.01), a more substantial benefit than for MF and MZ cells (Fig. 5A). With LPS alone, all cultures produced similar frequencies of IgM (230–450 AFC/10^6 cells) and IgG3 (4–9 AFC/10^6 cells) AFC (Fig. 5B). BAFF had little effect on the frequencies of IgM AFC (150–300 AFC/10^6 cells) and did not alter IgG3 AFC production by MF or MF B cells (4–12 AFC/10^6 cells) (Fig. 5B). However, BAFF’s effect on the generation of IgG3 AFC by im/T1 B cells was significant with frequencies increasing ≥3-fold (p = 0.01) (Fig. 5B).

**Efficient plasmacytic differentiation by im/T1 B cells in vivo**

Rapid IgM and IgG AFC responses in vitro to LPS or CpG by BL/6 and CD154^−/− im/T1 B cells suggest a capacity for Tt, Ab responses. To determine whether im/T1 B cells can respond to microbes in vivo, we enriched (~90%) im/T1 and MF B cells from GFP-Tg mice (7) and adoptively transferred equal numbers into congenic BL/6 mice. Immediately after transfer, some recipients were immunized with bacterial vaccine; 5 days later, splenic GFP^+ B220^+ and GFP^+ B220^+ CD138^+ cells in individual recipients were enumerated by flow cytometry (Fig. 6). Whereas GFP^+ B220^+ cell numbers were comparable for all groups, 0.2–0.5% of splenocytes, substantial numbers of GFP^+ B220^+ CD138^+ cells developed only in immunized recipients (Fig. 6), im/T1 B cells produced CD138^+ plasmablasts/cytes as efficiently as MF B cells (4.7 vs 3.8%, respectively). We conclude that in response to bacterial Ags, im/T1 B cells proliferate and differentiate to AFC in vivo.

**AID expression in im/T1 B cells**

CSR in im/T1 B cells implies AID expression (20). To determine whether peripheral im/T1 B cells express AID, we double-sorted pro/pre-, im/T1, T2, MF, MZ, and GC B cells from the BM (pro/pre-B, im/T1) and spleens (im/T1, T2, MF, MZ, and GC) of naive or immunized mice (9, 10, 21). RAG1 mRNA was abundant in...
pro- and pre-B cells, rare in im/T1 B cells, and absent from MF B cells (Fig. 7A). In contrast to RAG1, AID transcript levels were higher in im/T1 B cells than in the less mature IgM+ compartments. AID mRNA was generally undetectable in T2 and MF B cells but highly expressed in GC B cells. AID message in the im/T1 population did not represent contamination by phenotypically similar GC B cells (13), because GC B cells contain BCL-6 mRNA (22) and AID+ im/T1 B cells do not (Fig. 7A). In naive mice, BM and splenic im/T1 B cells express comparable levels of AID mRNA (Fig. 7A).

To quantify AID expression, we amplified Igβ, β-actin, and AID cDNA from equal numbers of double-sorted pro/pre-B, im/T1, T2, MF, and MZ B cells using a quantitative PCR (Fig. 7B) (1, 6). As comparators, splenic im/T1, T2, MF, and MZ B cells cultured with LPS (5 μg/ml; 24 h) and the 103/Bcl2 pre-B (23) and WEHI-231 im B cell (24) lines were also evaluated. LPS induces AID expression in mature B cells and WEHI-231 cells express low amounts of AID (25).

Whereas β-actin cDNA levels showed little change relative to Igβ during B cell development (data not shown), AID cDNA levels varied by as much as 105-fold (Fig. 7B). AID expression in pro/pre-B cells was ~70-fold higher than in MF B cells (p = 0.009) and was increased another 7-fold in im/T1 B cells (BM, p = 0.001; spleen, p = 0.03). Trace amounts of AID cDNA were present in MZ B cells (p = 0.03) but undetectable in T2 (p = 0.22) B cells. As expected, AID expression was greatest in GC (day 16) B cells; in GC B cells, AID message was ~2 × 105 times more abundant than in MF B cells. Whereas AID message in GC B cells is much elevated (~50-fold) compared with im/T1 B cells, LPS-activated im/T1, T2, MF, or MZ B cells and the 103/Bcl2 and WEHI-231 cell lines express AID mRNA comparably to im/T1 B cells (Fig. 7B).

The lower levels of AID mRNA in im/T1 B cells vs GC B cells could reflect lower frequencies of AID+ cells, diminished AID transcription, or both. We double-sorted im/T1, MF, and GC B cells to high purity (~99%) from immunized BL/6 mice and determined the frequencies of single AID+ Igβ+ cells in each compartment (Table I). The efficiency of single-cell RT-PCR for Igβ was highest in MF B cells (~80%) with lower frequencies in im/T1 (54%) and GC (42%) B cells. No AID+ Igβ+ mature B cells were detected, whereas AID+ Igβ+ im/T1 and GC B cells were equally common (12 and 11%, respectively). Therefore, AID expression in im/T1 B cells is low compared with GC B lymphocytes.

AID expression in im/T1 B cells is intrinsic

In MF B cells, AID is elicited by BCR/CD40 and/or TLR/ cytokine signals (6). To determine whether these signals are required for AID transcription in im/T1 B lymphocytes, we isolated im/T1 and mature B cells from the BM and spleens of T cell-deficient mice (nude and LAT−/−; Ref. 4), CD154−/− (3), IRAK4−/− (5), H50GTg (8), and their congenic BL/6 controls (Fig. 8). AID expression in im/T1 B cells from nude, LAT−/− (data not shown), CD154−/−, and BL/6 mice was comparable (Fig. 8A). AID mRNA was abundant in CD21+ cells from the BM of IRAK4−/− mice (Fig. 8B) and expressed equally in λ− and λ+ im/T1 B cells from H50GTg mice (Fig. 8C). The λ− Ab of H50GTg mice is specific for the NP, whereas λ− Abs have diverse specificities including

### Table I. Frequencies of AID+ B lymphocytes by single-cell RT-PCR

<table>
<thead>
<tr>
<th>RT-PCR</th>
<th>MF</th>
<th>im/T1</th>
<th>GC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Igβ+</td>
<td>68/84 (81%)</td>
<td>50/93 (54%)</td>
<td>35/84 (42%)</td>
</tr>
<tr>
<td>AID+</td>
<td>2/84 (2%)</td>
<td>10/93 (11%)</td>
<td>7/84 (8%)</td>
</tr>
<tr>
<td>AID+ Igβ+</td>
<td>0/68 (0%)</td>
<td>6/50 (12%)</td>
<td>4/35 (11%)</td>
</tr>
</tbody>
</table>

FIGURE 6. In vivo, im/T1 B cells efficiently differentiate into CD138+ plasmacytes in response to a bacterial vaccine. im/T1 and MF B cells from GFP-Tg mice were enriched by magnetic sorting (~90% purity) and transferred by i.v. injection to BL/6 recipients; 15 min later, recipients were injected i.v. with 5 × 107 heat-killed E. coli or PBS. After 5 days, splenocytes were harvested and labeled for B220 and CD138 expression. Frequencies of GFP+B220+ splenic cell populations (upper panels) and GFP+B220- CD138+ plasmacytes/blasts (lower panels) were determined by flow cytometry. Typical experimental (n = 3) results illustrated.

FIGURE 7. AID expression in im/T1 B cells. A, Semiquantitative RT-PCR (4-fold serial dilutions) for AID, RAG1, BCL-6, Igβ, and β-actin was performed on subsets of developing B cells. AID+ GC B cells (GL7high CD93−B220+np) from immunized mice were used as positive controls. cDNA quantities were normalized to Igβ message levels. B, Quantitative RT-PCR of AID mRNA at various stages of B cell development using SYBR Green Real-time PCR. Each point represents the expression of AID relative to Igβ mRNA (mean ± SE) calculated by the Ct method (x = 2 − (CtAID − CtIgβ)) (pro/pre-B, im/T1, T2, MF, and MZ, n = 6–10; GC, n = 3; pro/pre-B, im/T1, MF, and MZ plus LPS, n = 2; T2 plus LPS, n = 1 (♀); WEHI-231, n = 4 (♂); and 103/Bcl2, n = 8 (●)).
B cells from BL/6, CD154

B strains was compared. Relative expression of AID, n

Points represent mean (SD) values for duplicate

Ig from naive C57BL/6 mice. Expression of F

B cells (MF, and MZ B cells. Freshly isolated splenic im/T1

are NP specific, whereas

in MZ and some pro/pre-B cells. Freshly isolated splenic im/T1 B cells (●) from CD154−/− mice express levels of AID and γ3 CT that are 10− to 106-fold higher than that of MZ (▲) and MF (■) B cells. After 72 h of culture, AID and γ3 CT levels become equivalent in all groups. Relative expression of AID, β-actin, and γ3 CT was determined by quantitative RT-PCR before and after stimulation of cells with 5 μg/ml LPS. Points represent mean (±SD) values for duplicate samples (n = 2). C, Quantification of γ2a, γ2b, and α CT by RT-PCR in im/T1, T2, MF, and MZ B cells from naïve C37BL/6 mice. Expression of γ2b and α CT in freshly isolated im/T1 B cells was elevated (>10-fold) in comparison to T2, MF and MZ B cells; quantities of γ2a CT are equally abundant in im/T1 and T2 B cells but ∼10-fold lower in MF and MZ B cells. Points represent mean (±SD) of duplicate samples (n = 2).

We next compared the quantities of message for AID, γ3 CT, and β-actin to Igβ mRNA in im/T1, MF, and MF B cells from CD154−/− mice by quantitative RT-PCR (Fig. 5B). Message levels were determined for freshly recovered cells (0 h) and cells cultured with LPS for 24, 48, and 72 h (6). Immediately ex vivo, both AID expression and γ3 CT levels were dramatically higher in im/T1 B cells than in MF or MZ B cells. The initial levels of AID message and γ3 CT in im/T1 B cells were reached by MZ and MF B cells only after exposure to LPS for 24 and 48 h, respectively (Fig. 9B). By 72 h, AID mRNA quantities in MF B cells were ∼3 logs higher, and γ3 CT levels were 10-fold higher than in freshly isolated im/T1 cells. Intrinsic γ3 CSR levels in im/T1 B cells are ∼10% of those in MF B cells fully activated by LPS (Fig. 9B).

The relative abundance of γ3 CT in unactivated im/T1 is also reflected in γ2a, γ2b, and α CT; additional RT-PCR indicated that μ−→γ2a, γ2b, and α class-switch excision circles were 10- to 1000-fold more abundant in im/T1 than in MF and MZ B cells (Fig. 9C).

self-Ags (8). AID transcription in im/T1 B cells occurs independent of the signals, cognate T cell help, CD40-CD154 signaling, and activation via TLR, known to elicit AID in MF B lymphocytes.

CSR in im/T1 B cells

To determine whether early AID expression might mediate CSR in resting im/T1 B cells, we examined splenic im/T1 B cells from CD154−/− mice for γ3 GLT and γ3 CT. γ3 GLT predict IgM→IgG3 CSR (26, 27), and γ3 CT indicate recent and/or active CSR (14). CD154 deficiency eliminates T cell-dependent CSR without affecting B cell development (3, 28).

γ3 GLT were detected in splenic im/T1 and MZ B cells, but not MF B cells, from naïve and adjuvant-immunized animals (Fig. 9A). Only trace levels of γ3 GLT could be demonstrated in im/T1 B cells from the BM, a difference that may reflect the preponderance of im (IgMhi) over T1 (IgMlow) cells in this im/T1 compartment (Fig. 1). Message for the BLIMP-1 differentiation factor was detected in splenic im/T1 B cells as well as in MZ B cells but not in MF B cells (29) (Fig. 9A). The presence of BLIMP-1 in im/T1 cells is consistent with their capacity for rapid plasmacytic differentiation and Ab secretion (Fig. 4, A and B).

We next compared the quantities of message for AID, γ3 CT, and β-actin to Igβ mRNA in im/T1, MF, and MF B cells from CD154−/− mice by quantitative RT-PCR (Fig. 5B). Message levels were determined for freshly recovered cells (0 h) and cells cultured with LPS for 24, 48, and 72 h (6). Immediately ex vivo, both AID expression and γ3 CT levels were dramatically higher in im/T1 B cells than in MF or MZ B cells. The initial levels of AID message and γ3 CT in im/T1 B cells were reached by MZ and MF B cells only after exposure to LPS for 24 and 48 h, respectively (Fig. 9B). By 72 h, AID mRNA quantities in MF B cells were ∼3 logs higher, and γ3 CT levels were 10-fold higher than in freshly isolated im/T1 cells. Intrinsic γ3 CSR levels in im/T1 B cells are ∼10% of those in MF B cells fully activated by LPS (Fig. 9B).

The relative abundance of γ3 CT in unactivated im/T1 is also reflected in γ2a, γ2b, and α CT; additional RT-PCR indicated that μ−→γ2a, γ2b, and α class-switch excision circles were 10- to 1000-fold more abundant in im/T1 than in MF and MZ B cells (Fig. 9C).
**Discussion**

Inflammation mobilizes developing lymphocytes from BM and expands some, but not all, developing B cell subsets in the spleen (Fig. 2 and Ref. 1). This extramedullary B lymphopoiesis is T cell independent (Fig. 2 and Ref. 16) and places expanded populations of im/T1 B cells constitutively expressing AID, BLIMP-1, and IgG and IgA CT (Figs. 7–9) in anatomic sites that maximize exposure to exogenous Ags (30). Does this peripheral expansion of im/T1 B cells represent a significant and physiological response?

Much to our surprise, we found that im/T1 B cells are capable of mounting humoral responses: they express TLR (Fig. 3) and respond to microbial TLR ligands by proliferation, rapid plasma-cytic differentiation, and the accelerated production of IgM and IgG Ab (Figs. 3 and 4). These responses, especially proliferation and IgG production, were substantially enhanced by BAFF (Fig. 5), a B cell survival factor that is elevated by inflammation (31). In vivo, transferred im/T1 B cells efficiently responded to a bacterial vaccine by the generation of CD138+ plasmacytes (Fig. 6). The significant increase of peripheral im/T1 B cells in response to inflammation (Fig. 2) and their capacity to respond to microbial products suggest that im/T1 B cells, like B1 and MZ B cells, may represent a transient B cell compartment specialized for innate humoral immunity. Indeed, the rapidity with which im/T1 B and MZ B cells differentiate into AFC suggests a similar physiologic role (29) for these distinct compartments (Fig. 4). In an environment rich in BAFF, microbial Ags could select locally produced im/T1 B cells for expansion and Ab production. These populations could provide local IgM, IgG, and IgA Ab for opsonization, neutralization, and epithelial transfer.

Mature, Ag-specific B cells respond to Ags with cognate T cell help and initiate AID-dependent SHM and CSR (6). SHM and CSR enhance the protective capacity of Ab by increasing affinity for Ag and entry into extravascular sites. Whereas the predominant site for SHM and CSR is the CD154-dependent GC reaction (32, 33), expression of AID in im/T1 B cells does not require T cell help, CD154, or signaling via IRAK-4 (Fig. 8) but is sufficient to drive low levels of CSR (Fig. 9).

The trace levels of AID message in pro/pre-B cells increase ~10-fold at the im/T1 stage and then fall to the limit of detection in the T2 and MF compartments (Fig. 7). It is highly unlikely that we have misidentified AID+ im/T1 B cells; these cells do not express BCL-6, a transcription factor necessary for the GC reaction (Ref. 22 and Fig. 7), and appear in CD154+/− and IRAK4+/− mice that cannot form GC (3, 28) or respond to most TLR signals (5) (Fig. 8). im/T1 B cells are CD93−B220low, a phenotype that excludes B1 B cells, a minor population of activated MF B cells, and most plasmacytes (9, 11, 12).

The abundance of AID message in im/T1 B cells (Table I) is comparable to that of LPS-activated (24 h) MF B cells and the 103/Bcl-2 pre-B (23) or WEHI-231 im B cell (24) lines, but only ~2–3% of the levels in GC B cells (Fig. 7). Nonetheless, these levels support IgM→IgG and IgA CSR (Fig. 9). We note that Melamed et al. (34) have demonstrated that CSR in B cell precursors rescues B cell development in autoimmune prone μMT mice. This highly selective environment reveals that even the 7-fold lower level of AID expression in pro-B cells is sufficient for rare Ig CSR events.

Does the low level of AID expression in im/T1 B cells also support SHM? WEHI-231 cells are reported to exhibit limited SHM and express low levels of AID (Fig 7 and Ref. 25) as do AID+ pre-B cells transformed by the Abelson murine leukemia virus (AMuLV) (35). In our hands, AID message levels in WEHI-231 cells and in the AMuLV transformant, 103-Bcl2, are similar to those present in im/T1 B cells (Fig. 7). Mao et al. (20) have reported AID expression and SHM in im B cells from mice with restricted Ab diversity but not C57BL/6 mice. Indeed, we have observed low, but statistically significant, levels of SHM in im/T1 B cells from CD154+/− mice compared with AID−/− im/T1 B cells (16.1 × 10−4 vs 1.6 × 10−4 mutations/bp sequenced; data not shown). Our experiments indicate that AID expression levels and Ig SHM vary significantly between mouse strains, an observation concordant with the “leakiness” of the μMT phenotype on the BALB/c, but not C57BL/6, genetic backgrounds (36). We do not understand the genetic basis for this variability and the role, if any, for SHM in the im/T1 compartment. Experiments to analyze the genetic basis for variable AID expression by im/T1 cells are in progress.

Constitutive AID expression in im/T1 B cells drives CSR, but to what purpose? Recently, Gourzi et al. (35) concluded that AMuLV-induced AID expression in pro-B cells caused sufficient genomic damage to limit infection by the up-regulation of the Rae-1 NKG2D ligand. This is an intriguing idea. However, AID expression in the 103/Bcl-2 AMuLV pre-B cell line is comparable to that of normal im/T1 B cells (Fig. 7), and we note that Gourzi et al. defined virus-induced AID expression by comparison to whole BM cell populations containing relatively few im/T1 B cells (35). While AID expression in im/T1 B cells is likely genotoxic—an effect that may be mitigated by BAFF (Fig. 5)—it seems unlikely to us that developmentally regulated AID expression normally acts to control im/T1 B cell numbers by NK-mediated apoptosis.

Alternatively, T, responses by AID− im/T1 B cells might produce Ab specificities, including autoreactive paratopes, unavailable in the MF compartment (37). The ability to produce self-reactive Ab could be beneficial since one mechanism used by microbes to evade adaptive immunity is the mimicking of host Ags (38). Autoantibody production by im/T1 B cells might not endanger the host as responses by im/T1 B cells are substantially dependent on BAFF (Fig. 5); reduced BAFF levels, e.g., by the resolution of infection, would likely end autoantibody production (18). Alternatively, autoantibody produced by peripheral im/T1 B cells might be important for the efficient clearance of cellular debris from infection sites.

Finally, if AID drives CSR in im/T1 B cells, how is it that newly formed MF B cells express IgM? CSR in im/T1 cells is undoubt-edly rare, and it is likely that elevated BAFF levels are required for im/T1 B cells to survive AID-dependent genomic change (Fig. 5). The low level of AID expression in im/T1 B cells ensures that many, perhaps most, T1 B cells are undamaged and mature to cells expressing germline IgM. Under conditions that elevate BAFF and/or other survival factors, e.g., infection or inflammation (31), we predict that increasing numbers of class-switched T1 B cells will survive and mature. Indeed, CSR optimally rescues B cell development in Fas-deficient, autoimmune-prone mice (34).

Inflammation expands AID− im/T1 B cells numbers at sites exposed to exogenous Ags. In an environment with high levels of BAFF, activated AID− im/T1 B cells survive, respond to TLR ligands and Ag, and efficiently produce IgM and IgG Ab. We propose that these phenomena represent an inflammation-induced humoral response to infections that lie beyond the reach of IgM Ab that is unable to diffuse out of the vasculature.

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Disclosures
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