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During a T cell-dependent Ab response, B cells undergo Ab class switching and V region hypermutation, with the latter process potentially rendering previously innocuous B cells autoreactive. Class switching and hypermutation are temporally and anatomically linked with both processes dependent on the enzyme, activation-induced deaminase, and occurring principally, but not exclusively, in germinal centers. To understand tolerance regulation at this stage, we generated a new transgenic mouse model expressing a membrane-tethered \(\gamma_{2a}\)-reactive superantigen (\(\gamma_{2a}\)-macrosel self Ag) and assessed the fate of emerging IgG2a-expressing B cells that have, following class switch, acquired self-reactivity of the Ag receptor to the macrosel-Ag. In normal mice, self-reactive IgG2a-switched B cells were deleted, leading to the selective absence of IgG2a memory responses. These findings identify a novel negative selection mechanism for deleting mature B cells that acquire reactivity to self-Ag. This process was only partly dependent on the Bcl-2 pathway, but markedly inefficient in MRL-\textit{Fas}\textsuperscript{lpr} lupus mice, suggesting that defective apoptosis of isotype-switched autoreactive B cells is central to \textit{Fas} mutation-associated systemic autoimmunity. \textit{The Journal of Immunology}, 2010, 185: 1015–1027.

Foreign Ags trigger the growth and differentiation of Ag-specific memory B cells in peripheral lymphoid organs (1). Upon rechallenge, memory B cells produce high-affinity isotype-switched Abs with diverse effector functions and thus prime the immune system for rapid and effective responses. Somatic hypermutation (SHM), required for generating high-affinity changes, however, may also lead to the acquisition of self-reactivity (2–4), thereby raising the question of how this potentially harmful event is controlled. Receptor editing, clonal deletion, and anergy in early B cell development constitute the first level by which the precursor frequency of autoreactive B cells is restrained (5–9), but the mechanism involved in censoring mature B cells that acquire self-reactivity through SHM is, to a large extent, unknown.

Early experiments using an in vitro 4-hydroxy-3-nitrophenyl acetyl (NP)-hapten model that mimics responses to T-dependent Ags suggested that memory B cells are susceptible to tolerance induction (10). This postulate was later reinforced by several in vivo studies, some of which demonstrated apoptosis-mediated deletion of germinal center (GC) B cells that had acquired heightened capacity to bind the immunizing Ags (11–14). These studies suggested two plausible mechanisms for censoring newly created autoreactive GC B cells during ongoing immune responses (15). The first proposes that soluble self-Ag interferes with the interaction between centrocytes and Ag bound to follicular dendritic cells (FDCs), a process critical for the survival of the developing nant-scent memory B cells and also for selecting B cells that have acquired higher affinities. The second hypothesizes that, at a later stage, autoreactive centrocytes do not obtain the T cell help necessary for continued survival and undergo apoptosis. Self-reacting T cells are deleted or rendered incapable of responding to self-Ag through central and peripheral tolerance mechanisms, which provides a second layer of protection. Other studies on Ab responses to self-Ags in normal or BCR transgenic (Tg) mice have also suggested that memory B cells are biased away from self-reactivity, but the mechanisms involved and the developmental stages at which this bias can occur were not defined (16–20).

The extreme heterogeneity of lymphocyte specificity and the low precursor frequency of Ag-specific lymphocytes make Ag receptor Tg mouse models indispensable in the study of lymphocyte biology and tolerance. However, memory B cell tolerance cannot be easily assessed experimentally in these quasi-monoclonal immune systems, because tolerance is already imposed at early B cell developmental stages. Tolerance during an ongoing immune response can, however, be analyzed in polyclonal immune systems in which, with appropriate experimental tools, the fate of autoreactive B cells can be assessed precisely when SHM and class switch recombination (CSR) occur. We recently developed such a novel experimental system based on the transgenic expression of a membrane-bound synthetic superantigen, dubbed macrosel Ag (Ag) (21, 22). This model exploits the striking propensity of B cells to exchange the genes encoding Ig-constant regions during developmental progression.

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The online version of this article contains supplemental material.

Abbreviations used in this paper: AFC, Ab-forming cell; B6, C57BL/6; cIg, cytoplasmic Ig; cIgA, cytoplasmic IgA; CSR, class-switch recombination; FDC, follicular dendritic cell; GC, germinal center; (G4S)\textsuperscript{3}, linker codons in one-letter amino acid code GGGGSGGGGSGGGGS; HA, hemagglutinin; KLH, keyhole limpet hemocyanin; Li, Ig L chain leader exon and first intron; NP, 4-hydroxy-3-nitrophenyl acetyl; pURF, \(\gamma_{2a}\)-macrosel Ag Tg construct; SMH, somatic hypermutation; Tg, transgenic or transgenic.

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With the macroself Ag, engineered to react with a defined C region of mouse Ig, BCRs can be specifically triggered when the reactive class or subclass appears on the cell surface. In this study, a membrane-bound macroself Ag specific to an allogeneic transgenic mouse IgG2a was generated by single chain Fv Ab engineering technology and expressed as a transgene. Because B cells require antigenic stimulation to undergo CSR, we reasoned that an IgG2a specific macroself Ag should only engage Ag-experienced B cells, thereby allowing normal preimmune development. Furthermore, SMH and CSR occur concurrently during the Ag-driven immune response, such that the cells undergoing CSR are often those that hypermutate their Ab genes (23). We show in this study that ubiquitously expressed anti-IgG2a-macroself Ag promotes tolerance and rapid deletion of autoreactive isotype-switched B cells emerging after immunization with a T-dependent Ag. These and additional results provide direct evidence for the negative selection of mature B cells that acquire self-reactivity during ongoing immunity, suggesting a novel mechanism for deleting GC B cells that bind surface-expressed self-Ags, and show that lupus-prone MRL-Fas* mice are defective in this process.

Materials and Methods

**Mice and immunization protocol**

C57Bl/6 (B6), B6-igH9, B6-Cd45.1 and MRL/Fas* mice were obtained from the Jackson Laboratories (Bar Harbor, ME). MRL-Fas* Ig2a-macroself Ag Tg (pURF-Tg) mice were generated by backcrossing B6-igH9 pURF-Tg mice with MRL-Fas* mice for at least seven generations. EmuBcl-2-22 Tg (Bcl2-Tg) mice (24) were kindly provided by A. Strasser and A.W. Harris (Walter and Eliza Hall Institute, Melbourne, Australia). Mice were immunized with 400 µg keyhole limpet hemocyanin (KLH) in RIBI adjuvant (Sigma-Aldrich, St. Louis, MO). All mice were bred and maintained in The Scripps Research Institute Animal Resources facility according to Institutional Animal Care and Use Committee guidelines.

**Generation of Ig2a-macroself Ag gene constructs**

The VIJ1 and DJH1 variable gene families were amplified by PCR using as templates the plasmids containing genomic DNA of the anti-mouse IgG2a (a gift from M. Shlomchik, Yale University) derived from the 20.8.3 hybridoma (25). To generate a single-chain Ab gene, a PCR sewing approach was taken using the following oligonucleotide primers: primer 1 (5’-ACGCACGTGCAATGGATACATCTGAGACAA-3’), primer 2 (3’-ACGCACGTGCAATGGATACATCTGAGACAA-5’), primer 3 (5’-ACGCACGTGCAATGGATACATCTGAGACAA-3’), primer 4 (3’-ACGCACGTGCAATGGATACATCTGAGACAA-5’). The overlapting sequences are underlined; those recognized by restriction endonucleases are in italics. PCR products corresponding to the LVJL and the VDJH region were initially amplified with the primers 1, 2 and 3, 4, respectively. The single-chain Fv gene, including the intervening flexible peptide codons (Gly4-Ser)3, was assembled by a second PCR step with the outer primers 1 and 2, using 5’-GCTCGCAGGTACAGCTGAAAGAGTCAGG-3’ as a NruI/XmaI restriction fragment downstream of the Igha region were amplified with the primers 1, 2 and 3, 4, respectively. The reverse IgG2a primer was 5’-GGCTGTTAGAAGCACAGTGACAAATCGAGCTTGCATTATTTAT-3’ and the PCR products size were 472 bp for the IgG2a germline and 459 bp for the IgG2a PCR products were 472 bp for the IgG2a germline and 459 bp for the IgG2a PCR products were 472 bp for the IgG2a germline and 459 bp for the IgG2a germline.

**Spleen transplantation chimeras**

Recipient mice were pURF-Tg or littermate controls; all carried the CD45.1 allele, whereas spleen donors were CD45.2+. Recipients received 5 x 107 BM cells from the spleen as previously described (21). Five million cells were surface stained with the following mAbs: FITC-conjugated anti-CD4, CD8, Tcrβ, F4/80, Gr1, IgM, IgD (Dum channel), and PerCP-conjugated anti-CD45R/B220 (RA3-6B2). Surface-stained cells were fixed and permeabilized using a kit (Cytofix/Cytoperm; BD Biosciences, San Jose, CA) and stained according to the manufacturer’s instructions with the following Abs: anti-mouse IgG2a (RMG2a-62; Biologic, San Diego, CA), anti-mouse IgG2b (R12-3), and anti-mouse IgG1 (A85-1). After two washes, spleen cells were incubated with a PE-conjugated rat anti-mouse IgG (187.1) and allophycocyanin-conjugated streptavidin. Stained cells were acquired on a FACScalibur flow cytometer (BD Biosciences), and results were analyzed using the FlowJo software package (Tree Star, Ashland, OR) using 0.5% and 2% contour plot on logarithmic graphic displays. For direct analysis of Ag-specific B cells, ex vivo cells were labeled for 45 min with FITC-A85.1 (anti-IgG1), biotin-8.3 (anti-IgG2aa), or biotin-5.7 (anti-IgG2ab), and then washed and blocked with rat and mouse serum for 15 min before addition of the appropriate labeling mix combination containing FITC- or biotin-11.26 (anti-IgD), NP-APC, Cy7PE-6B2 (anti-B220, Biologic), PE-281.2 (anti-IgG2a), Cy5PE-H129.19 (anti-CD4), 53-6.7 (anti-CD8), and then streptavidin-Cy7APC. Stained cells were analyzed on an FACS Vantage SE (BD Biosciences). All reagents and Abs were purchased from BD Biosciences, unless indicated.

**PCR and quantitative real-time PCR**

Total RNA was purified from the spleens of immunized IgH* non-Tg and pURF-Tg mice using the RNaseasy Mini kit and RNase-Free DNase Set (Quagen, Valencia, CA) and cDNA was generated with the SuperScript III First-Strand Synthesis System for RT-PCR kit (Invitrogen) according to the manufacturers’ instructions. PCR reactions were performed in a final volume of 50 µl containing 4-fold cDNA serial dilutions. Vh-yr2a PCR products were generated using a sense primer specific for the majority of mouse Vh genes (5’-AGGTTGACGTGCAGGAGTGCTG-3’), primer 3 (5’-GGCTGTTAGAAGCACAGTGACAA-3’, respectively. The reverse IgG2a primer was 5’-GCTCGCAGGTACAGCTGAAAGAGTCAGG-3’ and the PCR products size were 472 bp for the IgG2a germline and 459 bp for the IgG2a germline.

**Production of pURF Tg mice**

The 4 kb pURF transgene construct was separated from bacterial vector sequences by a digestion with HindIII/Not1 and agarose gel electrophoresis. The fragment was isolated and purified for microinjection as previously described (21). Tg mice were produced by classical microinjection techniques at the TSRI Mouse Genetics Core Facility (La Jolla, CA).

**Flow cytometric analysis**

HEK 293T transfected cells were harvested using 1X PBS and 0.5 mM EDTA, washed twice and incubated with either a biotin-conjugated mouse anti-rat IgG1, a mouse IgG2a*, or a mouse IgG2b* mAb. Cells were incubated with a biotin-coupled rat anti-mouse IgG2a,b to assess Fv binding specificity. Biotin-coupled Abs were revealed with streptavidin-PE. For the analysis of splenic cells ex vivo, nucleated cell suspensions were prepared from the spleen as previously described (21). Five million cells were surface stained with the following mAbs: FITC-conjugated anti-CD4, CD8, Tcrβ, F4/80, Gr1, IgM, IgD (Dum channel), and PerCP-conjugated anti-CD45R/B220 (RA3-6B2). Surface-stained cells were fixed and permeabilized using a kit (Cytofix/Cytoperm; BD Biosciences, San Jose, CA) and stained according to the manufacturer’s instructions with the following Abs: anti-mouse IgG2a (RMG2a-62; Biologic, San Diego, CA), anti-mouse IgG2b (R12-3), and anti-mouse IgG1 (A85-1). After two washes, spleen cells were incubated with a PE-conjugated rat anti-mouse IgG (187.1) and allophycocyanin-conjugated streptavidin. Stained cells were acquired on a FACScalibur flow cytometer (BD Biosciences), and results were analyzed using the FlowJo software package (Tree Star, Ashland, OR) using 0.5% and 2% contour plot on logarithmic graphic displays. For direct analysis of Ag-specific B cells, ex vivo cells were labeled for 45 min with FITC-A85.1 (anti-IgG1), biotin-8.3 (anti-IgG2a*), or biotin-5.7 (anti-IgG2b*), and then washed and blocked with rat and mouse serum for 15 min before addition of the appropriate labeling mix combination containing FITC- or biotin-11.26 (anti-IgD), NP-APC, Cy7PE-6B2 (anti-B220, Biologic), PE-281.2 (anti-IgG2a), Cy5PE-H129.19 (anti-CD4), 53-6.7 (anti-CD8), and then streptavidin-Cy7APC. Stained cells were analyzed on an FACS Vantage SE (BD Biosciences). All reagents and Abs were purchased from BD Biosciences, unless indicated.

**Serum Ig determinations and NP-specific ELISPOT**

For total IgG2a, IgG1, and IgG1* serum Ab, polyvinylchloride microplates (Falcon; BD Biosciences) were coated with the following Abs: polyclonal goat-anti-mouse IgG2a or anti-mouse IgG1. After washing and blocking, sera (diluted in PBS plus 1% BSA) were incubated 3h at room temperature, and bound Abs were detected using biotinylated anti-mouse IgG2a,
IgG2ab, IgG1a, and IgG1 Ab levels were comparable to those of high and further elevated upon immunization (Fig. 2 A) IgG2ab mAbs. For NP-specific Ab titers, microplates were coated with NP7-specific IgG2aa and IgG2ab was performed with 96-well multi-spectra max250 model reader (Sunnyvale, CA). ELISPOT for detection of the chromogenic substrate 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (Sigma-Aldrich) in McIlvain’s buffer (84 mM Na3PO4/48mM citrate, pH 4.6). Absorbance was measured on a Molecular Devices

Statistical analysis

Group comparisons were analyzed by two-tailed Student t test unless otherwise indicated. p < 0.05 was considered significant.

Results

Generation of the γ2a-macroself Ag constructs and Tg mice

To express an artificial autoantigen reactive to mouse IgG2a (γ2a-macroself Ag; Fig. 1A), we engineered the Tg construct (pURF) depicted in Fig. 1B. The antigenic specificity was obtained by generating a single-chain Fv gene derived from the heavy and L chain variable genes of the 20.8.3 mAb, which binds specifically to mouse IgG2a,d,e,f,g,h,j,n,o, but not to mouse IgG2ab,c,p (25). The construct also includes the hinge and FC domains of the rat IgG1 and the transmembrane and cytoplasmic tail region of the H-2Kb gene, which promote ubiquitous cell surface expression (21). A plasmid carrying the chimeric gene under the control of the human ubiquitin C promoter was transiently transfected in human embryonic kidney 293T cells, and the chimeric gene encoded the predicted cell surface protein with binding specificity for the mouse IgG2a Ab (Fig. 1C).

To analyze the effect of the ubiquitously expressed γ2a-macroself Ag on IgG2a-expressing B cells in vivo, Tg mice expressing the pURF construct were generated. Five B6 Tg lines were derived with distinct γ2a-macroself Ag expression levels ranging from high to intermediate, as measured by flow cytometry (data not shown). One Tg line was selected for further studies, because this line expressed high levels of the γ2a-macroself Ag on the surface of virtually all cells tested (Fig. 1D).

Absence of IgG2a response in γ2a-macroself Ag Tg mice

To follow tolerance of B cells whose self-reactive receptors emerged after activation, we immunized mice carrying or lacking γ2a-macroself Ag with NP-KLH in RIBI adjuvant (27), boosted on d28, and assessed the IgG2a Ab response by ELISA assay and flow cytometry. Mice used in these experiments were IgHa/b, providing at least three separate animals.

Cell surface staining was used initially to follow B cells responding to Ig. We gated on viable splenocytes that were negative for CD4, CD8, and IgD and assessed their binding to NP along with other markers of interest. Total NP-specific B cell numbers were similar in immunized Tg and littermate mice. Similarly, the total numbers of NP-specific IgG1 cells were comparable between the macroself Ag TgS and control mice after priming and recall immunization (Fig. 3A). By contrast, we observed a significant reduction of NP-specific-, B220⁺-, IgG2a⁺-expressing cells after Ag priming, and this was even more pronounced after recall, consistent with a block in the development of memory B cells (Fig. 3B). Collectively, these results support the notion that GC IgG2a⁺ B cells were...
selectively deleted in γ2a-macroself Ag Tg mice after priming and recall immunization with NP-KLH in RIBI adjuvant.

IgG2aa B cells are deleted in γ2a-macroself Ag Tg mice

Because the absence of Ag specific IgG2aa B cells in γ2a-macroself Ag Tg mice immunized with NP-KLH could have been the result of Ag receptor downregulation rather than cellular depletion, we used a flow cytometric approach to detect both surface and intracellular isotype-switched Igs (Fig. 4A). Spleen cells, obtained at 14 d post-immunization from IgH^b littermate control mice (non-Tg, open squares), and IgH^b pURF-Tg mice (black circles) after priming and recall immunization. B, Mean ± SD of NP-specific-IgG2aa, -IgG2ab, -IgG1a, -IgG2a, and -IgG1 Ab titers measured by ELISA assays on serum derived from IgH^b pURF-Tg (black circles) and littermate control mice (open squares) as indicated. The average concentrations of serum Ab and days after priming and recall immunization are indicated on the y-axis (logarithmic scale) and x-axis, respectively. Three to five mice were analyzed per group at each time point.

FIGURE 2. Absence of IgG2aa Ab response in IgH^b γ2a-macroself Ag Tg mice after priming and recall immunization with NP-KLH in RIBI adjuvant.

A, Mean ± SD of total IgG2aa, IgG2ab, IgG1a, IgG2a, and IgG1 Ab titers measured by ELISA assays of serum derived from B6 (open lozenges), B6-Igh^a congenic (gray lozenges), IgH^b littermate control mice (non-Tg, open squares), and IgH^b pURF-Tg mice (black circles) after priming and recall immunization. Mean ± SD of NP-specific-IgG2aa, -IgG2ab, -IgG1a, -IgG2a, and -IgG1 Ab titers measured by ELISA assays on serum derived from IgH^b pURF-Tg (black circles) and littermate control mice (open squares) as indicated. The average concentrations of serum Ab and days after priming and recall immunization are indicated on the y-axis (logarithmic scale) and x-axis, respectively. Three to five mice were analyzed per group at each time point.

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These experiments were extended to investigate whether autoreactive B cells in γ2a-macroself Ag Tgs were present at any time after priming with NP-KLH. In these experiments, Tg and
FIGURE 3. Absence of IgG2a+ memory B cell formation in IgH<sup>μ</sup>/γ2a-macroself Ag Tg mice. A. Flow cytometric analysis of spleen cells derived from pURF-Tg and non-Tg littermate control mice 7 d after priming and 5 d after recall immunization with NP-KLH in Ribi adjuvant. NP-specific B220<sup>hi</sup>/CD138<sup>-</sup> and B220<sup>hi</sup>/CD138<sup>+</sup> cells were visualized by successive gating on a broad forward scatter versus side scatter window to include B cell blasts, then on PI<sup>-</sup>, Dump<sup>-</sup> (CD4<sup>-</sup>, CD8<sup>-</sup>, IgD<sup>-</sup>) to exclude dead cells, T cells, and naive B cells. NP-binding IgG1-positive cells were visualized by gating on B220<sup>hi</sup> cells. The histograms on the right represent the mean numbers of NP-specific and NP-specific B220<sup>hi</sup>/IgG1<sup>+</sup> cells in non-Tg (gray bars) and pURF-Tg mice (black bars). B. Flow cytometric analysis of NP-specific, B220<sup>hi</sup>/IgG2a<sup>+</sup> and B220<sup>hi</sup>/IgG2a<sup>-</sup> cells in pURF-Tg and non-Tg mice after priming and recall immunization. The histograms on the bottom represent the average numbers of NP-specific IgG2a<sup>+</sup>, IgG2a<sup>-</sup>-positive cells and the IgG2a<sup>-</sup>/IgG2a<sup>+</sup> cell ratio in littermate control (gray bars) and pURF-Tg (black bars) mice. The percentage of positive cells (± SEM) is indicated in each gate. Three mice were analyzed per group at each time point except for the non-Tg mice after Ag recall (n = 2).
littermate control mice of homozygous IgHva allotype were used because on this genetic background, the γ2a-macroselAg reacts with all IgG2a-expressing B cells, thereby allowing detection of IgG2aβ B cells with an mAb specific to an epitope different from that recognized by the macroselAg. This approach revealed that IgG2a-expressing B cells were rare in the spleens of Tg mice at all time points (Supplemental Fig. 1). In contrast, the average numbers of IgG1 and IgG2b B cells were increased 2- to 3-fold in Tg mice. These results indicate that polyclonal and Ag-specific B cells reactive to the γ2a-macroselAg are promptly deleted in the spleen after priming and CSR.

Molecular analysis of IgG2a CSR

To test further the notion that γ2a-macroselAg led to B cell clonal elimination, we quantified by RT-PCR germline and mature IgG2a transcripts present in RNA from spleens of unimmunized and NP-KLH immunized mice. Transcripts containing the H chain V region, the CH1, and hinge regions of the mouse IgG2a gene segment were greatly reduced in Tg mice (Fig. 5 A,5B). Consistent with these results, little or no γ2a mRNA was detected by quantitative real-time PCR in the spleens of Tg mice (Fig. 5C). We also quantified the germline γ2a-specific transcripts driven by the λ1 promotor (λ1-γ2a), which is a product of CSR. Because the λ1 promotor is still active after isotype switch, the amount of transcripts composed of the λ1 exon spliced onto the γ2a 5’-exon correlates with the number of cells that survive after CSR to the IgG2a isotype, despite some transcripts arising from switching of the nonproductive allele. After Ag priming, increasing levels of germline γ2a transcripts driven by the λ1 promotor were detected in littermate control mice but, these transcripts remained poorly induced in γ2a-macroselAg Tg mice (Fig. 5A, 5D). Seven days after Ag priming, when γ2a postswitched transcripts reached their highest levels in spleens of littermate control mice, a 6-fold lower amount was observed in Tg mice. Finally, we quantified γ2a germ-line transcripts driven by the Iγ2a promoter (Iγ2a-γ2a), which is induced in B cells before CSR (Fig. 5A, 5E). The Iγ2a-γ2a germ-line transcripts were induced in the spleens of Ag-primed γ2a-macroselAg Tg mice with a pattern similar to non-Tg littermates, although somewhat lower at the peak of the response. These results indicate that the artificial autoantigen selectively induces deletion of reactive B cells soon after CSR and transcription of functional γ2a mRNA.

Adoptive transfer studies

Because the γ2a-macroselAg is expressed on all cells tested, including B cells, we assessed tolerance induction after primary immunization with a T cell-dependent Ag in an adoptive transfer model in which γ2a-macroselAg Tg mice served as hosts of wild type IgHvb donor spleen cells. As with nonmanipulated Tg mice, 2 wk after NP-KLH priming, surface IgG2aβ B cells were largely depleted, whereas nonreactive IgG2aβ B cells were readily detected in the spleens of Tg mice (Fig. 6A, 6B). Similarly, using ELISPOT assays, NP-specific IgG2aβ Ab-forming cells (AFCs) were not detected in the spleen of Tg hosts, whereas the numbers of IgG2aβ AFCs were similar to those found in non-Tg chimeras (Fig. 6C). These results supported the notion that wild type isotype-switched autoreactive B cells developing in γ2a-macroselAg Tg mice undergo rapid deletion, presumably by an apoptotic mechanism.

FIGURE 4. Absence of cytoplasmic IgG2aβ-expressing B cells after Ag priming in γ2a-macroselAg Tg mice. A, Flow cytometric analysis of B220+, cytoplasmic IgG2aβ, IgG2aβ, IgG1, and IgG2b expressing spleen B cells identified by successive gating on side scatter versus forward scatter, Dump (CD8β, CD4β, F4/80β, Gr1β, IgMβ, IgDβ) and cytoplasmic IgG2aβ (clgk) cells, 14 d after priming with NP-KLH in RIBI adjuvant. The frequency of positive cells, rounded to 1%, is indicated next to each gate. B, Histograms representing the mean ± SE percentages and numbers of Dump, clgkβ, IgG2aβ, IgG2aβ, IgG2bβ, IgG1β, and IgG2bβ cells in littermate control (open bars) and pURF Tg (dark bars) mice. The numbers of mice analyzed are indicated in parenthesis. clgk, cytoplasmic IgG2aβ.
Enforced expression of Bcl2 reduces the deletion of autoreactive IgG2a-switched B cells

Splenocytes overexpressing Bcl2 in the B cell compartment (24) were tested by adoptive transfer to determine whether enforced expression of this anti-apoptotic molecule would suppress the deletion of macroself Ag-reactive IgG2a switched B cells. Indeed, IgG2a⁺ cells were substantially rescued in the macroself Ag recipients that received Bcl2 Tg cells (Fig. 6A, 6B). Moreover, although the average numbers of NP-specific IgG2a⁺ AFCs were one third of those in macroself Ag-free hosts of Bcl2 Tg cells, they were those obtained with non-Tg recipients of wild type spleen cells (Fig. 6C). In the absence of macroself Ag, chimeras reconstituted with Bcl2 Tg cells had elevated numbers of surface IgG2a⁺ and IgG2a⁺ B cells compared with wild type mice. These differences also correlated well with the increased numbers of NP-specific IgG2a⁺ and IgG2a⁺ AFC by ELISPOT assays. We conclude that the survival-enhancing effects of Bcl2 partially inhibit deletion of autoreactive isotype-switched B cells, thereby promoting the development of Ag-specific autoreactive plasma cells.

MRL-Faslpr mice fail to delete autoreactive IgG2a B cells

We next tested whether lupus-prone MRL-Faslpr mice are capable of eliminating γ2a-macroself Ag-reactive memory B cells by introducing the Tg to this background. Similar to above, 6-wk-old mice were injected with NP-KLH and analyzed 14 d later for clgG2a⁻ and clgG2b-expressing B cells. In non-Tg controls, MRL-Faslpr had up to 20-fold greater numbers of clgG2a⁻ and clgG2b-expressing B cells compared with B6-Igha⁺ mice, consistent with Fasl⁻/⁻-associated B cell expansion (Fig. 7A; Table I). Furthermore, as previously shown, the numbers of IgG2a-expressing B cells in both the memory B (B220⁺/clgG2a⁺) and plasma cell (B220⁺/clgG2a⁺/cIgG2a⁺) compartments were markedly reduced in macroself Ag Tg compared with non-Tg B6-Igha⁺ mice. In striking contrast, clgG2a⁺ B cells were abundantly present in MRL-Faslpr macroself Ag Tg mice with a similar number of memory B cells and a 2- to 3-fold greater number of plasma cells detected. Consistent with these results, IgG2a anti-NP and anti-chromatin were detected in the sera of NP-KLH-immunized MRL-Faslpr γ2a⁺-macroself Ag Tg mice (Fig. 7B). Moreover, total IgG2a levels in Tg were similar to non-Tg MRL Fas-deficient mice, suggesting that loss of tolerance to the γ2a-macroself Ag occurred prior to immunization.

A possible explanation not requiring loss of tolerance, however, is that maternal transfer of IgG2a⁺ through the placenta or milk could have bound to and neutralized the γ2a-macroself Ag in offspring of MRL-Faslpr mice. Indeed, low levels of cell surface γ2a-macroself Ag not bound by IgG2a were detected when peripheral blood cells from adult MRL-Faslpr γ2a-macroself Ag Tg mice were incubated...
with fluorescent-conjugated IgG2a and fluorescent-labeled–anti-rat IgG1 (data not shown). To address this issue and to document spontaneous loss of tolerance to the \( \gamma_2a \)-macroself Ag, we measured serum allotype-specific IgG2a in 1- to 8-wk-old F1 littermates from female B6-\( \text{Faslpr} \)(IgG2ab) crossed with MRL-\( \text{Faslpr} \)(IgG2aa) males heterozygous for the \( \gamma_2aa \)-macroself Ag transgene; whereby mothers are IgG2ab and non-Tg, and offspring are IgG2aa/b with approximately half being \( \gamma_2aa \)-macroself Ag-positive (Fig. 7C). In the non-Tg (B6-\( \text{Faslpr} \times \text{MRL-} \text{Faslpr} \))F1 pups, IgG2aa was initially detected at 3 wk of age with levels progressively increasing at 4 and 8 wk. Similarly, IgG2aa was first detected at 3 wk in \( \gamma_2aa \)-macroself Ag Tg mice, but initially at lower concentrations (3 and 4 wk) until 8 wk when levels were comparable to non-Tg littermates. In contrast, IgG2ab levels in both groups were similar throughout and, as expected because of maternal transfer of IgG, were present at the earliest time point (1 wk). During this same period, surface expression of the \( \gamma_2aa \)-macroself Ag was detected by a monoclonal anti-rat IgG1 and was present at similar or greater levels as controls at 1–2 wk and in peripheral blood cells, but was reduced at 8 wk in the thymus (Supplemental Fig. 2A, 2B). In contrast, the capacity of macroself Ag to bind IgG2aa in the (B6xMRL)F1-\( \text{Faslpr} \) Tg mice, although similar to B6-Tg mice at 1 and 2 wk, was significantly reduced by 4 wk and essentially nil by 8 wk (Supplemental Fig. 2C), consistent with the initial detection and subsequent rise of serum IgG2aa from weeks 3 to 8. A similar breach of tolerance to the \( \gamma_2a \)-macroself Ag was also observed in Fas-deficient B6-\( \text{Igh}a \)-macroself Ag Tg mice, but appeared less complete. Serum IgG2a, although present, was at significantly lower concentrations in Tg compared with non-Tg B6-\( \text{Faslpr} \) mice at 80 d (Fig. 7D, Supplemental Fig. 3). These findings indicate that loss of tolerance to the \( \gamma_2a \)-macroself Ag in Fas-deficiency occurs spontaneously despite high levels of macroself Ag (similar to levels in Tg B6 mice), can be detected at the earliest appearance of IgG2aa, is not caused by blocking of the \( \gamma_2a \)-macroself Ag by maternal transmission of IgG2a, and is enhanced by MRL background genes.

### Discussion

Memory B cell differentiation in response to T-dependent Ags poses a great challenge for self and nonself discrimination, because the V-gene somatic mutations that promote affinity maturation can also render cells self-reactive. Somatic mutation and class switching in...
response to T cell-dependent immune reactions are believed to occur mainly, but not exclusively, in GCs (28–33). Regardless of where such reactions take place, however, their consequences for self-reactivity need to be controlled. The dynamic nature of the GC reaction, the low frequency of lymphocyte precursors, and the extraordinary heterogeneity of BCR specificities have made in vivo assessment of memory B cell tolerance difficult. Perhaps, the greatest difficulty has been to target tolerogens to newly acquired B cell specificities following SHM and CSR.

In this study, we describe a novel Tg mouse model carrying an anti-IgG2a–reactive artificial autoantigen (γ2a-macroself Ag) that induces memory B cell tolerance in normal mice during immune response to a T-dependent Ag. Ubiquitous expression of the membrane-bound γ2a-macroself Ag or even on non-bone marrow-
derived cells induces specific deletion of Ag-specific IgG2a B cells soon after primary immunization. Moreover, IgG2a B cells are effectively absent at any time after Ag priming, even when assayed by intracellular staining. From these results it can be concluded that the absence of Ag-specific IgG2a B cells is not due to Ag receptor downregulation, that tolerance induction is solely dependent on expression of the IgG2a H chain, and that autoreactive primary plasma cells, post-GC memory B cells, and long-lived plasma cells are absent after Ag priming. Furthermore, RNA analysis shows that, in the presence of the macroself Ag, deletion of IgG2a B cells appears to be a rapid process, occurring early after Ag priming and CSR likely within or soon after emigration from the GC.

As a result of the macroself Ag expression, the memory B cell and plasma cell compartments are virtually completely purged of autoreactive B cells without compromising the amplitude of the Ag-specific Ab response. In fact, we observed compensatory increases of other nonautoreactive isotype-switched B cells presumably because of a relative excess in B cell trophic cytokines and reduced competition for T cell help and/or BCR binding to Ags on FDCs as a result of the deletion of the entire IgG2a isotype subset. This dual effect of autoreactive B cell deletion combined with simultaneous expansion of nonautoreactive B cells may be central to efficient memory responses for foreign Ags. Indeed, tolerance is sustained over time by the exclusion of autoreactive Ab-forming cells from the long-lived bone marrow plasma B cell pool, which is responsible for the continuous production of serum Abs (34, 35).

As noted, previous studies have suggested potential mechanisms by which newly formed autoreactive B cells might be deleted in GCs. One is the blocking of BCR-mediated B cell contact with FDCs by an excess of soluble self-Ag (14); the second is insufficient cognate T cell interaction because of tolerance of T cells to self-Ags (15, 36). In both instances, autoreactive B cells succumb to insufficient survival signals. In this study, we show that despite adequate FDC and T cell function, as indicated by the normal development of other Ag-specific IgG isotype-expressing B cells following immunization, B cells that bind to macroself Ag are nonetheless deleted. Our findings thus identify a selection mechanism that deletes autoreactive B cells that have acquired self-reactivity to surface-expressed self-Ags during the process of class switching. Because class switch to IgG2a occurs in the GC and during the same time frame as SHM, our findings suggest a model for studying the fate of B cells that acquire reactivity to membrane bound self-Ags during SMH and CSR. There are several reasons to believe that deletion induced by the γ2a-macroself Ag might take place in the germinal center. First, the absence of IgG2a-expressing B cells after immunization indicates censorship by some process—deletion is the most likely—and this process must affect B cells that switched class in GCs. Second, the inability to detect IgG2a B cells in normal mice indicates that the censoring occurs rapidly. Third, the dependence of this process on Fas is consistent with this occurring in the GC, where B cells express high levels of Fas. This Fas-dependent deletion of B cells that have acquired self-reactivity would seem to cover a potential gap in the other aforementioned tolerance mechanisms, because it is conceivable that self-reactive B cells through engagement of membrane-expressed self-molecules could interact with both FDCs and activated T cells and escape deletion.

Several possible ways by which deletion occurs can be envisioned. First, because engagement of IgG2a class-switched receptors to the IgG2a-macroself Ag will occur regardless of affinity maturation, B cells that have switched class to IgG2a prior to affinity maturation will be at a competitive disadvantage with B cells expressing other isotypes in acquiring Ag for presentation to T cells. Thus, deletion will occur not because of a lack of T cell helper cells, per se, but because of the inability of self-reactive B cells to generate high-affinity receptors. Another related possibility is that B cells might be unable to adequately internalize Ag because of Ag-receptor binding to macroself Ag on surrounding cells, making them incapable of receiving T cell help. A third possibility is that B cells engaging cell surface self-Ags are deleted by negative selection. This possibility contrasts with the FDC and T cell-mediated tolerance mechanisms, whereby GC B cells undergo apoptosis because of insufficient survival signals (lack of positive selection). Moreover, while negative selection in early B cell development in the bone marrow and in the periphery for immature B cells (T1 subset) is determined solely by affinity threshold, selection in GCs must depend on an additional factor or factors, because BCRs with high affinity to foreign Ags are maintained and positively selected. Of possible relevance is a previous in vitro study showing that prolonged BCR cross-linking induced death of CD40-activated GC, but not similarly activated naive or memory, B cells (37). Although the basis for this differential effect was not determined, it nevertheless suggests an intrinsic difference in the response of GC B cells to BCR engagement. It is also possible that recent GC emigrants might be susceptible to BCR-mediated cell death similar to naive B cells (8, 38). In marked contrast with our findings with GC B cells, however, peripheral deletion of IgM1IgD+ B cells is not dependent on Fas (39).

Our findings contrast with an earlier study involving Tg mice expressing influenza hemagglutinin (HA) as a membrane-bound “neo-self” Ag, which concluded that specificity for self-Ags does not prevent differentiation of autoreactive memory B cells after viral infection (36). However, subsequent analyses by these investigators showed that expression of the HA neo-self Ag on lymphoid cells was low in this Tg line, thereby suggesting Ag ignorance. Indeed, when a line with high HA neo-self Ag expression was assessed, absence of HA-specific IgG Abs after immunization was observed (20), leading to reformulation of the previous conclusion, now in support of clonal deletion. The model, however, did not allow firm conclusions in this regard, because tolerance was also imposed at early B cell developmental stages.
The evidence that isotype-switched IgG2a B cells were deleted in the macroself Ag Tg mice included lack of cytoplasmic IgG2a B cells, absence of IgG2a AFC and NP-binding B cells, and reduction of IgG2a and postswitched germine Igα–γ2a transcripts in immunized mice. Accordingly, Bcl2 Tg B cells transferred into macroself Ag hosts showed increased survival of IgG2a-switched B cells following immunization. Thus, it appears that apoptosis is directly involved in the deletion of autoreactive isotype-switched B cells. This finding is consistent with previous observations that Bcl2 overexpression promoted the survival of B cells expressing a Tg BCR with dual reactivity for the hapten Arsenate and nuclear autoantigens (18, 19), as well as with the development of a lupus-like syndrome in Bcl2 Tg mice (24). By contrast, others showed that apoptotic death of GC B cells provoked by high doses of soluble Ags was not affected by Bcl2 overexpression (13, 14), raising the possibility that Bcl2 instead acts by promoting the survival of self-reactive primary plasma cells. In this case, primary Ab-forming cells and memory B cells were believed to derive from two distinct precursors (40). There is, however, no a priori reason for such differential effects of Bcl2, and indeed our findings support prosurvival effects for both self-reactive IgG2a primary plasma and memory B cells.

Apoptosis of autoreactive B cells can also be induced by an extrinsic pathway primarily through the interaction of the FasL with the Fas receptor (41). Indeed, Fas (lpr) or Fasl (gld) gene mutations in mice are associated with lupus-like manifestations, including high titers of somatically-mutated IgG autoAbs (42, 43).

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Earlier studies with MRL-Fas<sup>jpr</sup> (H-2<sup>k</sup>) Tg for anti-H-2Kk (39, 47) or Fas<sup>jpr</sup> double Tg for HEL (soluble or membrane bound) and anti-HEL BCR (48) showed no clear defects in B cell tolerance, although receptor editing and anergy are predicted to be less efficient (49, 50). These findings suggested that the Fas–FasL apoptotic pathway plays no major role in tolerance during the early B cell developmental stages. By contrast, in these same mice, high levels of IgG autoantibodies to lupus-associated Ags developed with time, suggesting that tolerance defects mapped to the class-switched compartment. Consistent with this conclusion, previous studies with Tg anti-sDNA BCRs showed that in normal mice these B cells were functionally silenced by central tolerance-mediated anergy (51) and by an undefined checkpoint in the GC (52), whereas in MRL-Fas<sup>jpr</sup> mice, such self-reactive B cells were present in the periphery and, through SHM and secondary L chain rearrangement, gained the ability to bind other autoantigens, such as dsDNA and cell nuclei, and thus become pathogenic (53).

More recently, specific targeted deletion of Fas in B cells during class-switch recombination was found to be sufficient to induce lymphoproliferation in (B6xMRL)F1-Fas<sup>jpr</sup> mice (54). Although this finding suggests that B cell tolerance during class switch might be defective, B cell tolerance was not examined (54). Other studies, however, focused on tolerance induction in B cells undergoing a T cell dependent Ab response have concluded that Fas played no role (12, 55, 56). The present findings, made possible by the development of a new macroself Ag model, clearly document that the Fas pathway is the main mechanism responsible for the elimination of autoreactive switched B cells shortly after their generation. Furthermore, because Fas is highly expressed in GC B cells (55) and participates in memory B cell repertoire selection (57), it can be concluded that escape from censoring of autoreactive IgG-switched B cells in Fas mutant mice most likely occurs at this site (51), and also outside GCs at the extrafollicular T zone-red pulp border where autoreactive B cell proliferation and SHM predominates in MRL-Fas<sup>jpr</sup> mice (58).

Importantly, our findings show that tolerance to the γ2-macroself Ag is affected not only by Fas, but also by MRL genes and Bcl-2, further suggesting that the Fas<sup>jpr</sup> and MRL background effects on tolerance are additive. Based on this finding, we believe that defective elimination of newly generated autoreactive B cells post-CR/SHM may be a major underlying cause of lupus and other autoimmune diseases.

An increased frequency of SHM-related low-affinity poly- and self-reactive B cells in the circulating IgG<sup>+</sup>, but not IgM<sup>+</sup> memory pools in normal human individuals, was reported (59, 60). This finding suggests differences in how these two compartments are established and raises the possibility that abnormalities in the activation of self-reactive IgG<sup>+</sup> memory B cells might contribute to the development of autoimmunity in susceptible individuals. In this study, we show that lupus-associated Fas<sup>jpr</sup> mutation results in loss of tolerance to autoreactive class-switched B cells, suggesting that autoimmunity, at least in this case, is more likely mediated by loss of tolerance to newly generated self-reactive B cells rather than the aberrant activation of self-reactive IgG<sup>+</sup> memory B cells.

In the γ2-macroself Ag model, a potential limitation is that interaction of the macroself-Ag with the BCR occurs at the C region rather than at the V region, where normal binding of Ag occurs. Ab binding to the C region, however, activates B cells similar to normal Ag-BCR interactions (61) and has been used to successfully study B cell development and tolerance in other mouse models (62–65). Thus, it is likely that the findings in this study are applicable to B cells that acquire self reactivity to membrane-bound self-Ags during SHM/CSR.

This study with a novel system documents that autoreactive memory B cells emerging after an immune reaction to T-dependent Ags are normally tolerized following isotype CSR and SHM, a finding that is consistent with the existence of a “window of tolerance” during memory B cell repertoire formation (10, 60). It further demonstrates that Fas mutation and associated humoral autoimmunity is characterized by defects in late checkpoints pertaining to memory B cell tolerance. It is likely that proliferation and differentiation of otherwise innocuous, low-affinity autoreactive B cells in the periphery of Fas-defective lupus-prone mice are induced through the simultaneous engagement of BCR and TLR by self-nucleic acids and related immune complexes in concert with dendritic cell and T cell activation (66–68). Considering that B cell tolerance defects are central to the pathogenesis of lupus in predisposed strains of mice (69) and humans (70, 71), the macroself Ag approach offers a versatile system by which these defects, from the earliest to the latest checkpoints, can be identified accurately.

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Disclosures
The authors have no financial conflicts of interest.

References
B CELL TOLERANCE AFTER CLASS SWITCHING


SUPPLEMENTAL MATERIAL

**Supplemental Figure 1.** Deletion of IgG2a-expressing B cells in IgHa γ2a-macroself Ag transgenic mice after antigen priming. (A) Spleen cells derived from C57BL/6-IgHa pURF-Tg and littermate control mice (non-Tg) were stained as described in Fig. 4 except that B220 versus cytoplasmic IgG2a expression was analyzed on Dump-, clgk+ with a rat anti-mouse IgG2a monoclonal antibody at different time points after priming with NP-KLH. Numbers above each set of panels indicate days after antigen priming. The percentage in each quadrant, rounded to the nearest 1%, is indicated in the lower right corners of each plot. (B) Mean±SD numbers of cytoplasmic IgG2a (left panel), IgG2b (middle panel) and IgG1 (right panel) positive B cells as quantified by flow cytometry analysis in spleens of pURF-Tg and littermate control mice (non-Tg). Numbers on the x-axis refer to the days after antigen priming. n=3-5 mice/group at each time point.

**Supplemental Figure 2.** Expression and IgG2aa binding of γ2a-macroself Ag in (B6-Fas(npr)xMRL-Fas(npr))F1-pURF-Tg (lpr-Tg) and non-Tg (lpr-nTg) mice. (A) Surface expression of γ2a-macroself Ag in 1, 2, 4, and 8 wk-old mice. Thymocytes (1 and 2 wk-old mice) or peripheral blood cells (4 and 8 wk-old mice) were stained with biotinylated anti-rat IgG1/streptavidin-FITC and MFI determined by flow cytometry. Each panel represents a separate experiment. MFI levels in thymocytes from 3 day-old pups were 3537±70 for B6-Tg (n=2), 4034±601 for lpr-Tg (n=3) and 491±15 for B6-nTg (n=2) mice. B6-Tg mice were IgHb. (B) Comparison of surface expression of γ2-macroself Ag on peripheral blood cells and thymocytes in 8.5 wk-old mice. Isolated peripheral blood cells and thymocytes were stained and analyzed as above. P<0.05 between lpr-Tg and either B6-Tg or lpr-nTg groups. (C) IgG2aa binding to γ2a-macroself Ag.
Cells, as above, were stained with biotin-conjugated anti-H-2Dq (clone KH117)/streptavidin-FITC. P<0.05 between B6-pURF Tg and (B6-FasIprxMRL-FasIpr)F1-pURF-Tg mice are indicated (unpaired, 2-tailed t-test).

**Supplemental Figure 3.** Loss of tolerance to γ2a-macroself Ag in B6-FasIpr Ighα mice. (A) Serial serum IgG2a concentrations in Tg FasIpr/lpr (Tg lpr/lpr), Tg FasIpr/+ (Tg lpr/+), and non-Tg lpr/+ B6 background mice. IgG2a concentrations determined by ELISA. P<0.05 for Tg lpr/lpr compared with either Tg lpr/+ or non-Tg lpr/+ groups; and between Tg and non-Tg lpr/+ mice. (B) γ2-macroself Ag expression on spleen cells from Tg lpr/lpr, Tg lpr/+, and non-Tg lpr/+ B6 mice at different ages (left panel) and mean±SEM of data (right panel). Isolated spleen cells were stained with anti-rat IgG1 (BD, clone RG11/39.4) and MFI determined by flow cytometry. P<0.05 for Tg lpr/lpr compared with either Tg lpr/+ or non-Tg lpr/+; and between Tg and non-Tg lpr/+ mice.
Supplemental Figure 1
Supplemental Figure 2