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Deletion of IgG-Switched Autoreactive B Cells and Defects in $\text{Fas}^{lp}$r Lupus Mice

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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 $\gamma_2$-reactive superantigen ($\gamma_2$-macroself 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 macroself-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-\text{Fas}^{lp}$r$ lupus mice, suggesting that defective apoptosis of isotype-switched autoreactive B cells is central to $\text{Fas}$ mutation-associated systemic autoimmunity. 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 naive 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 macroself Ag (Ag) (21, 22). This model exploits the striking propensity of B cells to exchange the genes encoding Ig-constant regions during development progression.
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 allelic variant of mouse IgG2a was generated by single chain Fab engineering technology and expressed as a transgene. Because B cells require immunogenic 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 depletion 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<sup>+</sup> mice are defective in this process.

**Materials and Methods**

**Mice and immunization protocol**

C57BL/6 (B6), B6-Igh<sup>+</sup> H-2<sup>b</sup> and MRL-Fas<sup>+</sup> mice were obtained from the Jackson Laboratories (Bar Harbor, ME). MRL-Fas<sup>+</sup> mice were backcrossed to C57BL/6 for at least seven generations. EmbrC3-Tg2-22 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 γ2a-macroself Ag gene constructs**

The V1J and DJ H chain variable genes were amplified by PCR using as templates the plasmids containing genomic DNA of the anti-mouse IgG2a<sup>2a,m</sup> mAb (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′-GCTCGCAGGTACAGCTGAAAGAGTCAGG-3′); primer 2 (3′-GCCACATTGCA-ATCCTAGAGTCACCGAGG-5′); primer 3 (5′-GCCACGGAAGGGGTCCCTGACGCGGAGGCCGAGG-3′); primer 4 (3′-GCCCACTAGTAACTGTAGCTGGTGG-5′); primer 5 (5′-GCCGGCCGGAAGGGGTCCCTGACGCGGAGGCCGAGG-3′); and primer 6 (5′-GCCGGCCGTGCTGCTGCTGGTGG-3′). The overlap- ping sequences are underlined; those recognized by restriction endonucleases are in italics. PCR products corresponding to the L<sub>vi</sub> and V<sub>hi</sub> region were amplified by the primers 1, 2, 3, and 4, respectively. The single-chain Fab gene, including the intervening flexible peptide codons (GLΥ<sub>2</sub>Ser<sub>3</sub>), was assembled by a second PCR step with the outer primers 1 and 4, using as templates the L<sub>vi</sub> and V<sub>hi</sub> PCR products. The PCR product was further cloned as a NruI/Xmal restriction fragment downstream of the human ubiquitin C promoter and upstream of the rat-IgG Fe chain fused to the transmembrane, cytoplasmic exons, and 3′-UTR regions of the H<sub>2</sub>k<sup>+</sup> genomic DNA in the pBluescript II SK plasmid (21).

**Transplantation of human embryonic kidney 293T cells**

HEK 293T cells were cotransfected with PIRES-EFGP plasmid (Clontech, Mountain View, CA) and the plasmid containing the pURF transgene using Lipofectamine/Plus reagent (Invitrogen, Carlsbad, CA) on six-well plates according to the manufacturer’s recommendations. Transfected cells were harvested after 2 d of growth in complete IMDM medium for flow cytometric analysis.

**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).

**Spleen transplantation chimeras**

Recipient mice were pURF-Tg s or littermate controls; all carried the C<sub>57</sub>B/6J background. Mice were injected i.v. with 3 × 10<sup>7</sup> donor splenocytes. The day after transplantation, mice were analyzed for expression of Thy1 and CD45.2+. CD45.2<sup>+</sup> cells were identified using 1× PBS and 0.5 mM EDTA, washed twice and incubated with either a biotin-conjugated mouse anti-rat IgG1, a mouse IgG2a<sub>2a</sub> or a mouse IgG2a<sub>2b</sub> 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 mouse 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 (DUMP channel), and PerCP-coupled anti-CD45R/B220 (RA3-6B2). Surface-stained cells were fixed and permeabilized using a kit (Cytofix/Cytoperm; BD Biosciences, San Jose, CA) and staining according to the manufacturer’s instructions with one of the following: biotin-conjugated anti-IgG2a<sub>2a</sub>-IgG2a<sub>2b</sub>, anti-IgG2a<sub>2a</sub>-IgG2b<sub>2b</sub>, or anti-IgG2a<sub>2b</sub>-IgG2b<sub>2a</sub>. Flow cytometric analysis

**Flow cytometric analysis**

HEK 293T transplanted cells were harvested using 1× PBS and 0.5 mM EDTA, washed twice and incubated with either a biotin-conjugated mouse anti-rat IgG1, a mouse IgG2a<sub>2a</sub>, or a mouse IgG2a<sub>2b</sub> 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 mouse 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 (DUMP channel), and PerCP-coupled anti-CD45R/B220 (RA3-6B2). Surface-stained cells were fixed and permeabilized using a kit (Cytofix/Cytoperm; BD Biosciences, San Jose, CA) and staining according to the manufacturer’s instructions with one of the following: biotin-conjugated anti-IgG2a<sub>2a</sub>-IgG2a<sub>2b</sub>, anti-IgG2a<sub>2a</sub>-IgG2b<sub>2b</sub>, or anti-IgG2a<sub>2b</sub>-IgG2b<sub>2a</sub>. Flow cytometric analysis

**Real-time PCR and quantitative real-time PCR**

Total RNA was purified from the spleens of immunized IgH<sup>+</sup> non-Tg and pURF-Tg mice using the RNeasy Mini kit and RNase-Free DNase Set (Qiagen, Valencia, CA) and cDNA was generated with the SuperScript III First-Strand Synthesis System for RT-PCR kit (Invitrogen) according to the manufacturer’s instructions. PCR reactions were performed in a final volume of 50 µl containing 4-fold cDNA serial dilutions. V<sub>i</sub>γ<sub>2a</sub> PCR products were generated using a sense primer specific for the majority of mouse V<sub>1i</sub> genes (5′-AGGTTGACGACTGCAAGGAGGCTG-3′) and an IgG2a specific antisense located in the hinge region (5′-ATCATGAGTCTCGAAGGAGGC-AGGC-5′). For the detection of IgG2a<sub>2a</sub> and IgG2a<sub>2b</sub> transcripts, the following forward and primers were used: 5′-CTCTGGCCTCCTGATGGACATGTTAATTTCCAACCTGCTGCTGG-3′; primer 4 (3′-V<sub>i</sub>γ<sub>2a</sub>) 5′-GCCAGCCCCCTGGGTCCCTGACGCGGAGGCCGAGG-3′; primer 5 (5′-V<sub>i</sub>γ<sub>2a</sub>) 5′-GCCAGCCCCCTGGGTCCCTGACGCGGAGGCCGAGG-3′; and primer 6 (3′-V<sub>i</sub>γ<sub>2a</sub>) 5′-GCCAGCCCCCTGGGTCCCTGACGCGGAGGCCGAGG-3′. The overlap- ping sequences are underlined; those recognized by restriction endonucleases are in italics. PCR products corresponding to the L<sub>vi</sub> and V<sub>hi</sub> region were amplified by the primers 1, 2, 3, and 4, respectively. The single-chain Fab gene, including the intervening flexible peptide codons (GLΥ<sub>2</sub>Ser<sub>3</sub>), was assembled by a second PCR step with the outer primers 1 and 4, using as templates the L<sub>vi</sub> and V<sub>hi</sub> PCR products. The PCR product was further cloned as a NruI/Xmal restriction fragment downstream of the human ubiquitin C promoter and upstream of the rat-IgG1 Fe chain fused to the transmembrane, cytoplasmic exons, and 3′-UTR regions of the H<sub>2</sub>k<sup>+</sup> genomic DNA in the pBluescript II SK plasmid (21).

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IgG2ab, IgG1a, and IgG1 Ab levels were comparable to those of high and further elevated upon immunization (Fig. 2A).

Spectra MAX250 model reader (Sunnyvale, CA). ELISPOT for detection 48mM citrate, pH 4.6). Absorbance was measured on a Molecular Devices

Biotinylated Abs were

membrane and cytoplasmic regions of the antigen: IgG2ab, IgG1, IgG2b, IgG3. In the sera of macroself Ag Tg all time points after immunization, whereas, as expected, IgG2aa levels in littermate control C57BL/6— except IgG2ab (5.7) and incubated with serial serum dilutions mixed with biotinylated IgG2aa or IgG2ab mAbs. For NP-specific Ab titers, microplates were coated with NP,-BSA (10 µg/ml). After incubation with serial serum dilutions, the plates were treated with biotinylated anti-IgG2aa, anti-anti-IgG2ab, anti-IgG2a, anti-IgG1, or anti-IgG1 monoclonal Abs. Biotinylated Abs were revealed using streptavidin-peroxidase (Sigma-Aldrich) followed by addition of the chromogenic substrate 2, 2’-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (Sigma-Aldrich) in McIlvain’s buffer (84 mM Na2PO4/48mM citrate, pH 4.6). Absorbance was measured on a Molecular Devices Spectra MAX250 model reader (Sunnyvale, CA). ELISPOT for detection

NP-specific IgG2aa and IgG2ab was performed with 96-well multi-screen membrane filtration plates (Millipore, Billerica, MA) coated with NP,-BSA, washed, and blocked; next, 50,000 to 500,000 spleen cells per well were cultured at 37°C in 5% CO2 for 18 h in RPMI 1640 supplemented with 5% FCS. The wells were washed before addition of biotinylated anti-mouse IgG2aa or IgG2ab. After washing, the wells were incubated with streptavidin-HP (Biogen Legend). ELISPOTs were developed using a kit (BD ELISPOT AEC Substrate Set; BD Biosciences). The plates were washed with water, and positives were scored manually using a dissection microscope. Each assay was done in triplicate from at least three separate animals.

**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 IgG2aa,b (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. 1C).

**Absence of IgG2aa 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 IgG2aa response by ELISA assay and flow cytometry. Mice used in these experiments were IgHbb, providing the following control IgG isotypes that do not react with the superantigen: IgG2aa, IgG1, IgG2b, IgG3. In the sera of macroself Ag Tg mice, total IgG2aa Ig levels were essentially at background levels at all time points after immunization, whereas, as expected, IgG2aa levels in littermate control C57BL/6- Ighb/ab (B6-Ighb/ab) mice were high and further elevated upon immunization (Fig. 2A). By contrast, IgG2aa, IgG1, and IgG1 Ab levels were comparable to those of littermate control mice. Similar results were obtained in assays of NP-specific Abs (Fig. 2B). These results indicated that γ2a-macroself Ag specifically affected the generation of serum IgG2aa.

**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 Tg and control mice after priming and recall immunization (Fig. 3A). By contrast, we observed a significant reduction of NP-specific-, B220+-, IgG2aa-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 IgG2aa B cells were...
selectively deleted in γ2a-macroself Ag Tg mice after immunization with a T-dependent Ag.

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 IgHb congeneric (gray lozenges), IgHb littermate control mice (non-Tg, open squares), and IgHb pURF-Tg mice (black circles) after priming and recall immunization. B, Mean ± SD of NPγ-specific-IgG2aa, -IgG2ab, -IgG1a, -IgG2a, and -IgG1 Ab titers measured by ELISA assays on serum derived from IgHb 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 IgHb 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-Ighb congenic (gray lozenges), IgHb littermate control mice (non-Tg, open squares), and IgHb pURF-Tg mice (black circles) after priming and recall immunization. B, Mean ± SD of NPγ-specific-IgG2aa, -IgG2ab, -IgG1a, -IgG2a, and -IgG1 Ab titers measured by ELISA assays on serum derived from IgHb 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\textsuperscript{a/b}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\textsuperscript{high}/CD138\textsuperscript{−} and B220\textsuperscript{high}/CD138\textsuperscript{+} cells were visualized by successive gating on a broad forward scatter versus side scatter window to include B cell blasts, then on PI\textsuperscript{−}, Dump\textsuperscript{−} (CD4\textsuperscript{−}, CD8\textsuperscript{−}, IgD\textsuperscript{−}) to exclude dead cells, T cells, and naive B cells. NP-binding IgG1-positive cells were visualized by gating on B220\textsuperscript{high} cells. The histograms on the right represent the mean numbers of NP-specific and NP-specific B220\textsuperscript{high}/IgG1\textsuperscript{+} cells in non-Tg (gray bars) and pURF-Tg mice (black bars). B, Flow cytometric analysis of NP-specific, B220\textsuperscript{high}/IgG2a\textsuperscript{+} and B220\textsuperscript{high}/IgG2a\textsuperscript{−} 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\textsuperscript{+}, IgG2a\textsuperscript{−}-positive cells and the IgG2a\textsuperscript{+}/IgG2a\textsuperscript{−} 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 IgH<sup>a</sup> allotype were used because on this genetic background, the γ2a-macroself Ag reacts with all IgG2a-expressing B cells, thereby allowing detection of IgG2a<sup>b</sup> B cells with an mAb specific to an epitope different from that recognized by the macroself Ag. 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-macroself Ag are promptly deleted in the spleen after priming and CSR.

**Molecular analysis of IgG2a CSR**

To test further the notion that γ2a-macroself Ag 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. 5A). Consistent with these results, little or no γ2a mRNA was detected by quantitative real-time PCR in the spleens of Tg mice (Fig. 5B). We also quantified the germine γ2a-specific transcripts driven by the I<sub>μ</sub> promoter (I<sub>μ</sub>-γ2a), which is a product of CSR. Because the I<sub>μ</sub> promoter is still active after isotype switch, the amount of transcripts composed of the I<sub>μ</sub> 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 germ-line γ2a transcripts driven by the I<sub>μ</sub> promoter were detected in littermate control mice but, these transcripts remained poorly induced in γ2a-macroself Ag 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-macroself Ag 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-macroself Ag 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-macroself Ag Tg mice served as hosts of wild type IgH<sup>b</sup> donor spleen cells. As with nonmanipulated Tg mice, 2 wk after NP-KLH priming, surface IgG2a<sup>a</sup> B cells were largely depleted, whereas nonreactive IgG2a<sup>b</sup> B cells were readily detected in the spleens of Tg mice (Fig. 6A, 6B). Similarly, using ELISPOT assays, NP-specific IgG2a<sup>a</sup> Ab-forming cells (AFCs) were not detected in the spleen of Tg hosts, whereas the numbers of IgG2a<sup>b</sup> AFC 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-macroself Ag Tg mice undergo rapid deletion, presumably by an apoptotic mechanism.
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<sub>α</sub> cells were substantially rescued in the macroself Ag recipients that received BcI2 Tg cells (Fig. 6A, 6B). Moreover, although the average numbers of NP-specific IgG2a<sub>α</sub> AFCs were one third of those in macroself Ag-free hosts of BcI2 Tg cells, they were similar to those obtained with non-Tg recipients of wild type spleen cells (Fig. 6C). In the absence of macroself Ag, chimeras reconstituted with BcI2 Tg cells had elevated numbers of surface IgG2a<sub>α</sub> and IgG2a<sub>β</sub> B cells compared with wild type mice. These differences also correlated well with the increased numbers of NP-specific IgG2a<sub>α</sub> and IgG2a<sub>β</sub> 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-Fasl<sup>pr</sup> mice fail to delete autoreactive IgG2a B cells**

We next tested whether lupus-prone MRL-Fasl<sup>pr</sup> mice are capable of eliminating γ2a-macrosel f 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-Fasl<sup>pr</sup> had up to 20-fold greater numbers of clgG2a- and clgG2b-expressing B cells compared with B6-Igh<sup>α</sup> mice, consistent with Fasl<sup>pr</sup>-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<sup>low</sup>/clgG2a<sup>low</sup>) and plasma cell (B220<sup>high</sup>/clgG2a<sup>high</sup>) compartments were markedly reduced in macroself Ag Tg compared with non-Tg B6-Igh<sup>α</sup> mice. In striking contrast, clgG2a B cells were abundantly present in MRL-Fasl<sup>pr</sup> 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-Fasl<sup>pr</sup> γ2a<sub>α</sub>-macrosel f 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<sub>α</sub> through the placenta or milk could have bound to and neutralized the γ2a-macrosel f Ag in offspring of MRL-Fasl<sup>pr</sup> mice. Indeed, low levels of cell surface γ2a-macrosel f Ag not bound by IgG2a were detected when peripheral blood cells from adult MRL-Fasl<sup>pr</sup> γ2a-macrosel f 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 γ2a-macroself Ag, we measured serum allotype-specific IgG2a in 1- to 8-wk-old F1 littermates from female B6-Faslpr (IgG2ab) crossed with MRL-Faslpr (IgG2aa) males heterozygous for the γ2aa-macroself Ag transgene; whereby mothers are IgG2ab and non-Tg, and offspring are IgG2aa/b with approximately half being γ2aa-macroself Ag-positive (Fig. 7C). In the non-Tg (B6-FaslprxMRL-Faslpr) F1 pups, IgG2a was initially detected at 3 wk of age with levels progressively increasing at 4 and 8 wk. Similarly, IgG2a was first detected at 3 wk in γ2aa-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 γ2aa-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 IgG2a in the (B6xMRL)F1-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 IgG2a from weeks 3 to 8. A similar breach of tolerance to the γ2aa-macroself Ag was also observed in Fas-deficient B6-Igha macroself Ag Tg mice, but appeared less complete. Serum IgG2a, although present, was at significantly lower concentrations in Tg compared with non-Tg B6-Faslpr mice at 80 d (Fig. 7D, Supplemental Fig. 3). These findings indicate that loss of tolerance to the γ2aa-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 IgG2a, is not caused by blocking of the γ2a-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-

FIGURE 7. MRL-Fas<sup>lpr</sup> γ2a-Tg mice fail to delete IgG2a-expressing B cells after NP-KLH priming. Six week-old MRL-Fas<sup>lpr</sup> and MRL-Fas<sup>lpr</sup> pURF-Tg mice were analyzed by flow cytometry for cytoplasmic IgG2a and IgG2b expression 2 wk after immunization with NP-KLH in RIBI adjuvant. Age-matched B6-Igh<sup>a</sup> pURF-Tg and littermates (non-Tg) were used as controls. A, Spleen cells from the indicated mice were analyzed by flow cytometry for B220<sup>+</sup>, cytoplasmic IgG2a, and IgG2b-expressing spleen B cells as described in Fig. 4 except that an anti-TcRβ was added in the Dump channel to counter select double-negative T cells. Top panels are Dump<sup>−</sup> cells. The percentage in each gate, rounded to the nearest 1%, is indicated. B, Mean ± SE of total, NP-specific, and anti-chromatin IgG2a Abs in the serum of naive and immunized littermate B6-Igh<sup>a</sup> and MRL-Fas<sup>lpr</sup> controls and pURF-Tg mice, 14 d after Ag priming. Three to five mice were analyzed in each group. C, Allotype-specific serum IgG2a in (B6-Fas<sup>lpr</sup>× MRL-Fas<sup>lpr</sup>)F1 pURF mice born to IgG2a<sup>a</sup> allotype (B6-Fas<sup>lpr</sup>) mothers. IgG2a levels below the detection limit are indicated as <1 μg/ml. p < 0.0001 between nTg and Tg mice. D, Allotype-specific serum IgG2a and IgG1 in Fas-deficient B6-Igh<sup>a</sup> pURF Tg mice. Littermates for this experiment were (B6-Igh<sup>a</sup> Faslpr/× B6-Igh<sup>a</sup> Faslpr/+ pURF Tg)F1 mice. IgG2a concentrations <1 μg/ml were below detection. n = 5–9 mice, except 35 d Tg lpr/+ where n = 2. p < 0.05.
derived cells induces specific deletion of Ag-specific IgG2α B cells soon after primary immunization. Moreover, IgG2α 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 IgG2α B cells is not due to Ag receptor downregulation, that tolerance induction is solely dependent on expression of the IgG2α 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 IgG2α 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 IgG2α isotype subset. This dual effect of autoreactive B cell deletion combined with simultaneous expansion of nonautoreactive B cells may be central to efficient deletion of IgM+IgD+ B cells is not dependent on Fas (39).

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 FDCs and activated T cells and escape deletion. Several possible ways by which deletion occurs can be envisioned. First, because engagement of IgG2α class-switched receptors to the IgG2α-macroself Ag will occur regardless of affinity maturation, B cells that have switched class to IgG2α 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-Ag 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.

Table I. IgG2α-switched B cells escape tolerance in MRL-Fas<sup>bw</sup> γ2a-macroself Ag mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of Positive Cells/10&lt;sup&gt;6&lt;/sup&gt; B Cells (Mean ± SE)</th>
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<tbody>
<tr>
<td></td>
<td>B220&lt;sup&gt;hi&lt;/sup&gt;/IgG2α&lt;sup&gt;low&lt;/sup&gt;</td>
</tr>
<tr>
<td>Non-Tg</td>
<td>1 ± 0.2</td>
</tr>
<tr>
<td>pURF-Tg</td>
<td>15 ± 4.5</td>
</tr>
<tr>
<td>pURF-Tg</td>
<td>0.03 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MRL-Fas&lt;sup&gt;bw&lt;/sup&gt; (5)</td>
<td>25 ± 6</td>
</tr>
</tbody>
</table>

Boldface indicates significant (p < 0.05) differences between MRL-Fas<sup>bw</sup> compared with B6-Igh<sup>b</sup> mice in nontransgenic and transgenic mouse. Number of mice analyzed indicated in parenthesis.

<sup>a</sup>p < 0.05 between pURF transgenic compared with wild type littermate of the same genetic background.
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 Fas 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). Similarly, autoimmune accompanies Fas or Fasl mutations in humans with the autoimmune lymphoproliferative or Canale-Smith syndromes (44). Functional complementation studies have clearly shown that absence of Fas expression on B cells is responsible for most of the serologic and histologic abnormalities in MRL-Fas lpr mice (45, 46). Earlier studies with MRL-Fas lpr (H-2k) Tg for anti-H-2Kk (39, 47) or Fasl double Tg for HEL soluble 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-dsDNA 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 lpr 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 became 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 (B6×MRL)F1-Fas lpr 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 lpr 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 lpr and MRL background effects on tolerance are additive. Based on this finding, we believe that defective elimination of newly generated autoreactive B cells postCSR/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+, but not IgM+ 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+ memory B cells might contribute to the development of autoimmunity in susceptible individuals. In this study, we show that lupus-associated Fas lpr 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+ 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 tolerated 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
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-, cIgk+ 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-lpr×MRL-Fas-lpr)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) IgG2aα 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-\textit{Faslpr}x\textit{MRL}-\textit{Faslpr})F1-pURF-Tg mice are indicated (unpaired, 2-tailed t-test).

**Supplemental Figure 3.** Loss of tolerance to γ2a-macroself Ag in B6-\textit{Faslpr} Igha mice. (A) Serial serum IgG2a concentrations in Tg \textit{Faslpr/lpr} (Tg lpr/lpr), Tg \textit{Faslpr/+} (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 3