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Chronic Graft-Versus-Host in Ig Knockin Transgenic Mice Abrogates B Cell Tolerance in Anti-Double-Stranded DNA B Cells

Debora R. Sekiguchi,* Sandra M. Jainandunsing,§ Michele L. Fields,† Michael A. Maldonado,* Michael P. Madaio,‡ Jan Erikson,† Martin Weigert,§ and Robert A. Eisenberg‡*

Anti-dsDNA Abs are specific diagnostic markers of systemic lupus erythematosus, and are also implicated in kidney pathology. Anti-dsDNA B cells have been shown to be tolerated in nonautoimmune mice. The immunodysregulation that causes these cells to break tolerance is presumably part of the fundamental defects in systemic lupus erythematosus. To explore these mechanisms, we used the chronic graft-versus-host model mediated by MHC class II differences. Induction of chronic graft-vs-host in anti-DNA H chain knockin (3H9.KI) transgenic mice on a nonautoimmune background resulted in specific activation of anti-dsDNA B cells, as evidenced by high titers of soluble Ab in sera and a high frequency (70%) of anti-dsDNA B cell clones recovered as hybridomas. In addition, the λ†-anti-dsDNA B cells developed increased expression of cell surface activation markers, and concentrated in the T cell area of the follicle with an Ab-forming cell-compatible phenotype. Genetic analysis of the hybridoma clones showed strong evidence of secondary rearrangements of the L chain associated with anti-dsDNA reactivity. Thus, our study indicates that alloreactive T cell help can break tolerance in a complex manner, involving several events. The Journal of Immunology, 2002, 168: 4142–4153.

Systemic lupus erythematosus (SLE) is an autoimmune disease that affects multiple organ systems in association with a spectrum of autoantibodies that target normal proteins and nucleic acids. Anti-dsDNA Abs in particular are characteristic of the syndrome and in fact are a diagnostic marker, since these Abs are rarely found in individuals who do not suffer from this disease. In addition, some of the pathology of SLE, especially nephritis, is most likely mediated by anti-dsDNA Abs (1). For these reasons, the elucidation of the mechanisms of tolerance failure for this specificity is critical for understanding the underlying immunodysregulation of SLE. Investigations conducted on Ig transgenic mice demonstrate that anti-DNA B cells are actively regulated (tolerized) in nonautoimmune mice (2–8). In spontaneous autoimmune mice, such tolerance is lost (9–12).

To define the mechanisms of tolerance loss in SLE B cells, we used the chronic graft-vs-host (cGVH) model. In our model, SLE is induced by transferring alloreactive splenic cells from nonautoimmune bm12 mice into coisogenic, nonautoimmune B6 recipients (bm12→B6) (13). The donor and recipient cells differ by 3 aa on their MHC class II molecules, and this difference is sufficient to make the two strains fully alloreactive. Previous work showed that cognate recognition of recipient B cells by alloreactive donor CD4 T cells produces a gVH reaction specifically characterized by high titers of autoantibodies of the typical SLE specificities (14–23) and proliferative glomerulonephritis (13, 24, 25). The experimental nature of this system enabled us to time precisely, and to characterize, tolerance failure in B cells.

As a means of determining how allo-T help induces tolerance loss in anti-DNA B cells, specifically those that recognize dsDNA, we combined the cGVH model with an H chain Ig transgene (tg), 3H9. This H chain plays a dominant role in determining anti-DNA specificity. Since most mouse κ-chains sustain DNA binding in combination with the 3H9-H chain (30% both ssDNA and dsDNA; an additional 30% only ssDNA) (26), the 3H9tg alone is informative, and allows us to study anti-DNA B cell regulation in the context of B cells with other specificities.

Our study utilized the 3H9 knockin (3H9.KI) model, in which the chromosomal JH locus has been replaced with the rearranged V(D)J 3H9tg. In contrast to conventional tgs, this KI system permits the transgenic locus to undergo normal editing (6), isotype switching, and somatic mutation. This enables us to analyze, under more physiologically accurate conditions, how allo-T help affects these and other B cell processes, as well as at which stages such intervention occurs.

In previous studies using this KI, one of us (M.W.) showed that a population of anti-dsDNA B cells was tolerized by undergoing receptor editing mainly at the L chain, generating peripheral B cells that carry a different specificity (7). By inducing cGVH in the 3H9.KI, we disrupted anti-DNA B cell tolerance, and were thereby able to begin to dissect in detail the mechanisms by which this T cell help induces B cell autoreactivity characteristic of SLE.
Our data indicated that secondary rearrangements at the L chains played a crucial role in generating anti-dsDNA B cells, and that cGVH induced tolerance failure in the periphery. This raises the possibility that the anti-dsDNA B cell population arose as a product of reediting in the periphery, as was the case in 3H9.KI.MRL/lpr mice (11). It is equally possible that some anergic cells generated during rearrangement were activated in the periphery. We also found that allo-T help activated the entire B cell population, but, due to the restriction of Ab production under cGVH (16, 17), this did not appear sufficient to produce Abs, thereby suggesting that Ag recognition is required. Focused analysis of a specific anti-dsDNA population (\(\lambda^\delta\)) showed that allo-T help activated these cells, inducing high expression of molecules involved in T-B cell interaction. Parallel histological studies indicated that under cGVH, these anti-dsDNA-specific cells, including those that had differentiated to Ab-forming cells (AFC), migrated to the T cell area within the spleen. These results begin to clarify the events characteristic of the loss of B cell tolerance in systemic autoimmune.

**Materials and Methods**

**Mice**

The development of 3H9.KI (or site-directed) transgenic mice has been previously described (6). The tg has been backcrossed onto the nonautoimmune C57BL/6 (B6) background for at least six generations (unless otherwise stated) to engender 3H9.KI.B6 mice (3H9(+) ). The presence of the tg was determined by PCR amplification of tail DNA with primers specific for 3H9 (2).

Nonautoimmune B6 and coisogenic B6.C-H2-H-10 (H11001/KhEg (bm12) mice, as well as B6-IgG Km12/H11003 mice, were originally obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were 2–5 mo at the time of cGVH initiation. In all cases, age-matched B6 mice or 3H9.KI-negative littermates (3H9(–)) were used as controls. All mice were bred and maintained in our mouse colony at the University of Pennsylvania Medical Center.

**Experimental cGVH disease protocol**

cGVH disease was induced as previously described (13). Briefly, recipient mice on a B6 background were injected i.p. with single-cell suspensions of \(1 \times 10^8\) bm12 donor splenocytes, prepared by pressing donor spleens through a wire mesh screen in HBSS.

All experimental GVH mice developed anti-sDNA and anti-dsDNA autoantibodies. All negative control mice were negative for autoantibodies (n = 15).

**Follow-up of mice**

Blood samples were obtained from experimental mice just before the induction of cGVH disease and at 1- to 4-wk intervals thereafter. Sera were stored at \(-20^\circ\)C for later analysis.

**Detection of autoantibodies in sera by ELISA**

Sera were tested at a dilution 1/500, unless otherwise stated.

**Anti-dsDNA**

Expression of anti-dsDNA Abs was determined via solid-phase ELISAs similar to those previously described (25, 27). Plates were coated with optimal concentrations of autoantigens. Biotinylated goat anti-mouse IgG (Fc-specific; Jackson ImmunoResearch Laboratories, West Grove, PA) was added as a secondary Ab. The following autoantigens were used.

- dsDNA: calf thymus DNA. Before the event characteristic of the loss of B cell tolerance in systemic autoimmunity.

C for 10 min and cooled on ice quickly. 2) dsDNA: calf thymus DNA.

Anti-dsDNA-\(\lambda^\delta\). See below.

**Allootype-specific anti-dsDNA**

The allootypes of IgG2a anti-dsDNA Abs were tested by assays similar to that for anti-dsDNA, except that the sera were diluted 1/250. The assays were developed with rabbit anti-mouse preadsorbed allootype reagents (anti-IgG2a\(\delta\) or anti-IgG2a\(\delta\); Accurate Chemical & Scientific, Westbury, NY) and detected with alkaline phosphatase (AP) anti-rabbit IgG Ab (Jackson ImmunoResearch Laboratories).

**Total serum IgG2a**

Total serum IgG2a of the a or b allotype was measured by coating the plates with anti-mouse F(ab’)2, at a concentration of 0.2 \(\mu\)g/well. The sera were diluted to 1/50,000. The assays were developed as above (allotype-specific anti-dsDNA).

**Evaluation of nephritis**

The presence and severity of nephritis were determined by light microscopy on H&E-stained sections, as previously described (28). The severity of glomerular, tubular, and vascular lesions was determined independently in a blinded manner by one of us (M.P.M.) and scored on a semiquantitative scale (0−4+: absent, mild, moderate, severe). Multiple sections at a minimum of two different levels were observed. Each section typically involved evaluation of over 50 glomeruli and >25 blood vessels, and the interstimus contained two to three longitudinal sections of kidney.

**Immunofluorescence staining**

The following conjugated Abs were purchased from BD PharMingen (San Diego, CA): FITC/PE/biotin anti-B220 (RA3-6B2), FITC anti-CD21 (7G6), FITC anti-IgA1/2 (R26-46), FITC anti-IgG1 (A85-1), PE anti-B7-2 (GL1), PE anti-CD23 (B3B4), PE anti-CD24 (M1/69), PE anti-IgD\(\delta\) (217-170), PE anti-IgM\(\delta\) (DS-1), biotin anti-Fas (Jo2), biotin anti-IgD\(\delta\) (AMS 9.1), biotin anti-IgM (II/41), and streptavidin-CyChrome. Biotin anti-class II (3137) and anti-FcγRc (2.4G2, used for blocking) were grown in our laboratory, as was 1.209, an anti-idiotypic Ab specific for 3H9 CH in combination with most \(\kappa\) L chains (4) (J. Erikson, unpublished data). PE anti-\(\alpha\) (JC5) was a gift from Dr. J. Kearney (University of Alabama, Birmingham, AL).

Cell surface staining was routinely performed with age- and sex-matched controls, as previously described (29). A total of 1.5 × 10^6 cells was blocked with 2.4G2. The cells were then incubated with directly labeled Abs for 30 min and washed. An additional 20-min incubation with streptavidin-CyChrome was performed to detect biotinylated Abs. Cells were fixed in PBS containing 1% paraformaldehyde and analyzed on a BD Biosciences FACScan (Mountain View, CA). Relative fluorescence intensity was plotted on a logarithmic scale using CellQuest software.

In analyzing the \(\lambda^\delta\) cells, we took into consideration that the majority of IgA B cells in mice carrying the conventional 3H9tg are IgA1. Although this has not been tested in the 3H9.KI mice, we followed Mandik-Nayak et al. (8, 12) by using pan anti-IgA reagents to identify 3H9/91 B cells.

**Generation of hybridomas**

To induce cGVH, a 3H9.KI mouse (backcrossed three times onto the B6 background) was injected with bm12 spleens following standard protocols (see above). The presence of anti-DNA Abs in sera was corroborated 10 wk postinjection, and hybridomas were generated as described elsewhere (5). In brief, spleen cells from the immunized mice were fused without further manipulation to the Sp2/0 myeloma line. Hybridomas were plated at limiting dilution, and wells bearing single colonies on 96-well plates were expanded. Hybridomas secreting Ig were selected for further study.

**ELISA on hybridomas**

**Ig secretion in supernatants.** Isotype and Ig concentration in culture supernatants were determined using a solid-phase ELISA, as described previously (30). In brief, to determine the isotype, plates were coated with optimal concentrations of uncoated anti-total Ig, and serial dilutions of supernatants from individual hybrids were added. Binding was detected through further incubation with anti-IgM, or anti-IgG coupled to AP and developed with p-nitrophenyl phosphate. Ig concentration was determined by comparing samples with a standard curve generated by a titrated isotype-matched Ab.

**DNA binding.** Binding to dsDNA was measured by a two-step fluid-phase binding ELISA, as described previously (3, 31). The Ig concentration was normalized for each tested sample. Biotinylated dsDNA was obtained as described by Radic et al. (3). Appropriate concentrations of Ab and
biontinated dsDNA were mixed, incubated, and transferred to avidin-coated microtiter plates. Bound DNA-Ab complexes were detected with AP-conjugated anti-mouse isotype-specific Ab.

To test λ-anti-dsDNA in sera, the same procedure was followed, but serial dilutions of sera and biontinated dsDNA were mixed. The DNA-Ab complex was detected with AP-conjugated anti-λ Ab.

**PCR assays on hybridoma DNA**

Genomic DNA was purified from individual hybrids, as described elsewhere (30). A total of 100 ng DNA was used in each reaction. Primers and conditions for H and L chain PCR assays were as detailed previously (6, 30, 32).

**H chain PCR assay.** The presence of 3H9 H chain tg was identified by PCR amplification using primers complementary to the 3H9 H chain leader exon (the LD3H9) and the complementarity-determining region 3 (CDR3) sequence (2).

**k L chain PCR assay.** For the Jk typing PCR assays, V Schlissel (33) or L5 forward Vκ primers were used with Jk2, or Jk5 reverse-Jk primers (34). The V Schlissel PCR primer should amplify 80–90% of Vκ genes. The size of the PCR products corresponds to the Jk segment participating in the rearrangement event (32).

**λ L chain PCR assays.** λ Rearrangements were amplified using the VA1/2 + JA1 primer combination (30).

**Sequence analysis of H chain genomic DNA**

The H chain V regions were sequenced from DNA according to protocols described previously (11). In brief, DNA was isolated and amplified by PCR. The PCR product was gel purified and sequenced employing an automated sequence analyzer. The 5’ primers used were the LD3H9, which binds V genes with leader sequences similar to those used by the 3H9tg, in combination with a primer located in the J3H9-Cμ intron. This PCR is not as specific as the LD/CDR3 PCR and amplifies 3H9.KI-tgs with mutations in CDR3 region as well as VH replacements in which the invading VH gene uses a 3H9-like leader sequence.

**Immunohistochemistry**

Spleens were processed as previously described (8). Sections were stained with anti-CD22 (Cy3.1-FITC or biotin) and anti-CD4 (GK1.5-FITC or biotin; BD PharMingen), and anti-λ-AP (Southern Biotechnology Associates, Birmingham, AL). FITC- and biotin-conjugated reagents were then detected with the secondary Abs anti-FITC-AP (Sigma-Aldrich) or anti-FITC-HRP (Chemicon, Temecula, CA), and streptavidin-AP or streptavidin-HRP (Southern Biotechnology Associates), respectively.

**Spleen/bone marrow (BM) transfer**

Recipient IgM+ mice were sublethally irradiated with 300 rad to avoid the host-vs-graft reaction (35), and injected i.v. with single-cell suspensions of 5 × 10^5 spleen or BM cells from 3H9.KI mice, prepared by pressing donor spleens through a wire mesh screen in HBSS.

**Statistics**

Statistical significance was determined using an unpaired nonparametric Mann-Whitney U statistic test to determine kidney disease; χ^2 for our hybridoma analysis; and t test for analysis of our ELISAs.

**Results**

Three mechanisms of B cell regulation have been demonstrated in Ig transgenic mice: receptor editing, clonal deletion, and functional inactivation (anergy). To determine which mechanisms are affected, and how anti-DNA B cells lose tolerance through allo-T cell help, we induced cGVH in 3H9.KI mice that had been backcrossed onto the B6 background for at least six generations. Unirradiated 3H9.KI recipients were injected with bm12 spleen cells from mice of the same sex (bm12→3H9(+)). Our two negative control groups consisted of unmanipulated 3H9.KI mice (3H9(+) and 3H9.KI mice that received syngeneic B6 spleen cells (B6→3H9(+)). The 3H9-negative littersmates that had received bm12 spleen cells (bm12→3H9(−)) were also included as a positive cGVH control.

**Induction of anti-DNA Abs in transgenic mice by cGVH**

To establish the validity of the model, the above groups were bled monthly, and the sera were tested by ELISA for the presence of anti-ssDNA and anti-dsDNA IgG Ab. The data shown in Fig. 1 represent one of two comparable experiments, each utilizing five mice per group. Within the first 8 wk after cGVH induction, high levels of both anti-ssDNA (p ≤ 0.05) and anti-dsDNA (p ≤ 0.05) Abs were found in the 3H9.KI mice (bm12→3H9(+)), as compared with non-cGVH controls. No anti-DNA Abs were detected in any of the negative control groups. Thus, the provision of allo- genetic T cell help induced a failure of anti-DNA B cell tolerance in the 3H9.KI mice.

Fig. 1A shows that levels of anti-ssDNA Abs remained similar in the bm12→3H9(+) mice and in the positive cGVH controls (bm12→3H9(−)) throughout the peak response time of the experiment (4–8 wk). In contrast, the peak of anti-dsDNA Abs was significantly higher in the bm12→3H9(+) group than in the nontransgenic cGVH control, bm12→3H9(−) (Fig. 1B; also Fig. 1C) (p ≤ 0.05). This suggests that the presence of a population of anti-DNA B cells in the 3H9.KI mice allowed allogenic T cell help to induce anti-dsDNA IgG Abs selectively.

To define more precisely the kinetics of anti-dsDNA Ab induction during the first 2 mo of cGVH, mice in a separate experiment were bled weekly. The peak time of serum anti-dsDNA was 2 wk and showed levels even higher than those found in the sera of diseased MRL/lpr mice used as positive controls (OD = 0.9) (Fig. 1C).

**Development of kidney disease in cGVH transgenic mice**

Anti-dsDNA IgG Ab production has been implicated in renal disease in SLE (1). Mice were therefore sacrificed at peak anti-DNA Ab titer (2–3 wk), and their kidneys were evaluated for the presence and severity of nephritis (0–4+ scale) (28). Glomerular, interstitial, and vascular scores were each significantly higher in the bm12→3H9(+) mice than in the unmanipulated 3H9(+) animals (p ≤ 0.05; Fig. 2). Overall, the severity of kidney disease in the experimental mice (bm12→3H9(+)) was comparable to that seen in the concomitant nontransgenic cGVH controls (bm12→3H9(−)). This means that neither the higher titers of anti-dsDNA Abs in the sera of bm12→3H9(+), nor the significant increase in the frequency and number of anti-dsDNA B cells, influenced the severity of end-organ damage.

The effect of cGVH on the expression of 3H9 H chain in the 3H9.KI mice

The 3H9.KI system allows B cells to undergo secondary rearrangement at the H chain (6). Thus, the 3H9tg can either be replaced by another V region from the same chromosome, or it can be inactivated, and the other allele expressed. To assess the persistence of this H chain tg, we stained spleen cells with the 1.209 anti-idiotypic mAb that recognizes the 3H9 H chain in conjunction with most, but not all, L chains (4) (data not shown). FACS analysis showed that in unmanipulated 3H9.KI mice, the great majority of splenic B cells expressed the 3H9 H chain. This did not change under cGVH (Fig. 3A). In addition, staining with allotypic anti-IgD reagents confirmed that both before and after cGVH, nearly all B cells expressed the transgenic (knocked in) a-allotype locus uniquely (Fig. 3B), rather than the b-allotype from the other chromosome. These data therefore indicated that the general B cell repertoire is largely determined by the 3H9tg with its strong bias toward DNA reactivity.

**Phenotype of B cells in the 3H9.KI mice after cGVH induction**

We next determined the phenotypic changes that occurred in B cells during cGVH. Previous work has shown that 60% of the L...
FIGURE 1. Induction of anti-DNA Abs in 3H9.KI mice by cGVH. Groups tested: experimental GVH group, bm12 spleen cells injected into 3H9.KI recipients (bm12–>3H9(+), ●). Positive GVH control: bm12 spleen cells injected into 3H9.KI-negative littersmates (bm12–>3H9(−), △). Negative controls: syngeneic B6 spleen cells injected into 3H9.KI-positive mice (B6–>3H9(+), □); B6 spleen cells injected into 3H9.KI-negative littersmates (B6–>3H9(−), ○); 3H9.KI without injection (3H9(+), *). The presence of anti-DNA Abs in sera was tested by ELISA. Results represent means ± SEM. * Statistical difference (p < 0.05) from the appropriate non-cGVH-negative control. In A and B, sera were assessed monthly. Presence of anti-ssDNA IgG Abs was measured in A and presence of anti-dsDNA IgG Abs in B. When the titers of bm12–>3H9(+) mice were compared with those of our positive cGVH control, a significant difference was detected at wk 4 and 8 (p < 0.05), for dsDNA only. A second experiment yielded comparable results. Each experiment utilized n = 4–5 mice per group. C, A third experiment in which sera were assessed weekly to determine the peak time of anti-dsDNA Ab production. At 2, 3, 4, and 8 wk, anti-dsDNA titers in bm12–>3H9(+) were significantly higher than in bm12–>3H9(−) (p < 0.05). MRL/lpr sera tested at the same concentration gave an OD of 0.9 for anti-dsDNA. Experimental group, n = 11 mice; control groups, n = 5. In the experiment shown here (in A and B), a large percentage of bm12–>3H9(+) mice died, resulting in the termination of this group after 20 wk. This result was not reproducible.

Our study also followed CD21 and CD23, since changes in these molecules had been reported in other murine lupus models (12, 40–43). Both CD21 and CD23 showed decreased expression following cGVH. The change in the CD23low B cell population may be especially significant. Following cGVH induction in normal B6 mice, a large population of these cells was noted. A more dramatic increase was observed after the induction of cGVH in 3H9.KI mice (Fig. 4B). Furthermore, when our study targeted a specific anti-dsDNA B cell population (λ− cells; see Fig. 7), an even greater percentage of these CD23low cells was found, both before and after cGVH induction. Thus, the decreased levels of CD23 may be relatively specific for those B cells that are autoreactive. These data indeed correlate with reports in other lupus models (12, 44, 45).

Secondary rearrangements of L chains in cGVH

Secondary rearrangement at the L chain has been shown to occur in transgenic mice whose tg codes for autoantibodies. This process is manifested by the prominence of λ L chain B cells in anti-H-2k/H-2k transgenic mice (46), and by a bias of B cells employing the downstream Jκ5 segment in 3H9 transgenic mice (3, 7).

To investigate the use of L chain genes in the cGVH reaction, we generated monoclonal hybridomas from the splenic cells of a bm12–>3H9(+) mouse. We tested for secondary rearrangements using a previously described series of PCR assays (6, 30, 32). Most clones produced anti-dsDNA Abs, and the majority of the anti-dsDNA+ cells (65%) used the 3H9tg (3H9tg(+)). (These data, as well as the 3H9tg(−) clones, are further analyzed and discussed in

chains that bind the 3H9 H chain give rise to anti-DNA B cells (26); 40% of the L chains will therefore create non-DNA B cells. This predicts that if the non-DNA population were resting, it would be distinguished by our analysis. However, in the 3H9.KI undergoing cGVH, the entire B cell population showed an activated phenotype (see Fig. 4A). The presence of anti-DNA Abs in sera was tested by ELISA. Results represent means ± SEM. * Statistical difference (p < 0.05) from the appropriate non-cGVH-negative control. In A and B, sera were assessed monthly. Presence of anti-ssDNA IgG Abs was measured in A and presence of anti-dsDNA IgG Abs in B. When the titers of bm12–>3H9(+) mice were compared with those of our positive cGVH control, a significant difference was detected at wk 4 and 8 (p < 0.05), for dsDNA only. A second experiment yielded comparable results. Each experiment utilized n = 4–5 mice per group. C, A third experiment in which sera were assessed weekly to determine the peak time of anti-dsDNA Ab production. At 2, 3, 4, and 8 wk, anti-dsDNA titers in bm12–>3H9(+) were significantly higher than in bm12–>3H9(−) (p < 0.05). MRL/lpr sera tested at the same concentration gave an OD of 0.9 for anti-dsDNA. Experimental group, n = 11 mice; control groups, n = 5. In the experiment shown here (in A and B), a large percentage of bm12–>3H9(+) mice died, resulting in the termination of this group after 20 wk. This result was not reproducible.

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Among the anti-dsDNA⁺ 3H9tg⁺ hybridomas, 7 (64%) of 11 of the typosable functional rearranged Vκ segments were joined to Jκ5. This frequency was significantly higher than the Jκ5 frequency (6–25%) found in sets of hybridomas derived from nonautoimmune mice (47, 48) (p ≤ 0.05) or in our anti-dsDNA⁻ 3H9tg⁻ clones (p ≤ 0.05; Table I). In addition, the same anti-dsDNA⁺ 3H9tg⁺ population also showed an increase in Jκ usage (29%). It is important to clarify that 30 of the 93 Vκ regions, as well as Vα1, can sustain dsDNA binding when paired with the 3H9 H chain (Ref. 26 and our unpublished data). Therefore, if any L chain at random were to generate anti-dsDNA cells, we should see a Vκ:Vα ratio of 30:1 within the subpopulation. Instead, we found a ratio significantly higher, i.e., 3:1, which suggests that molecular mechanisms favored rearrangement toward λ-chains. Overall, both the increased Jκ and the increase in Jκ5 strongly support the conclusion that this population has undergone extensive secondary rearrangements.

Analysis of λ⁺ peripheral B cells

The skewing of the hybridomas toward the 3H9/A1 population in cGVH is of particular interest, as the 3H9/A1 combination is known to bind dsDNA (31). In nonautoimmune mice carrying the conventional 3H9tg, cells with this B cell receptor appear to be regulated based on the persistence in the periphery of λ⁺ cells with an altered phenotype and localization, and the absence of λ⁻-anti-dsDNA⁻ Abs in sera (8). In the present model, these cells emerged as important producers of anti-dsDNA Abs, so we analyzed them in more detail. Since we had shown that the 3H9 H chain is expressed in most splenic B cells in our model (Fig. 3A, see bm12→3H9(+) mice), and we also knew that the great majority (83%) of the λ⁺ cells in our hybridomas paired with the 3H9 H chain (Table I), we reasoned that most A1 cells in this study would in fact be 3H9/A1. We therefore used specific λ⁺ reagents to follow these cells (see Materials and Methods).

We first confirmed that the λ⁺ B cells in vivo in bm12→3H9(+) mice were actually secreting anti-dsDNA Abs (Fig. 5). In fact, the levels of λ⁺-anti-dsDNA⁺ Abs in the sera of bm12→3H9(+) mice were significantly higher than those seen in our positive cGVH control group (bm12→3H9(−)) (p ≤ 0.05). We then compared the phenotype of the λ⁺ cells before and after cGVH induction (Fig. 6). In the tolerant 3H9.KI mice (which, as expected, did not produce λ⁺-anti-dsDNA Abs; Fig. 5), the λ⁺ B cells were present in the periphery with levels of surface Igλ decreased ~3-fold relative to the B6 control (mean fluorescence intensity; Fig. 6, 18 vs 52). Following cGVH, these cell surface Ig levels remained reduced. The continued reduction of Igλ levels in our model suggested that these cells were still chronically encountering Ag under the cGVH reaction (8, 49–51). Fig. 6 shows, additionally, that the percentage of λ⁺ B cells increased after cGVH in 3H9.KI. This was consistent with the high frequency of λ⁺ cells found in our hybridoma data.

Activation status and localization of λ⁺ cells after cGVH induction

We used the same phenotypic reagents as previously (Fig. 4) to investigate the activation/developmental status of the λ⁺ B cells. Before cGVH, λ⁻ B cells in the 3H9.KI showed an activated phenotype: increased size, and higher levels of class II and B7-2 compared with the B6 control mice (Fig. 7). These data were consistent with previous findings in other tolerant 3H9 transgenic mice, and probably reflect some activation by Ag encounter (8). The λ⁺ cells in the 3H9.KI also seemed mature; they expressed levels of B220 and CD24 similar to those seen in the B6 mouse, and were IgD⁺ (Fig. 6).

After cGVH induction, levels of class II, B7-2, and Fas on λ⁺ cells were elevated in the 3H9.KI. The fact that these cells also increased in size suggested that allo-T help resulted in a high level

FIGURE 2. Kidney disease in 3H9.KI mice following GVH. Recipient mice were sacrificed 2–3 wk after cGVH induction. H&E-stained kidney samples were examined by light microscopy and scored blindly for disease on a semiquantitative scale from 0 (normal) to 4 (maximum) for the glomeruli (A), interstitia (B), and vessels (C). Each symbol represents an individual animal. Black horizontal bars indicate mean values for each group. Scores for both GVH groups were significantly higher than those for negative controls (p ≤ 0.05). Scores of bm12→3H9(+) mice did not differ significantly from those of our positive GVH control mice (p ≤ 0.05). Symbols for each group are the same as in Fig. 1.
of activation in 3H9/λ cells (Fig. 7). The up-regulation of molecules such as B7-2 and class II may enable the λ⁺ B cells to interact more strongly with the T cells and provide increased signaling. These data are consistent with two different interpretations: either anergic λ⁺-anti-dsDNA cells are further activated by allo-T cells, or the λ⁺-anti-dsDNA cells are created de novo and, at the same time, fully activated by the cGVH process. At this point it is difficult to determine which of these possibilities is correct.

Finally, we conducted studies to determine whether any alterations in the splenic architecture correlated with the loss of B cell tolerance (Fig. 8). For the most part, B and T cells are located in discrete areas in the spleen, called the B cell follicle and periarteriolar lymphoid sheath (PALS), respectively. Spleen sections from 3H9(+) and bm12→3H9(+) mice were stained with anti-B220 to determine the position of these anti-dsDNA B cells, with anti-CD22 to mark B zones, and with anti-CD4 to mark T zones. In all tolerant (non-cGVH) 3H9.KI mice, most λ⁺ B cells have been found clustered at the T-B interface, as was also the case in nonautoimmune BALB/c mice carrying the conventional 3H9tg (8). Consistent with the FACS data, an apparent increase in λ⁺ B cells was observed 2 wk after the induction of cGVH in bm12→3H9(+) mice. Additionally, a high concentration of darkly stained λ⁺ cells was present in the PALS, as well as in the bridging channels of the red pulp. This pattern of darkly staining cells, which presumably reflects intracytoplasmic Ig, has been correlated with AFC in several models (12, 52–55). Curiously, the same pattern was also observed in bm12→3H9(−) mice. In addition, both the spleens and the follicles within them were larger in cGVH mice.

**Initial location of B cell tolerance breakdown under cGVH**

To determine whether the abnormal T help of cGVH breaks tolerance in the BM or in the spleen, we transferred BM or spleen cells from 3H9.KI into IgM knockout mice (IgM−/−) on the following day. In both types of recipient, the production of IgG2a (KI-allotype) Abs confirmed that the donor 3H9-B cells engrafted appropriately (data not shown). Spleen cell recipients produced anti-dsDNA Abs 2 wk after cGVH. These initial data strongly suggest that splenic B cells can and do lose tolerance under cGVH, producing SLE Abs (Fig. 9). By contrast, BM cells failed to react. This could be due to an insufficient quantity of transferred BM cells, or perhaps the cells required more time to develop and should be tested at a later time point. However, it
Table I. L and H chain usage in 3H9.KI hybridomas after cGVH induction* (38 clones)

<table>
<thead>
<tr>
<th>Anti-dsDNA+ (n = 26 (68.5%))</th>
<th>Anti-dsDNA– (n = 12 (31.5%))</th>
</tr>
</thead>
<tbody>
<tr>
<td>J1 (1 (6%))</td>
<td>J1 (1 (11%))</td>
</tr>
<tr>
<td>J2 (3 (17%))</td>
<td>J2 (2 (22%))</td>
</tr>
<tr>
<td>J3 (1 (11%))</td>
<td>J3 (1 (11%))</td>
</tr>
<tr>
<td>J4 (1 (11%))</td>
<td>J4 (1 (11%))</td>
</tr>
<tr>
<td>J5 (7 (41%))</td>
<td>J5 (2 (22%))</td>
</tr>
<tr>
<td>A1 (5 (29%))</td>
<td>A1 (0)</td>
</tr>
<tr>
<td>Untyped (3 (17%))</td>
<td>Untyped (3 (33%))</td>
</tr>
</tbody>
</table>

**L chain usage**

<table>
<thead>
<tr>
<th>3H9g+ (n = 17 (65%))d</th>
<th>3H9g– (n = 9 (35%))d</th>
<th>3H9g+ (n = 3 (25%))d</th>
<th>3H9g– (n = 9 (75%))d</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4 (44%)</td>
<td>1 (33%)</td>
<td>1 (11%)</td>
</tr>
<tr>
<td>0</td>
<td>1 (11%)</td>
<td>1 (11%)</td>
<td>2 (66%)</td>
</tr>
<tr>
<td>0</td>
<td>1 (11%)</td>
<td>1 (11%)</td>
<td>2 (66%)</td>
</tr>
</tbody>
</table>

* Monoclonal hybridomas were derived from unmanipulated 3H9.KI spleen cells from a single mouse 10 wk after cGVH induction. Ig-secreting hybridomas were assayed for dsDNA binding, persistence of the 3H9g and L chain DNA rearrangement. Preliminary data from 3-wk hybridomas shows a similar skew to downstream L chain usage in the 3H9g+ population. Fifty-three percent of these hybridomas are IgM and 47% are IgG. Both groups show a similar tendency in secondary L chain rearrangement in anti-dsDNA B cells.

**Discussion**

The autoimmune syndrome of cGVH results from cognate recognition of host B cells by alloreactive donor T cells (14, 15). The subsequent loss of B cell tolerance leads to production of typical SLE autoantibodies (16, 17). To investigate this process of tolerance breakdown, we focused on anti-dsDNA B cells, a population strongly implicated in SLE. Our approach was to induce cGVH in anti-DNA.KI mice (a system that has the crucial advantage of sustaining processes vital to B cell definition: secondary rearrangement, isotype switching, and somatic mutation). We first determined the process by which anti-dsDNA B cells arose in the general B cell population under the reaction and then defined the effects of allo-T help on targeted anti-dsDNA B cells.

**Activation steps involved in the breakdown of B cell tolerance in cGVH**

By distinguishing stages in the process of B cell activation, our study begins to elucidate the complex linkage between Ag exposure and tolerance breakdown under cGVH. On the one hand, the FACS data (Fig. 4A, top row) showed an initial activation of the entire B cell population following allo-T help provision. Since the 3H9.KI repertoire contains non-DNA-binding as well as DNA-binding B cells, part of the activation of the host B cells by the donor T cells was presumably not constrained by the specificity of the individual B cells. In fact, the same pattern also occurred in the classical (nontransgenic) cGVH model (Fig. 4A, bottom row). In addition, we observed similar changes in the hen egg lysozyme model, given cGVH, in both class II and size (40).

On the other hand, autoantibody specificity in cGVH is restricted. Autoantibodies characteristic of SLE, such as those directed to nuclear Ags and DNA, have been found to be consistently present in sera of cGVH mice, while autoantibodies not typical of SLE, such as those directed at thyroglobulin and insulin, or other tissue-specific Ags are absent (16, 17). This restriction of autoantibody specificity has been interpreted as indicating an active role for Ag. Our sera and hybridoma data also supported this interpretation. Serological data showed that titers of the Abs that recognized dsDNA increased disproportionately to those that recognized ssDNA, pointing to selective activation by Ag. Hybridoma data showed strong skewing to anti-dsDNA in this (65% of our hybridomas bound dsDNA) and a related KI-transgenic cGVH model (manuscript in preparation).

These studies suggest, therefore, that the B cell activation that characterizes the systemic autoimmune syndrome of cGVH is a complex, multistep process. The phenotypic changes most likely correspond to a lower level of polyclonal activation affecting all the B cells, while the actual secretion of Ab may correspond to a higher level requiring a specific additional stimulus associated

**FIGURE 5.** Induction of λ-anti-dsDNA Abs in 3H9.KI mice by cGVH. Prebleed (0 wk) and peak (2 wk, 3 wk) anti-dsDNA sera from the mice used in the experiment described in Fig. 1C were further analyzed to test for the presence of λ-anti-dsDNA Abs. Symbols represent individual mice. Gray horizontal bars indicate the mean value for each group. The phenotypic changes most likely correspond to a lower level of polyclonal activation affecting all the B cells, while the actual secretion of Ab may correspond to a higher level requiring a specific additional stimulus associated.
with Ag. This predicts an Ag-driven stage, one likely correspondent with oligoclonal expansion and IgG isotype switching. In fact, additional data from analyses of the hybridomas provide evidence for this stage (manuscript in preparation).

Secondary L chain gene rearrangements occur during the generation of anti-dsDNA B cells in cGVH

Analysis of Ig gene usage in hybridomas from the bm12→3H9(+) mice indicated that a very high frequency of the anti-dsDNA B cells retaining the 3H9tg had undergone secondary L chain rearrangement. Forty-six percent of all anti-dsDNA clones showed evidence of repeated L chain rearrangements: 27% used the most downstream Jγ region, Jγ5, and 19% utilized λ. This suggestion of L chain rearrangement is consistent with previous reports on MRL/lpr mice (9), as well as with data from SLE patients showing evidence of secondary rearrangement in peripheral B cells (56). Together these findings point toward a paradoxical possibility: that secondary rearrangements may play a crucial role not only in tolerizing, but also in generating autoreactive B cells during autoimmunity.

Perhaps the most important questions here are: at what point does this secondary rearrangement occur? Specifically, does it occur before or after the cGVH reaction is initiated? At what stage of maturity are the B cells that lose tolerance as a result of allo-T help? We propose three major possibilities that, although distinct, may not be mutually exclusive. Indeed, all of these may play some role in cGVH.

The first scenario derives from the idea that allo-T cell help could function not only by providing activation signals, but also by increasing recombination-activating gene (RAG) expression, thereby actually inducing B cells to reinitiate rearrangement in the periphery. Several groups have shown that immunization increases RAG expression in B cells in peripheral organs (38–60). Although it remains unclear whether this occurs through gene reexpression or through peripheralization of immature cells (11, 58, 59, 61–65), these results raise the possibility that RAG could be reinduced in mature or Ab-secreting B cells. Our data may support this possibility. When we transferred spleen cells into IgM−/− (B cell-deficient) recipients, and then induced cGVH, we saw anti-dsDNA Abs as soon as 2 wk after cGVH induction, suggesting that tolerance loss can take place in the periphery under cGVH. Additionally, the frequency of secondary rearrangement of L or H chains in hybrids was higher than that found in the mature

FIGURE 7. Phenotypic analysis of splenic \( \lambda^+ \) B cells in 3H9.KI before and after cGVH induction. Spleen cells from mice in Fig. 2 were gated on lymphocytes by scatter, B220, and \( \lambda^+ \) (as indicated in Fig. 6A), and analyzed for: class II, cell size, B7-2, Fas, B220, CD24, IgD, or CD23. Results are representative of five mice. bm12→3H9(+) (heavy line), 3H9.KI (dotted histogram), and B6 (dotted line). Activation markers are shown in the top panel, and developmental markers are shown in the bottom panel.
population by FACS. Frequency of $\lambda^+$ B cells was 13% in hybrids vs 4.4% by FACS; 35% of our hybridomas were $3H9_{tg}$, compared with a much smaller percentage of $3H9$ idiotype-negative B cells recorded by FACS. Given that our FACS analysis does not recognize AFC, these results could indicate that the secondary rearrangements occurred in a selected population of activated peripheral cells: those that fuse into hybridomas, and perhaps those that convert into AFC. Finally, work in humans (57) as

![Figure 8](image8.png)

**FIGURE 8.** Localization of $\lambda^+$ B cells before and after cGVH induction in $3H9_{KI}$ mice. Histological spleen sections from Fig. 2 mice are grouped by column: bm12→$3H9(-)$ mice (left), $3H9(+)$(middle), and bm12→$3H9(+)$(right). In each column, the top two photographs are from one mouse and the bottom two photographs are from another of the same group. For each mouse, both sections were stained with Abs against $\lambda$ (blue), the upper section with Abs against CD22 (B cells, red), and the lower with Abs against CD4 (T cells, red). In tolerant nonautoimmune $3H9_{KI}$ mice, $\lambda^+$ B cells accumulate at the T-B interface. The darkly stained cytoplasm in some $\lambda^+$ cells corresponds to findings in AFC. Sections are representative of results from all mice used in Fig. 2 ($n = 5$ mice/group). Original magnification, $\times 100$.

![Figure 9](image9.png)

**FIGURE 9.** Transfer of B cells susceptible to loss of tolerance through cGVH. $IgM^{-/-}$ mice (B cell deficient) were reconstituted with either BM or spleen (Spl) cells from $3H9_{KI}$ mice; and cGVH was induced on the following day (bm12→$IgM^{-/-}$ BM$^{H_{10}}$, and bm12→$IgM^{-/-}$ Spl$^{H_{10}}$, respectively.) As negative controls, $IgM^{-/-}$ mice were reconstituted with either BM or spleen cells and injected with syngeneic B6 spleen cells (B6→$IgM^{-/-}$ BM$^{H_{10}}$, and B6→$IgM^{-/-}$ Spl$^{H_{10}}$, respectively). As another negative control, unreconstituted $IgM^{-/-}$ mice were injected with bm12 spleen cells (bm12→$IgM^{-/-}$, □). The $3H9_{KI}$ mice were injected with spleen cells from bm12 mice (bm12→$3H9(-)$, ○) as a positive control. The production of total IgG2a Ig confirmed that the donor B cells engrafted appropriately (data not shown). $IgG$ (A) and $IgG2a$ allotype (B and C) anti-dsDNA in sera was tested by ELISA. $n = 5$ group for bm12→$IgM^{-/-}$ BM$^{H_{10}}$ and bm12→$IgM^{-/-}$ Spl$^{H_{10}}$; $n = 3–4$ group for all other groups. In B, no statistical difference was observed between bm12→$IgM^{-/-}$ Spl$^{H_{10}}$ and bm12→$3H9(+)$. At wk 3 and 4, a, Statistical difference (p ≤ 0.05) from the negative control group B6→$IgM^{-/-}$ Spl$^{H_{10}}$.
well as autoimmune mice (11) points to peripheral loss by reediting mechanisms.

Within the scope of this model, several differences help explain why the ubiquitous allo-T help provided by cGVH may affect only SLE Abs, making them more likely to trigger autoimmunity. First, since editing ordinarily occurs in immature B cells, the edited anti-dsDNA B cells should have encountered the self-Ag in the BM milieu. Therefore, tolerance loss in the periphery by cGVH re-editing (as proposed above) could generate de novo creation of anti-DNA B cells, which had never gone through a stage of tolerance. Second, the ability of Abs to acquire de novo reactivity for DNA Ags in the periphery is facilitated at a molecular level. Many proteins and Abs can bind DNA through active basic amino acids, especially arginine. Since the codon bias in the CDRs favors mutation to arginine, there is a high probability of their becoming anti-DNA during clonal expansion or rearrangement (66). Finally, SLE Ags seem to possess a different structure than normal proteins; their backbones and multivalent epitopes may facilitate interactions with multiple B cell receptors, leading to strong signaling that can occur in the periphery. Therefore, it is possible that both the characteristics of DNA binding, as well as the diversity of, and rearrangement process in, Ig genes may explain the prevalence of these specificities in induced autoimmunity.

A second, possibly complementary scenario, also consistent with the data presented above, is that anergic B cells in the spleen could actually be rescued and stimulated to secrete autoantibodies. Based on the absence of $\lambda^\nu$-anti-dsDNA Abs in sera and the presence of $\lambda^\nu$ cells in the periphery, it has been suggested that $\lambda^\nu$ cells in 3H9 transgenic mice are regulated by anergy (8). In this scenario, the allo-T help of cGVH would further stimulate these cells to secrete autoantibodies, counteracting mechanisms leading toward their death by the induction of survival factors. Indeed, in vitro studies do show survival and activation of these cells with T help reagents. Additionally, hybridomas stimulated with T help reagents appear to rescue these cells (67). Taken together, these data make this scenario a distinct possibility.

Under the third scenario, receptor editing in the BM would generate some B cells possessing an autoreactive specificity. Regulation of anti-dsDNA B cells by editing was shown in different anti-DNA transgenic mouse models that began with an abnormally high precursor frequency of anti-DNA B cells, and possessed a higher dsDNA affinity (3–5, 7). The established efficiency of receptor editing (3–5, 7, 46, 68, 69) implies that, in the course of this process, only cells with special characteristics (for example, $\lambda^\nu$ and other cells that are final products of editing) could arise as autoreactive. Alternatively, some cells may not be subject to tolerization by editing. The data shown in Fig. 9B are also consistent with the possibility that these rare edited B cells can exit to the periphery as transitional cells. If these cells were autoreactive, rather than being deleted by Ag, they could be triggered to produce autoantibodies in the presence of allo-T help. Still, whether immature and transitional cells can actually be affected to produce Abs is not yet known.

The loss of anti-dsDNA B cell tolerance can be tracked in a population expressing $\lambda$ L chains

Finally, to determine more precisely the effects of allo-T help on anti-dsDNA B cell regulation, we monitored the subset of cells expressing A-chains. This specific population is known to produce anti-dsDNA Ab in combination with the 3H9 H chain. It had three advantages: it had been previously defined (31); it could be effectively identified within this system (8, 12); and it was most likely the product of secondary rearrangement at the L chain. We compared nonautoimmune (B6) 3H9.KI mice, in which these anti-dsDNA B cells are tolerated, with those mice in which autoimmunity was induced by cGVH. We looked at serum autoantibodies, as well as changes in surface phenotype, splenic localization, and ability to differentiate into AFC.

Our findings in the 3H9.KI on a nonautoimmune background (B6) confirmed what had been previously reported in another non-autoimmune (BALB/c) background using the conventional 3H9tg (8), namely that these anti-dsDNA B cells are regulated. This was indicated by the absence of the $\lambda^\nu$-anti-dsDNA Abs in the serum. These tolerant cells were present in the periphery with a unique surface phenotype: uniformly low surface Ig (8) and features of activation. These results reproduced those of the above-mentioned study and were consistent with the hypothesis that these cells are tolerantized after being activated by Ag. On the other hand, there was a difference between our results and those from the conventional transgenic model. Whereas in the earlier experiments, anti-dsDNA B cells had been reported to be developmentally arrested, those in the 3H9.KI were mature (IgM+, IgD−, B220high, CD24low). Two factors may account for this: either constraints imposed by the different tgs (KI vs conventional) may have played a role, or the fact that our Ig was on a B6 rather than a BALB/c background may have allowed further development.

As explained above, the provision of allo-T cell help either generated these anti-dsDNA cells de novo, or altered their anergic fate. Under cGVH, they were no longer consistently tolerized, but some produced anti-dsDNA Abs. In this study, we have documented major phenotypic features that accompany this functional change. First, the $\lambda^\nu$ cells still showed low levels of surface Ig following cGVH induction. In this work, it is important to note that these Ig expression levels were similar to those observed in the tolerant control cells. Since the extent of down-regulation has been correlated with the amount of self Ag available (70, 71), these similar expression levels suggest that this anti-dsDNA population continued to encounter the same amount of Ag after allo-T help was provided (under cGVH). Second, these cells also showed surface markers consistent with an increased activation level. In conclusion, both these factors, the encounter with Ag at levels consistent with those found in the tolerized population, and increased activation, appear to be necessary conditions for the $\lambda^\nu$-anti-dsDNA cells to mature fully into AFC. By contrast, Ag recognition alone may not be sufficient, and would be expected to leave the $\lambda^\nu$-anti-dsDNA B cells in a state of incomplete activation.

Finally, our histological data revealed further interesting changes. In the tolerized state, the $\lambda^\nu$ B cells were mostly restricted to the T-B cell interface (Fig. 8) (8). Following cGVH induction and autoantibody secretion, an increased frequency of $\lambda^\nu$ cells was seen. These cells were found scattered in the PALS and appeared to be AFC. This occurred not just in the bridging channels, as would be expected (53, 54), but also in high concentration in the T cell area. The same pattern of AFC cell distribution has been observed previously by Marshak-Rothstein and colleagues (72) in another cGVH model, as well as in the conventional anti-DNA 3H9 transgenics on an autoimmune background (12, 55). Further investigation should help determine what causes these AFC to congregate in the T cell area.

Interestingly, a similar distribution of $\lambda^\nu$-AFC has been observed in our nontransgenic cGVH mice (bm12–$\sim$3H9 (−)). Although we do not know whether or not $\lambda^\nu$ cells in this control group are autoreactive, a high frequency of cells appears to undergo secondary L chain rearrangements in SLE (56, 73), and these may represent autoreactive B cells in addition to those that are anti-dsDNA.

In conclusion, this study has shown that the cognate allo-T help provided by cGVH breaks anti-dsDNA B cell tolerance in anti-
DNA.KI transgenic mice, and has characterized the events associated with that tolerance breakdown. The high frequency of anti-dsDNA B cells generated in our model was most likely the result of secondary rearrangements at L chains. This strongly suggests, contrary to expectation, that secondary rearrangement could play a critical role not only in tolerizing, but also in generating the autoreactive B cells implicated in SLE. Our second relevant observation is that, in the CGVH model, autoreactive cells lose tolerance in the periphery. Therefore, we propose that CGVH induces reediting in mature or Ag-producing cells, creating a new population of anti-dsDNA cells, and that allo-T help, in combination with Ag, activates them fully. At the same time, autoreactive cells in the periphery could also be stimulated by allo-T help. Secondary rearrangement at the L chain may result in an increased frequency of \( \lambda^+ \) cells, producing a population of anti-dsDNA B cells that is present in the periphery. Up-regulation of molecules involved in T-B cell interaction, as well as the ability of these cells to move to the T cell area of the follicle, appeared to be important factors in tolerance breakdown in this population. Future studies will seek to further characterize the mechanisms and steps involved in this process.

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References
