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Autoreactive T Cells Revealed in the Normal Repertoire: Escape from Negative Selection and Peripheral Tolerance

Jun Yan and Mark J. Mamula

Self-reactive T cells are known to be eliminated by negative selection in the thymus or by the induction of tolerance in the periphery. However, developmental pathways that allow self-reactive T cells to inhabit the normal repertoire are not well-characterized. In this investigation, we made use of anti-small nuclear ribonucleoprotein particle (snRNP) Ig transgenic (Tg) mice (2-12 Tg) to demonstrate that autoreactive T cells can be detected and activated in both normal naive mice and autoimmune-prone MRL lpr/lpr mice. In contrast, autoreactive T cells of nonautoimmune Tg mice are tolerated by Tg B cells in the periphery. In adoptive transfer studies, autoreactive T cells from MRL lpr/lpr mice can stimulate autoantibody synthesis in nonautoimmune anti-snRNP Tg mice. Transferred CD4 T cells migrate to regions of the spleen proximal to the B cell follicles, suggesting that cognate B cell-T cell interactions are critical to the autoimmune response. Taken together, our studies suggest that anti-snRNP B cells are important APCs for T cell activation in autoimmune-prone mice. Additionally, we have demonstrated that anti-snRNP B cell anergy in nonautoimmune mice may be reversed by appropriate T cell help. The Journal of Immunology, 2002, 168: 3188–3194.

T cell development in the thymus undergoes both positive and negative selective processes, in which they are programmed for self-MHC restriction and elimination of potentially self-reactive cells (1–4). However, studies in animal models of T cell-mediated autoimmunity show that these processes in the thymus may not be sufficient for controlling self-reactive T cells. For example, analysis of self-reactive TCR transgenic (Tg) mouse strains has established that as many as 25–40% of autoreactive T cells escape clonal deletion even in the presence of the deleting ligand (5). Studies of organ-specific autoimmune diseases such as multiple sclerosis and type-1 diabetes mellitus reveal that autoimmunity and potentially pathogenic self-reactive T cells are present in the normal peripheral T cell repertoire (6, 7). Therefore, these autoreactive T cells are not efficiently deleted during normal development in the thymus and/or periphery. However, one important implication of this concept is whether the remaining autoreactive T cells may be activated and functionally competent to create autoimmunity. In addressing this hypothesis, we investigated the physiological status of autoreactive T cells in the normal repertoire of naive mice. In essence, what is the clinical significance of self-reactive T cells in the normal repertoire, and to what extent can these cells be activated and cause autoimmune disease? The importance of B cells as autoantigen presenting cells has been extensively studied in our laboratory and by other investigators (8–17). In B cell-deficient autoimmune-prone MRL lpr/lpr mice, populations of activated T cells are virtually eliminated as compared with wild-type (wt) MRL lpr/lpr mice (12), suggesting that B cells play a central role in the activation of autoreactive T cells. In the present studies, we have used Ig Tg mice, in which B cells have specificity for a target of lupus autoimmunity, the small nuclear ribonucleoprotein particle (snRNP; Ref. 18). We find that autoreactive snRNP-specific T cells can be activated from normal naive mice and MRL lpr/lpr mice. Anti-snRNP B cells tolerate T cells in Tg B10.A mice, but activate T cells in MRL lpr/lpr mice. Most importantly, we find populations of autoreactive T cells in the thymus of Tg B10.A mice while peripheral T cells are anergized, suggesting that tolerance induction is mediated by B cells in the periphery. Finally, autoreactive T cells from MRL lpr/lpr mice could provide a source of in vivo help for anti-snRNP B cells to make autoantibodies in Tg B10.A strains of mice. Thus, this system provides us the unique opportunity to examine how B cells can either activate or tolerate autoreactive T cells in the normal and autoimmune-prone repertoire.

Materials and Methods

Mice

Anti-snRNP Ig Tg mice were derived using the rearranged VDJ segment of the 2-12 anti-snRNP hybridoma cloned upstream in a vector containing the Cμ region gene segment (18). Tg mice have been backcrossed for >10 generations to C57BL/6, B10.A, or MRL lpr/lpr mice. The presence of the transgene was identified by PCR analysis of tail DNA using 2-12-specific primers 5′-GAGGTCCAGCTGCAGTCTGGA-3′ and 5′-GAGGTCCAGCTGCAGTCTGGA-3′ complementary to the XbaI site of JH4. Prior studies demonstrated that 92% of B cells possessed the transgene H chain (Ref. 18 and our unpublished observations). Animals are age- and sex-matched in all experiments, and are housed in a conventional facility at Yale University (New Haven, CT).

Purification of recombinant snRNP D protein (rSm-D) fusion protein

rSm-D expressed in Escherichia coli was used as stimulating Ag in T cell proliferation assays (19). rSm-D was purified by anion and cation exchange chromatography as previously described (20), and was absorbed for potential mitogens by anti-LPS column chromatography using agarose beads coated with polymyxin B (Pierce, Rockford, IL). In some experiments,
rSm-D was labeled with biotin for FACS staining or tissues-section staining.

**T cell proliferation assays**

Splenetic CD4 T cells were purified by negative selection, using anti-mouse B220, anti-mouse MHC class II, anti-mouse CD11b, and anti-mouse CD8 microbeads (Miltenyi Biotec, Auburn, CA), or positive selection using anti-mouse CD4 microbeads. Thymic CD4 T cells were purified by two-step selection. In brief, thymocytes were incubated with anti-CD8 microbeads and then run through the LS column. The depleted fraction was incubated with anti-CD4 microbeads. The thymic single-positive CD4 cells were positively selected by magnetic separation. B cells were purified by negative selection, using anti-mouse CD43 and anti-CD90 microbeads. The purity of CD4⁺ T cells or B cells was >94% as assessed by flow cytometry.

Conventional T cell proliferation assays were performed with CD4⁺ T cells (2 × 10⁵/well) and irradiated purified B cells (500 rad) as APCs at 5 × 10⁵/well. All assays were performed with triplicate samples and incubated with or without Ag for 3 days. Lymphocyte proliferation was assessed by ³H-thymidine incorporation (1.0 μCi/well, ICN Chemicals, Irvine, CA) during the last 18 h of culture. Sample wells were harvested onto filters, and incorporated radioactivity was counted in a Betaplate liquid scintillation counter (Wallac, Gaithersburg, MD).

**In vitro studies or in vivo administration of CFSE-labeled CD4 T cells**

CD4 T cells purified from B10.A or MRL lpr/lpr (>16-wk-old) Tg or non-Tg mice were labeled with the fluorescent dye CFSE (Molecular Probes, Eugene, OR, Ref. 21). Briefly, the cells were suspended at 5 × 10⁶/ml in prewarmed PBS containing a final concentration of 10 μM CFSE and incubated for 30 min at 37°C. The cells were washed once in FCS and twice in PBS before transfer. CD4 T cells (20 × 10⁶) were transferred i.v. via tail-vein injection. At 24 h or 5 days after transfer, spleens and lymph nodes were harvested from the recipients for tissue immunofluorescence and flow cytometry. For in vitro studies, CFSE-labeled CD4 T cells were cocultured with 2-12 Tg B cells at 5% CO₂ 37°C incubator. After 3 days incubation, cells were harvested for FACS analysis.

**Tissue immunofluorescence**

Spleens were suspended in OCT, frozen in 2-methyl-butane cooled with dry ice, sectioned, and fixed with acetone. The sections were blocked using PBS/3% BSA, and then stained with anti-B220-PE overnight at 4°C to detect follicular B cells. Following three washes with PBS/1% BSA, sections were mounted with Fluoromount-G and then visualized by laser scanning confocal fluorescence microscope (Zeiss Axiovert 100 M; Zeiss, Oberkochen, Germany).

**Flow cytometry**

Cells (1 × 10⁶) were surface-stained with various mAbs by conventional methodology. The following Abs were used: anti-B220-cy5, anti-heat-stable Ag-FITC, anti-CD4-PE, anti-B7-1-FITC, anti-B7-2-PE, and anti-IgM-FITC (BD Pharmingen, San Diego, CA). All samples were analyzed on a FACSCalibur flow cytometry (BD Biosciences, Mountain View, CA) using CellQuest software. Between 10,000 and 20,000 events were collected within a live lymphocyte gate set based on forward and side scatter.

**Results**

**Anti-snRNP 2-12 B cells are not deleted in either normal or autoimmune-prone mice**

The 2-12H transgene construct used to generate the Tg mice consists of the 2-12 VDJ cloned upstream of the Igα Allotype Cμ exon. An anti-snRNP B cell hybridoma, designated 2-12, was originally cloned from an MRL lpr/lpr mouse. Abs from the 2-12 hybridoma are specific for the D protein of murine snRNPs and to denatured DNA (22). To determine whether anti-snRNP autoreactive B cells develop and reach the periphery, three-color immunofluorescence of spleen cells with anti-B220, anti-IgM, and biotinylated rSm-D was performed. The percentage of IgM⁺ cells that stain with rSm-D ranges from 15–30% in B10.A, C57BL/6, and MRL lpr/lpr 2-12 Tg mice (Fig. 1), consistent with our previous studies performed in C57BL/6 mice (19). The specificity of the Tg B cells binding to snRNP D protein was confirmed with cold Ag competition (data not shown). In such assays, unlabeled snRNP reduces the binding of biotin-rSm-D by >80%. Control recombinant fusion protein failed to block snRNP D-specific binding to B cells. Thus, anti-snRNP Tg mice have significant numbers of snRNP D-specific B cells in the periphery, demonstrating that these cells are not actively deleted in either normal mice or in autoimmune-prone MRL mice.

**Autoreactive T cells in normal naive mice and MRL lpr mice are activated by 2-12 Tg B cells**

It has been suggested that B cells could promote T cell activation and inflammatory disease by several mechanisms. Because cognate T-B interactions are crucial for the development of lupus autoimmunity (23–27), we hypothesized that B cells may exert their effects on CD4⁺ T cells through autologous presentation. Toward this end, we used anti-snRNP B cells as APCs to examine the status of autoreactive T cells inhabiting the repertoire of normal and autoimmune-prone mice. T cell proliferation was examined with B cell APCs from anti-snRNP Ig Tg animals.

As illustrated in Fig. 2A, CD4 T cells from B10.A wt mice did not respond to rSm-D protein presented by autologous non-Tg APCs, but had a significant response to 2-12 Tg B cells either with or without rSm-D protein. This observation indicated that self-reactive T cells inhabit the normal repertoire, and can be activated by appropriate snRNP-presenting APCs. The ability of wt B10.A T cells to respond to Tg B cells is likely due to specific Ag-processing functions of these cells. We hypothesize that Tg B cells, by virtue of their surface receptor specificities, present a unique group of snRNP D peptides which T cells in the non-Tg mice have never contacted and which have never been deleted. CD4 T cells originating from B10.A Tg mice failed to respond to peptide presented by either non-Tg or Tg B10.A B cells, suggesting that these autoreactive T cell subsets have been anergized in Tg mice, presumably by Tg B cells. All T cell responses were inhibited by the presence of Abs to class II MHC (Ref. 19 and data not shown). It is unlikely that T cell responses in this study are due to autologous
mixed lymphocyte reaction because the final data panel (T cells and APCs from Tg B10.A mice) shows no detectable proliferative responses.

We next labeled CD4 T cells with CFSE in cultures with 2-12 Tg B cells to further confirm that autoreactive T cells from wt mice are able to divide in vitro from stimulation with 2-12 Tg B cells.

As seen in Fig. 2B, CD4 T cells from wt mice went through five rounds of division compared with no division of “tolerized” CD4 T cells from 2-12 Tg B10.A mice. Together, these results demonstrate that autoreactive T cells can be found in the normal repertoire of naive mice by activation with Ag-specific APC.

These observations were in contrast to similar experiments performed with the autoimmune-prone MRL lpr/lpr mice. As in B10.A mice, CD4 T cells from MRL lpr/lpr mice or Tg mice failed to respond to snRNP protein presented by B10.A wt APCs (Fig. 3A). However, T cells from MRL lpr/lpr mice or Tg mice significantly responded to peptide presented by B10.A Tg B cells (Fig. 3A). This observation is in sharp contrast to T cell responses of B10.A Tg mice, indicating autoreactive CD4 T cells are not tolerized or deleted by Tg B cells in MRL lpr/lpr Tg mice. This notion is supported by the analysis of T cell clones from 2-12 Tg MLP mice that respond to individual snRNP D peptides.4

T cells from either Tg or wt B10.A mice responded significantly to snRNP peptide presented by Tg MRL lpr/lpr B cells, but not from MRL lpr/lpr wt B cells (Fig. 3B). This outcome implies that autoreactive T cells are not deleted in B10.A Tg mice and that “tolerance” can be overcome in vitro by Tg MLP B cells as APCs.

**Autoreactive T cells are tolerized in the periphery of 2-12 Tg normal mice**

We next investigated whether the tolerance induction of autoreactive T cells is mediated by 2-12 Tg B cells and the sites where tolerance occurs. The expression of tolerance or autoimmunity was dependent on either the presence of anti-snRNP Tg B cells and/or the background, B10.A vs MRL. With these two variables in mind, we investigated whether tolerance was mediated centrally in the thymus or in the periphery. CD4 T cells were purified from the thymus or spleen of individual mice and examined for their response to snRNP autoantigen. As illustrated in Fig. 4A, both thymic and splenic CD4 T cells from wt mice responded to D protein presented by Tg B cells. As found in Fig. 2, splenic CD4 T cells from Tg mice failed to respond to D proteins. However, thymic CD4 T cells from the same Tg mouse had a significant response (~10-fold greater) to rSm-D protein presented by Tg B cells. This observation indicates that autoreactive T cells are not centrally tolerized in the thymus, but instead are tolerized by Tg B cells in the periphery.
the periphery. The level of TCR expression in all CD4 T cells is similar (data not shown).

**Dual TCR T cells contribute to the autoimmune repertoire to snRNP D protein**

Studies using TCR Tg mice have demonstrated that several mechanisms could be involved in preventing autoreactive T cells from being deleted (28–31). For example, it has been found that dual TCR-bearing T cells expressing self-specific TCRs escape central tolerance and are functionally competent to the “self” Ag in vivo. These studies suggest that dual TCR T cells with one autoreactive TCR may survive negative selection through the second, nonself, TCR (29, 30, 32, 33). It is still not clear whether dual TCR T cells constitute autoreactive T cell repertoire in normal naive mice.

To examine the contribution of dual TCR T cells to self-reactive T cell repertoire, CD4 T cells from TCR\(^{\alpha-\beta+}\) Tg mice as well as wt mice were cocultured with 2-12 Tg B cells in the presence of snRNP D protein. TCR\(^{\alpha-\beta+}\) mice are unable to generate two productively rearranged TCR\(^{\alpha}\) loci or two \(\beta\) loci; therefore, they have only single-specificity TCR T cells. As shown in Fig. 5, CD4 T cells from wt mice develop a significant response to the D protein as we described above (Fig. 2A). However, the response from TCR\(^{\alpha-\beta+}\) mice is reduced by 50% compared with that from the wt. The decreased response is specific and comparable because wt and TCR\(^{\alpha-\beta+}\) CD4 T cells respond identically to anti-CD3 stimulation (Fig. 2B). The reduced response of TCR\(^{\alpha-\beta+}\) CD4 T cells suggests that dual TCR T cells constitute part of the self-reactive T cell repertoire in the periphery. However, we have yet to clone and identify the peptide specificity of snRNP-reactive T cells from hemizygous mice to quantify the fraction of self-reactive T cells that may still inhabit this repertoire.

**Anti-snRNP autoantibody production in B10.A 2-12 Tg mice reconstituted with MRL lpr/lpr anti-snRNP Tg mice CD4 T cells**

Thus far, we have demonstrated T cell tolerance induction and no autoantibody production in 2-12 Tg B10.A mice. In contrast, anti-snRNP Tg cells are readily observed in Tg MRL lpr/lpr mice. Next, we examined whether T cell help from MRL lpr/lpr mice could drive autoantibody production in MHC class II-matched 2-12 Tg B10.A mice.

Toward this end, purified CD4 T cells from age-matched wt MRL lpr/lpr or 2-12 Tg MRL lpr/lpr mice were adoptively transferred into B10.A 2-12 Tg mice and wt littermates. MRL lpr/lpr animals with autoantibody production (16–30 wk of age) were used as the source of donor CD4 T cells. Anti-snRNP autoantibodies were detected in B10.A 2-12 Tg mice, but not in non-Tg mice 1 mo after transfer of T cells from MRL lpr/lpr Tg mice (Fig. 6). In contrast, donor CD4 T cells from B10.A Tg or non-Tg B10.A mice transferred into B10.A Tg mice failed to drive autoantibody production (data not shown). These latter observations demonstrate that minor MHC differences between donor and recipient mouse strains was not a basis for autoantibody production. More importantly, this observation suggests that Ag-specific autoreactive T cells can deliver helper signals for autoreactive Tg B cells of nonautoimmune-prone mice.

**FIGURE 4.** The induction of autoreactive T cell tolerance in B10.A Tg mice is mediated by Tg B cells in the periphery. CD4\(^{+}\) T cells purified from wt (A) or Tg (B) B10. A thymus and spleen were cocultured with B10.A Tg B cells for 72 h. Proliferation was quantified by uptake of \[^{3}H\]TdR.

**FIGURE 5.** The \(\alpha^{+}\beta^{+}\) CD4 T cell repertoire contains reduced autoreactive T cells against snRNP D protein compared with the wt repertoire. CD4 T cells purified from unimmunized C57BL/6 wt and TCR\(\alpha^{+}\beta^{+}\) mice and cocultured with 2-12 Tg B cells. The proliferation was measured after a 3-day culture. As control, anti-CD3 Ab 2C11 was added with B6 B cells to demonstrate CD4 T cell proliferation is nearly identical between mouse strains (B).
Transferred CD4 T cells can migrate to regions of lymphoid tissue proximal to follicles

T cell-dependent Ab responses typically require cognate interaction between Ag-presenting B cells and Ag-specific T cells. Ag-mediated cross-linking of the B cell receptor promotes B cell localization to the boundary between the T cell zones and follicles, promoting encounters between Ag-bearing B cells and Ag-specific T cells (34). We labeled purified CD4 T cells of different origins with CFSE for adoptive transfer into B10.A 2-12 Tg or wt mice. After 24 h, we defined the localization of the transferred cells by immunostaining frozen sections of the spleen. In striking contrast to the CD4 T cells either from B10.A 2-12 Tg or wt, transferred CD4 T cells from MRL 2-12 Tg mice migrated into the B cell follicles (Fig. 7, A and B). Transferred CD4 T cells from B10.A 2-12 Tg and wt mice were found to localize within the T cell zone, with only occasional cells migrating near follicles (Fig. 7, C and D). Similar patterns were seen at 2, 3, or 5 days after transfer. However, no localization pattern difference occurred in B10.A 2-12 Tg and non-Tg mice. These observations suggest that T cells from 2-12 MRL Tg mice can acquire the intrinsic ability to migrate to B cell follicles, and that this process is independent of the resident B cells. It also provides rationale for why MRL T cells drive autoantibody production through cognate B-T interactions in recipient mice.

Discussion

Self-reactive T cells are conventionally regarded to be eliminated by negative selection in the thymus or by the induction of tolerance in the periphery (4, 35). The results described here demonstrate that snRNP-reactive T cells can be detected and activated in normal naive mice by autoantigen-specific B cells as APCs. However, these autoreactive T cells are normally anergic because they cannot proliferate in vitro by coculturing with autologous nonspecific B cells and self peptides. In contrast, autoreactive T cells are spontaneously activated in lupus-prone MRL lpr/lpr mice. In addition, spontaneously activated autoreactive T cells can provide help to autoreactive B cells from normal mice (anti-snRNP Tg B10.A strains) for autoantibody production.

Autoreactive T cells inhabit the naive repertoire of normal mice

The importance of B cells as autoantigen presenting cells has been emphasized by previous studies in our laboratory and others (8, 10, 13, 36). There is an accumulating data suggesting that B cells are capable of presenting autoantigen in activating autoreactive T cells (12, 15). Direct evidence comes from studies of B cell-deficient MRL lpr/lpr mice which fail to generate spontaneously activated T cell populations that are normally found in wt MRL lpr/lpr mice.
It is also possible that the frequency of self-reactive T cells are decreased in the single TCR repertoire compared with dual TCR-bearing T cells. Enumerating snRNP-reactive T cells from both single and dual TCR T cells is presently underway and should resolve the latter question.

**T cell help drives autoantibody production in nonautoimmune-prone mice**

snRNP-specific, autoreactive T cells are actively tolerized in nonautoimmune Tg mice, but not in autoimmune-prone MRL mice. Furthermore, the transfer of CD4 T cells from MRL lpr/lpr Tg mice into B10.A Tg mice initiates an anti-snRNP autoantibody response. This observation demonstrates that Ag-specific T cells can provide help for anti-snRNP B cell of nonautoimmune origin to produce autoantibodies in vivo. We do not yet understand the factors that explain a lack of adequate CD4 T cell helper functions of wt B10.A mice when transferred into B10.A Tg mice. However, we presume that a high frequency of snRNP-specific T cells exist in Tg MRL mice because they are easily activated with Ag in vitro. This premise is also supported by recent studies demonstrating that MRL T cells are activated by lower thresholds of Ag stimulation as compared with normal background T cells (43).

Moreover, autoantibody production requires autoreactive T-B cell collaboration, and is supported by the colocalization of T cells and B cells in the follicles as presented here. The trafficking of transferred cells within secondary lymphoid tissues of the host was performed to help explain biology of T cell help for autoantibody production (44–47). CD4 T cells from MRL lpr/lpr Tg mice migrated to within the margins of B cell zones in the spleen. In contrast, transferred CD4 T cells from B10.A Tg or non-Tg mice failed to migrate to the B cell follicles consistent with their inability to drive autoantibody production under these conditions. Previous studies have demonstrated that Ag-specific Tg T and B cells initially remain localized without contact in their respective zones. Following Ag stimulation, these cells move in a synchronous fashion out of their respective sites for cognate interactions at the interface of these zones (34, 48, 49).

In conclusion, these studies illustrate the presence of autoreactive T cells both in normal naive mouse and MRL lpr/lpr mouse, a murine model of human systemic lupus erythematosus. The induction of autoimmunity relies on the presence and cognate interactions of specific T cell-helper functions with B cells. Perhaps more importantly, these studies illustrate that B cells as APCs play an important role both in the activation and tolerance induction of autoreactive T cells, depending on the background. These observations have implications for the understanding of autoreactive T cell tolerance or autoimmunity and the initiation of autoantibody responses in normal individuals in which aberrant autoreactive T cell activation may arise.

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**References**


