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Lupus-Specific Antiribonucleoprotein B Cell Tolerance in Nonautoimmune Mice Is Maintained by Differentiation to B-1 and Governed by B Cell Receptor Signaling Thresholds

Ye Qian,* Carlos Santiago,‡ Michelle Borrero,* Thomas F. Tedder,‡ and Stephen H. Clarke2*

Systemic lupus erythematosus is an autoimmune disease characterized by the presence of autoantibodies. One of the unique targets of the immune system in systemic lupus erythematosus is Sm, a ribonucleoprotein present in all cells. To understand the regulation of B cells specific to the Sm Ag in normal mice, we have generated an IgH chain transgenic mouse (2-12H Tg). 2-12H Tg mice produce B cells specific for the Sm that remain tolerant due to ignorance. We demonstrate here that anti-Sm B cells of 2-12H Tg mice can differentiate into Sm-specific peritoneal B-1 cells that remain tolerant. Differentiation to B-1 and tolerance are governed by the strength of B cell receptor signaling, since manipulations of the B cell receptor coreceptors CD19 and CD22 affect anti-Sm B cell differentiation and autoantibody production. These results suggest a differentiation scheme in which peripheral ignorance to Sm is maintained in mice by the differentiation of anti-Sm B cells to B-1 cells that have increased activation thresholds. The Journal of Immunology, 2001, 166: 2412–2419.

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4 Abbreviations used in the paper: RNP, ribonucleoprotein; Tg, transgenic; BCR, B cell receptor; PC, phosphatidyl choline; HEL, hen egg lysozyme.

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immunization with mouse small nuclear RNPs (snRNPs) induces an anti-Sm response, indicating that anti-Sm B cells are functional and ignorant of endogenous levels of Sm (39). Here, we report that 2-12H Tg mice have a high frequency of peritoneal anti-Sm B1 cells. The transfer of splenic B cells from 2-12H Tg mice to irradiated non-Tg littermates indicates that splenic anti-Sm B cells can differentiate to B-1. The differentiation of anti-Sm B cells to B-1 is sensitive to signal strength, as decreasing signal strength through the elimination of CD19 expression directs differentiation to B-2 and inhibits differentiation to B-1. Serum anti-Sm levels are also affected by changes in CD19 and CD22 expression; increasing the level of CD19 or eliminating CD22 increases serum anti-Sm levels. We suggest that differentiation to B-1 helps maintain ignorance to Sm by raising their activation threshold.

### Materials and Methods

**Mice**

2-12H Tg mice have been described previously (39) and are maintained in our colony at the University of North Carolina by backcrossing to C.B-17 mice. Offspring are identified by PCR analysis of tail genomic DNA as previously described (39). CD19+/− and CD22+/− mice, and hCD19 Tg mice were described previously (34, 37) and bred with 2-12H Tg mice.

CD19−/− and CD22−/− mice were identified by PCR analysis of tail genomic DNA. The primer pairs for the PCR were designed to flank the portion of the CD19 and CD22 genes that contained the neo insertion (34, 37). The PCR products from wild-type mice result in a 758-bp product for CD19 and 444 bp for CD22, whereas knockout mice result in no product. Forward primer GGT GGA TGG ATA GTC CCA GTG and reverse primer CGA CTG GAA AGC CTG TCA AGG C were used for identification of CD19−/− mice. Forward primer GTC CCA CCC AGA GCA GAC ACC and reverse primer CCG AGA CAT TGA GGT GAA TGG G were used for identification of CD22−/− mice.

hCD19 Tg mice were identified by analysis of PBLs. After lysis of RBC, the cells were incubated with anti-human CD19 PE-conjugated Ab for 30 min on ice and washed. The stained cells were analyzed by flow cytometry. hCD19 Tg mice were determined by positive staining with anti-human CD19 Ab using non-hCD19 Tg mice as control for background staining. Mean fluorescence intensity of hCD19+/− mice was twice as much as that of hCD19−/− mice.

**Flow cytometry**

The Abs used specific for IgM (DS-1), IgM (AF6-78), B220 (RA3-6B2), HAS (30-F1), CD23 (B3B4), CD43 (S7), and CD5 (53-7.3) were obtained from PharMingen (San Diego, CA) and were fluoresceinated, biotinylated, or conjugated to PE. In three-color experiments, directly fluoresceinated and PE-conjugated Abs were combined with a biotinylated Sm, which was revealed with streptavidin-PerCP. In four-color experiments, biotinylated ssDNA (Life Technologies, Grand Island, NY) was revealed with streptavidin-PerCP, and Sm was directly Cy5 (Molecular Probes, Eugene, OR) labeled and combined with directly fluoresceinated and PE-conjugated Abs. To detect membrane expression of various molecules, single-cell suspensions were prepared in RPMI 1640 medium (HyClone, Logan, UT) containing 0.1% sodium azide and 3.0% bovine calf serum (HyClone). Cells were incubated with previously determined optimal amounts of Ab in 50 μl of buffer for 20 min, after which they were washed three times with buffer and incubated with second-step reagents. After washing as before, the cells were analyzed using a FACSscan and FACS-Calibur (Becton Dickinson, Mountain View, CA). Data were analyzed using WinMDI (The Scripps Institute, La Jolla, CA). All data represent cells that fall within the lymphocyte gate determined by forward and 90° light scatter. One to 5 × 10^5 cells per sample were analyzed. All contour plots are 5% probability.

**Adaptive transfer**

Splenetic B cells from 2-12 mice were enriched using a mouse B cell recovery column kit (Cedarlane Laboratories, Hornby, Ontario, Canada) according to the manufacturer’s instructions. Approximately 2 × 10^7 cells were transferred i.v. into sublethally (500 rad) irradiated non-Tg littermates. Spleen and peritoneal cells were taken after 5 days for flow cytometry analysis. For cell division analysis, spleen cells were labeled with CFSE (Molecular Probes) as described by Lyons and Parish (40) before adoptive transfer. Briefly, the CFSE stock solution was prepared at 5 mM in DMSO. Spleen cells were harvested and washed with PBS three times and resuspended at 2 × 10^7 cells/ml in PBS. Immediately before labeling, the CFSE was thawed and diluted to 10 μM in a volume of PBS equal to that in which the cells were suspended. The two volumes were mixed together and incubated at room temperature for 10 min. An equal volume of calf serum was added to quench the labeling. The labeled cells were then washed three times with PBS and transferred i.v.

**Serum ELISA**

Quantitation of anti-Sm Abs and total IgM in mouse serum was done by ELISA as previously described (39). Briefly, 96-well PVC plates were
coated with either Sm or goat anti-mouse IgM (Southern Biotechnology Associates, Birmingham, AL) in borate-buffered saline, washed, and blocked with borate-buffered saline/BSA/Tween 20. Mouse sera were diluted serially in borate-buffered saline/BSA/Tween 20 and added to Ag-coated plates in duplicate and then incubated overnight. After washing, goat anti-mouse IgM alkaline phosphatase (Southern Biotechnology Associates) was added and the anti-mouse IgM was revealed by the addition of 1 mg/ml \( p \)-nitrophenyl phosphate (Sigma, St. Louis, MO) in 0.01 M diethanolamine buffer. OD 405 was determined after 90–120 min of incubation. Serum titers of anti-Sm were determined by generation of a standard curve for each assay using serial dilutions of mouse IgM of a known concentration.

**LPS stimulation**

Splenic and peritoneal exudate cells were harvested, washed, and resuspended at 1 \( \times \) 10^6 cells/ml in complete RPMI 1640 and incubated for 72 h in 5% CO_2 in the presence or absence of 50 \( \mu \)g/ml LPS. Supernatants from cultures were tested by ELISA for anti-Sm and total IgM production as described above.

**Statistical analysis**

Statistical analysis was performed using Student’s t test. A value of \( p < 0.05 \) was considered to be significant.

**Results**

**Anti-Sm B-1 cells are present in peritoneums of 2-12H Tg mice**

We previously demonstrated that 15–35% of splenic B cells in nonautoimmune 2-12H Tg mice are anti-Sm (Table I and Ref. 39). Immunofluorescence studies indicate that most of these B cells are transitional, as they are IgM<sup>high</sup>, HSA<sup>high</sup>, CD23<sup>−</sup>, CD43<sup>−</sup>, and CD5<sup>−</sup> (Fig. 1A and Ref. 39). They also express intermediate levels of CD21 consistent with transitional B cells, indicating that they are not marginal zone B cells. However, some appear to be mature B cells, since an IgM<sup>low</sup>, HSA<sup>low</sup>, CD23<sup>+</sup>, CD43<sup>−</sup>, and CD5<sup>−</sup> populations can be detected. We have not detected anti-Sm B cells in the spleen that have a B-1 phenotype. However, Fig. 1 shows that there is a large population of anti-Sm B-1 cells in the peritoneum. These cells were missed in the previous analysis (39) because they were inadvertently gated out. They are IgM<sup>high</sup>, CD23<sup>−</sup>, CD43<sup>−</sup>, and CD5<sup>−</sup> (Fig. 1A), and they are larger and more granular than the CD23<sup>+</sup> B cells of the peritoneum (Fig. 1B). They constitute ~30% of the B-1 subset (Table I). Nearly all of the peritoneal anti-Sm B cells express CD5, indicating that they are predominately B-1a. Interestingly, ~6% of the B-1 repertoire of non-Tg mice are anti-Sm (Table I and Fig. 1A), indicating that they are a significant component of the normal B-1 repertoire in mice.

B cell nonresponsiveness to LPS accompanies anergy (41–43). LPS stimulation of splenic B cells from 2-12H Tg mice induces secretion of anti-Sm Abs, suggesting that they are functional (39). Indeed, immunization with snRNPs induces an anti-Sm response. To determine whether peritoneal anti-Sm B-1 cells are also responsive to LPS, peritoneal B cells were cultured with LPS at 50

![FIGURE 1. Flow cytometry analysis of spleen and peritoneal B cells from 2-12H Tg and non-Tg littermates. A, Spleen and peritoneal cells were stained with B220-PE, Sm-biotin, and FITC-labeled IgM (IgM<sup>a</sup> for non-Tg and IgM<sup>b</sup> for 2-12H Tg mice), CD23, CD43, or CD5. All histograms are gated on B220<sup>+</sup> cells. B, The size (forward light scatter (FSC)) and granularity (side light scatter (SSC)) of peritoneal anti-Sm B-1 cells (bold line) and CD23<sup>−</sup> B-2 cells (thin line) from 2-12H Tg mice.](http://www.jimmunol.org/)

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µg/ml. Fig. 2 shows that peritoneal anti-Sm B cells of 2-12H Tg mice are responsive to LPS. IgM levels are similar to those of non-Tg littermates and the level of anti-Sm is 5-fold higher in 2-12H Tg cultures than in non-Tg cultures. In contrast, LPS stimulation of non-Tg mouse peritoneal B cells induces IgM Ab secretion, but little anti-Sm autoantibody secretion. Thus, by this criterion peritoneal anti-Sm B-1 cells are functional.

Splenic anti-Sm B cells have the potential to differentiate to B-1

To investigate the relationship between the predominantly transitional splenic anti-Sm B cells and the mature peritoneal anti-Sm B-1 cells, we transferred splenic B cells from 2-12H Tg mice to sublethally irradiated littermate mice. Both spleen and peritoneal B cells were examined 5 days later. Donor B cells were identified by IgM<sup>+</sup> staining, as endogenous B cells are IgM<sup>-</sup>. In the spleen, donor anti-Sm B cells are CD23<sup>+</sup>, CD43<sup>+</sup>, and CD5<sup>-</sup> and thus appear to be transitional (Fig. 3A). Donor B cells are also found in the peritoneum, including many that bind Sm. Interestingly, here the majority of anti-Sm B cells are B-1 (i.e., IgM<sup>high</sup>, CD23<sup>+</sup>, CD43<sup>+</sup>, and CD5<sup>-</sup>) (Fig. 3A).

To rule out that the appearance of peritoneal B-1 cells in recipient mice is due to the proliferation of an undetected population of splenic B-1 cells, we used an intracellular fluorescent labeling method to monitor in vivo cell division of the adoptively transferred splenic B cells. Spleen cells were transferred as described above after CFSE labeling. Fig. 3B shows that 5 days after transfer, nearly all of the donor peritoneal anti-Sm B cells, identified by their CFSE staining, have undergone only one or two divisions. These findings indicate that expansion of an undetected population of contaminating B-1 cells of donor origin cannot be responsible for the peritoneal anti-Sm B-1 cell population in recipient mice. Thus, splenic anti-Sm B cells have differentiated to B-1 in recipient mice. We also note that there is a large population of B-1 cells of donor origin that do not bind Sm, indicating that B cells of other specificities in 2-12H Tg spleens are selected into the B-1 subset.

Altered B cell coreceptor expression affects anti-Sm B cell differentiation

To investigate the effect of signal strength on anti-Sm B cell differentiation, we varied the levels of two B cell coreceptors, CD19 and CD22. 2-12H Tg/CD19<sup>-/-</sup> mice have 40% fewer B cells in the spleen than 2-12H Tg mice (<i>p</i> < 0.05, Table I), consistent with previous findings (37). The absence of CD19 has a similar effect on non-Tg mice. However, the frequency of anti-Sm B cells increases from 30% in 2-12H Tg mice to 47% in 2-12H Tg/CD19<sup>-/-</sup> mice (<i>p</i> < 0.05), a 57% increase, indicating that anti-Sm B cell development is favored in the absence of CD19. In contrast to 2-12H Tg mice in which splenic anti-Sm B cells are mostly CD23<sup>+</sup> transitional B cells, splenic anti-Sm B cells of 2-12H Tg/CD19<sup>-/-</sup> mice have a mature B-2 cell phenotype (i.e., CD23<sup>-</sup>, CD43<sup>-</sup>, CD5<sup>-</sup>, and HSA<sup>low</sup>) (Fig. 4).

The absence of CD19 also has dramatic effects on peritoneal anti-Sm B cells. The percentage of peritoneal B cells decreases 10-fold (70–7%) in 2-12H Tg with the elimination of CD19 (Table I and Fig. 4), consistent with the finding that CD19<sup>-/-</sup> mice are deficient in B-1 cell differentiation (37, 44). Moreover, the proportion of anti-Sm B cells decreases from 32.5% to 5% (Table I), and thus the total number of peritoneal anti-Sm B cells decreases...
Overexpression of CD19 increases the number of B-1 cells. The level of CD19 can be varied using mice hemizygous for the human CD19 transgene (hCD19<sup>+/−</sup>) and mice that are homozygous for the hCD19 transgene (hCD19<sup>+/+</sup>) (34). We have examined anti-Sm B cells in 2-12H Tg mice that are either hCD19<sup>−/−</sup> or hCD19<sup>+/+</sup>. The total number of splenic lymphocytes is reduced in 2-12H Tg/hCD19<sup>−/−</sup> mice and to a lesser degree in 2-12H Tg/hCD19<sup>+/−</sup> mice. This is consistent with previous reports of overexpression of CD19 (37). 2-12H Tg/hCD19<sup>+/−</sup> and 2-12H Tg/hCD19<sup>+/+</sup> mice have 38 and 58% fewer B cells than 2-12H Tg mice, respectively (Table I). Oddly, the frequency of anti-Sm B cells is lower in 2-12H Tg/hCD19<sup>+/−</sup> mice than in 2-12H Tg mice (Table I). Phenotypic analysis indicates that all of the anti-Sm B cells are B-1, as they are IgM<sup>hi</sup>, CD23<sup>−</sup>, CD43<sup>−</sup>, and CD5<sup>+</sup> (Fig. 4). As in 2-12H Tg mice, all anti-Sm B-1 cells in hCD19 Tg mice carrying the 2-12H transgene are CD5<sup>+</sup> and therefore B-1a.

CD22 is a negative regulator of B cell signaling through its association with SHP-1 (32). To determine how CD22 influences differentiation of anti-Sm B-1 and transitional B cells, we bred 2-12H Tg/CD22<sup>−/−</sup> mice. Although the frequency of splenic B cells in both 2-12H Tg and 2-12H Tg/CD22<sup>−/−</sup> mice is the same, the absence of CD22 decreases by 48% the number of splenic lymphocytes. A lack of CD22 also results in a decrease in the total number and frequency of anti-Sm B cells in 2-12H Tg mice (Table I). However, the anti-Sm B cells remain transitional (i.e., IgM<sup>hi</sup>, CD23<sup>−</sup>, CD43<sup>−</sup>, and CD5<sup>+</sup>) (Fig. 4).

In the peritoneum the number of lymphocytes increases 4-fold over CD22 intact mice, and the frequency of the B-1 cells increases from 78 to 98% in 2-12H Tg mice (Table I). Although the frequency of anti-Sm B cells decreases in 2-12H Tg as a result of the absence of CD22, the total number of anti-Sm B cells increases as a result of the increase in the total number of peritoneal B cells in CD22<sup>−/−</sup> mice (Table I). However, in CD22<sup>−/−</sup> mice they are B-1 (i.e., IgM<sup>hi</sup>, CD23<sup>−</sup>, and CD5<sup>+</sup>) (Fig. 4).

Serum anti-Sm levels in 2-12H Tg mice are affected by changes in coreceptor expression

Serum anti-Sm levels in 2-12H Tg mice were evaluated by ELISA. As we showed previously (39), serum anti-Sm levels in 2-12H Tg mice are not significantly different from those of non-Tg littermate mice, despite the substantially higher number of anti-Sm B cells in the spleens and peritoneums of 2-12H Tg mice (Fig. 5). However, 2-12H/CD22<sup>−/−</sup>, 2-12H/hCD19<sup>+/−</sup>, and 2-12H Tg/hCD19<sup>+/−</sup> mice have levels of anti-Sm that are significantly higher than those of 2-12H Tg mice (p < 0.05) and their non-Tg counterparts (p <
A 2-12H Tg MRL-Mplprlpr mice (Fig. 5). Conversely, 2-12H Tg/CD19−/− mice, which have no anti-Sm B-1 cells, have significantly decreased serum anti-Sm levels (p < 0.05) compared with 2-12H Tg mice. This suggests that changes in B-1 cell sensitivity to signaling affects serum anti-Sm levels.

Discussion

We have demonstrated the existence of anti-Sm B-1 cells in the peritoneum of nonautoimmune 2-12H Tg mice where they account for ~30% of the B-1 repertoire. This is in addition to the anti-Sm B cells in the spleens of these mice, the majority of which are transitional. We also show a direct relationship between the splenic and peritoneal anti-Sm B cells; adoptive transfer of splenic anti-Sm B cells results in their differentiation to B-1 and movement to the peritoneum. Thus, splenic anti-Sm B cells are capable of differentiation to B-1.

A B-2 to B-1 differentiative pathway was originally proposed by Wortis and colleagues (45), and we have demonstrated that B cells specific for phosphatidyl choline (PtC), which are exclusively B-1 in normal mice, follow this pathway of differentiation in vivo (46, 47). It remains to be determined whether differentiation to B-1 occurs throughout adult life. The peritoneal B-1 cell population develops early in life and maintenance of the B-1 population occurs by the self-renewal capacity of these cells (48, 49). It does not require continuous infusion of new B cells, as is required for the B-2 population (48, 49). However, this does not preclude that some cells enter the B-1 subset in adult life since not all B-1 cells may have the same half-life. Because anti-Sm transitional B cells are continuously generated by the adult bone marrow, there may be a constant flow of anti-Sm B cells to the B-1 subset throughout life. Alternatively, their differentiation may be blocked and they may instead undergo apoptosis, despite their capacity to differentiate to B-1. Such a block might be due to a negative feedback from already differentiated B-1 cells. In this regard, we note that there are 30 times as many anti-Sm transitional B cells as anti-Sm B-1 cells in 2-12H Tg mice (Table I). If the anti-Sm transitional B cells all differentiate to B-1, then by necessity the anti-Sm B-1 cells must be short-lived. Preliminary experiments suggest that the half-life of anti-Sm B-1 cells is not short and is equal to that of non-Tg B-1 cells. Thus, many transitional anti-Sm B cells probably undergo apoptosis in the spleen as previously suggested (39). Experiments are currently underway to resolve this issue.

Differentiation of anti-Sm B cells to B-1 appears to be governed by the strength of the signal delivered by Ag. Decreasing the strength of Ag receptor signaling by CD19 elimination resulted in B cell differentiation into B-2 cells rather than into B-1 cells. Thus, weak signals generated through the Ag receptor may result in transitional B cell differentiation into B-2 cells rather than undergoing apoptosis or differentiation to B-1. Since B-1 cell numbers are greatly reduced in CD19-deficient mice, the assumption was that signal strength was insufficient to support the long-term survival of B-1 cells (34). However, the current study suggests that appropriate signals generated through the Ag receptor are required for B cell development into B-1 cells. In support of this, overexpression of CD19 or elimination of CD22 significantly increased the number of 2-12H B-1 B cells, while the numbers of 2-12H B-2 cells in these mice were decreased (Table I and Fig. 4). Furthermore, consistent with B-1 as the origin of circulating IgM, serum anti-Sm autoantibody levels in 2-12H Tg/hCD19−/+ or −/− and 2-12H Tg/Cd22−/− mice were 4- to 5-fold higher than the levels in 2-12H Tg mice and non-Tg mice (Fig. 5). This is comparable to the levels in 2-12H Tg MRL-Mplprlpr mice (Fig. 5). Thus, activation thresholds regulate B-1 cell differentiation in addition to regulating tolerance and the levels of serum anti-Sm.

The differentiation of anti-Sm B cells to B-1 and the absence of higher serum anti-Sm in 2-12H Tg mice relative to non-Tg mice is paradoxical (Fig. 5). B cells are selected by Ag for recruitment into the B-1 cell subset, and B-1 cells are responsible for most of the circulating IgM in unmanipulated mice (13, 50). Indeed, anti-PtC Tg mice have ~100-fold more serum anti-PtC than non-Tg littermates due to the increase in anti-PtC B-1 cell numbers (51). Why then do 2-12H Tg mice not have higher serum anti-Sm levels than non-Tg littermates, given the higher number of anti-Sm B-1 cells? One possibility is that migration to the peritoneum removes these cells from contact with other cells or molecules required for activation and differentiation to Ab-secreting cells. For example, exposure to the activating Ag in the peritoneum may not be sufficient to drive differentiation of anti-Sm B-1 cells to Ab-secreting cells. Another possibility is that differentiation to B-1 is accompanied by changes in the B cell itself that decrease its sensitivity to Ag. One change induced by differentiation to B-1 relevant to this is the expression of CD5, a negative regulator of BCR signaling through its association with the phosphatase SHP-1 (52, 53). This association would increase the amount of Ag required for activation. Hippen et al. (54) have recently demonstrated that CD5 is expressed by anergic anti-hen egg lysozyme (HEL) B
cells and that it is responsible for the very low levels of circulating anti-HEL. In the absence of CD5, anergic anti-HEL B cells produce high levels of circulating anti-HEL similar to those found in mice with functional anti-HEL B cells. Thus, CD5 is instrumental in blocking activation of anergic B cells in this model. In an analogous way, CD5 expression could inhibit activation of anti-Sm B cells and account for the low levels of anti-Sm in unimmunized 2-12H Tg mice (Fig. 5 and Ref. 39). Thus, differentiation to B-1 may be an essential component in maintaining ignorance to Sm. Increasing the level of Sm Ag could overcome the negative effects of CD5 on BCR signaling and account for the ability to activate these cells upon immunization with snRNPs (39). This may relate to the finding that all anti-sm B-1 cells of 2-12H Tg mice express CD5, as do the peritoneal anti-sm B-1 cells of non-Tg littermates. Thus, regulation of CD5 expression may control the ability of B-1 cells to be activated and hence the autoantibodies secreted by B-1 cells.

In summary, we have demonstrated that anti-sm B cells differentiate to B-1 and that the strength of the signal provided by Ag determines whether an anti-sm B cell differentiates to B-2 or to B-1. In addition, alteration of the activation threshold increases or decreases the level of serum anti-sm, indicating that the activation threshold setting determines the level of circulating Ab. We suggest that differentiation to B-1 helps maintain ignorance to Sm in nonautoimmune mice through the expression of negative regulators of BCR signaling such as CD5. Alterations in the threshold setting or ability of B cells to differentiate to B-1 could be a predisposing factor in production of autoantibodies in disease. Thus, anti-sm B cells provide a new perspective on maintaining tolerance to self-Ags targeted in lupus.

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