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Mature B Cells Preferentially Lose Tolerance in the Chronic Graft-versus-Host Disease Model of Systemic Lupus Erythematosus

Arpita Choudhury,* Philip L. Cohen,*† and Robert A. Eisenberg‡*

Chronic graft-vs-host (cGVH) disease is a well-characterized systemic lupus erythematosus (SLE) model. Induction of cGVH in anti-DNA H chain knockin (3H9KI) transgenic mice results in specific activation of anti-dsDNA B cells. In this study, we show that B cells from 3H9KI mice were activated by cGVH even when adoptively transferred into irradiated JHT−/− recipients that lack endogenous B cells. This process of activation was reflected by high autoantibody titers and changes in phenotypic markers. We have used this system to characterize the particular B cell subsets that were responsible for secreting autoantibodies during cGVH response. We isolated splenic B cell subsets based on their expression of specific cell surface markers and used them in our adoptive transfer studies. We found that mature B cells were the most vulnerable to the allostimulus and were the major source of autoantibodies compared with immature B cells. The greater susceptibility of mature B cells to become activated and thereby lose tolerance was unanticipated and has implications for maintenance of peripheral tolerance and for the development of autoimmunity. Furthermore, of the mature B cells, marginal zone B cells were particularly responsible for mounting the initial response to the cGVH stimulus. This observation underscores the critical role of marginal zone B cells in activation and production of autoantibodies. *The Journal of Immunology, 2007, 179: 5564–5570.

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ystemic 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. The understanding of SLE has been greatly facilitated by the chronic GVH (cGVH) model. Transfer of Ia-incompatible spleen cells from nonautoimmune B6.C-H2b2m12/KhEg (bm12) mice into coisogenic C57BL/6 (B6) recipients results in cGVH reactions with features characteristic of SLE, including loss of B cell tolerance, high titers of autoantibodies and renal immunopathology (1).

To determine how allo-T cell help induces tolerance loss in anti-DNA B cells, we have combined the cGVH model with an H chain Ig transgene (tg), 3H9. The 3H9 is a “knockin” transgenic in which the normal chromosomal IgH locus was replaced with a rearranged V(D)J Ig, and this knockin (KI) H chain could combine with endogenous light chains to form both anti-dsDNA and non-autoantibody Abs (2, 3). Previous work from our laboratory showed that induction of cGVH in 3H9KI tg mice on a nonautoimmune B6 background resulted in specific activation of anti-dsDNA B cells (4). In a recent study using the cGVH model in anti-DNA H chain transgenic mice, 56R, Witsch et al. (5) have shown that B cells secreting pathogenic autoantibodies are probably located in the marginal zone (MZ) of the spleen. Although these studies helped in understanding some of the mechanisms by which allo-T cell help induces B cell autoreactivity, they do not unambiguously establish the precise timing and the B cell subset that is most affected by such intervention.

We have, therefore, developed an adoptive transfer model to address these issues in our present study. We have utilized the expression of different cell surface markers in peripheral B cells to isolate particular subsets. Immature B cells that emigrate from the bone marrow circulate as transitional B cells that are HSAhighIgMhighIgDB220lowIgD− and can be distinguished from mature splenic B cells by use of mAb AA4.1 that recognizes a protein of apparent m.w. 130–140, expressed exclusively on immature transitional cells (6). The mature B cells can be broadly classified as follicular (FO) or MZ B cells that differ in their localization and cell surface markers. FO recirculating B cells are IgMhighIgDhighCD21lowCD23highCD1−CD9low, and resident MZ B cells located at the junction of white and red pulps are IgMhighIgDlowCD21−CD23+CD1highCD9low (7). Based on these variations in cell surface markers, we have isolated different B cell populations from the anti-DNA H chain transgenic mice, 3H9, and transferred them into irradiated B6.JHT−/− recipients, which lack endogenous B cells. We then induced cGVH in the recipients.

Our data indicate that adoptively transferred B cells are susceptible to allo-T cell help and produce autoantibodies. Most importantly, mature B cells were more susceptible to allo-T cell help than immature B cells. Furthermore, of the mature B cell subpopulation, MZ B cells responded more vigorously initially than did FO B cells.
Materials and Methods

Mice

The generation of C57BL/6.3H9 (3H9) and C57BL/6.3H9/56R (56R) mice has been described previously (3, 8). The C57BL/6.1JH mice, which lack Jb segments and the intron enhancer in the IgH locus and therefore cannot generate B cells (9), were a gift from Dr. Sasha Tarakhovsky (Rockefeller University, New York City). C57BL/6J (B6) and coisogenic B6.C-H2*bm12/KthJg (bm12) mice, which differ by only three amino acids in the β-chain of I-A, were originally obtained from The Jackson Laboratory. All mice were bred and maintained in our colony at the University of Pennsylvania. Recipients and donors were sex and age matched within each experiment. All experimental procedures were conducted according to the guidelines of the Institutional Animal Care and Use Committee.

Adoptive cell transfers

JHT recipients were irradiated (3 Gy from a Cs-137 source) before adoptive cell transfers. All experiments were performed with 4–5 animals in each group. The experiments were repeated twice or thrice and had comparable results. Then 5–10 × 10⁶ cells were transferred i.v. cGVH was induced on the following day by transferring i.p. 1 × 10⁶ bm12 spleen cells. Blood samples were obtained from experimental mice before the induction of cGVH disease and at 2- to 4-wk intervals thereafter. Sera were stored at −20°C for later analysis.

Detection of autoantibodies and allotype specific antibodies

Autoantibodies were assessed by ELISA, as previously described (10). In brief, plates were coated with chicken erythrocyte-derived chromatin at 5 μg/ml; or with calf thymus-derived dsDNA at 3 μg/ml. Serum samples, diluted 1/250 in buffer, were added in duplicate and incubated overnight at 4°C. For the anti-dsDNA ELISA, plates were first coated with poly-l-lysine (1 μg/ml) (Sigma-Aldrich) before incubating with the autoantigen. Biotinylated goat anti-mouse IgG (pFc' specific; Jackson ImmunoResearch Laboratories) was added as secondary Ab. For reference, standard serum from a disrupted MRL/lpr mouse with high titers of autoantibodies was tested at serial 2-fold dilutions from 1/250 to 1/128,000. The plates were washed and incubated for 1 h at room temperature with avidin-alkaline phosphatase (Zymed Laboratories). The plates were washed again, and 1 mg/ml para-nitrophenyl phosphate substrate (Sigma-Aldrich) in 0.01 M diethanolamine (pH 9.8) was added. For allotype-specific ELISAs, the assays were similar to the one described above, except that they were developed with rabbit anti-mouse preabsorbed allotype reagents (anti-IgG2a or anti-IgG2b (also known as IgG2c)); Accurate Chemical and Scientific) and detected with avidin-alkaline phosphatase-conjugated anti-rabbit IgG Ab (Jackson ImmunoResearch Laboratories). For “a” allotype standard, serum from an old MRL/lpr was used; and for “b” allotype standard, serum from an old B6/lpr (IgHb) was used. Both the reference sera were used at serial 2-fold dilutions starting from 1/250 to 1/128,000. For estimation of total IgM, ELISA plates were coated with goat anti-mouse IgM (Jackson ImmunoResearch Laboratories) at 4 μg/ml. Biotinylated Bet-2 F(ab′)2 was used as the second Ab, and mouse IgM (clone CBPC 112) was used as standard in these assays. The plates were read at various time points with an automated ELISA reader (Molecular Devices).

Immunofluorescence staining

The following conjugated Abs and reagents were purchased from BD PharMingen: allophycocyanin anti-CD19 (1D3), FITC anti-CD21 (7G6), FITC anti-B7.1 (16–10A1), FITC anti-class II (AF6–120.1), PE anti-B7.2 (GL1), PE anti-CD23 (B3B4), and PE anti-Fas (Jo2). Anti-FcγR (2.4G2), used for blocking, was grown in our laboratory. Cell surface staining was routinely performed with age- and sex-matched controls, as previously described (4, 10). Cells were fixed in PBS containing 1% paraformaldehyde and analyzed on a BD Biosciences FACScan. Relative fluorescence intensity was plotted on a logarithmic scale using FlowJo software.

Cell separation using magnetic beads

Magnetic beads, anti-CD43, and anti-biotin were purchased from Miltenyi Biotec, and the AutoMACS was used for cell separation as described previously (10). Peripheral B cells were isolated by using anti-CD43 magnetic beads and the depletion cycle. Mature and immature B cells were further separated by staining with biotinylated mAb AA4.1 (obtained from Dr. Dave Allman, University of Pennsylvania) and anti-biotin magnetic beads. Mature B cells were AA4.1+ and immature B cells were AA4.1−. For separation of FO and MZ B cells, the CD43+ B cells were labeled with biotinylated anti-CD23, and AA4.1 Ab and anti-biotin beads were used for separation. The CD23AA4.1− fraction consisted of MZ B cells, and the CD23AA4.1+ fraction contained immature and FO B cells. The purity of cell separation was checked by flow cytometry.

Statistical analysis

Statistical analyses were performed using Student’s t test. A value of p < 0.05 was considered to be significant. The bars indicate SEs.

Results

We have previously established that induction of cGVH in 3H9KI tg mice resulted in specific activation of anti-dsDNA B cells (4). In the present studies, we have isolated B cell subsets to determine when, in ontogeny, B cell tolerance can be lost in the cGVH model.

Induction of cGVH in B cell knockout recipients

In our initial studies, we have used slgM knockout mice (μMT) as recipients in our adoptive transfer experiments. Unlike JHT mice, which completely lack the J locus, μMT mice lack only the IgM transmembrane exons, so their B cell development is blocked at the pro-B cell stage (11). Adoptive transfer of 3H9 spleen cells into lightly irradiated (3 Gy) μMT recipients, followed by transfer of 1 × 10⁶ bm12 spleen cells. Blood samples were obtained from experimental mice before the induction of cGVH disease and at 2- to 4-wk intervals thereafter. Sera were stored at −20°C for later analysis.
bm12 cells, resulted in production of IgG2a autoantibodies in the recipients, indicating activation of the donor 3H9 cells (data not shown). However, the control μMT recipients given only bm12 cells also made low levels of IgG2a Ig, although no autoantibodies (Fig. 1A). This suggested “leakiness” in μMT recipients, presumably due to the strong stimulus of cGVH, as has been reported in other experimental systems (12). We, therefore, used JHT mice as recipients in additional experiments, because these animals are incapable of producing Ig under any circumstances. The JHT recipients were also lightly irradiated (3 Gy) before being subjected to adoptive transfers. The experimental group of JHT recipients received 5–10×10⁶ spleen cells from 3H9 tg mice followed by 1×10⁸ bm12 cells. One control group of JHT recipients received only 3H9 spleen cells, whereas the other control group had only bm12 spleen cells. Each group consisted of 4–5 mice. Fig. 1B shows that the JHT recipients secreted Ig exclusively of the donor cell IgG2a allotype. Importantly, unlike μMT mice, JHT recipients with only bm12 cells did not produce any IgG of “a” allotype but only IgG2a IgG (presumably from the bm12 B cells), clearly illustrating the key point that JHT recipients do not show any leakiness (Fig. 1C). The experimental JHT recipients secreted total Ig of both allotypes, reflecting the presence of both 3H9 cells and bm12 cells; however, only the 3H9 tg-derived cells produced anti-dsDNA Abs (IgG2a allotype (Fig. 1D) but not IgG2aβ (Fig. 2E)). This is consistent with our previous observations that the cGVH response could be initiated only in the B cells recognized by alloreactive T cells (13, 14). These data establish that the process of loss of tolerance and induction of cGVH, as previously seen in 3H9 tg mice, could be reproduced in JHT recipients following adoptive cell transfer.

We have also previously found that the B cells in 3H9 tg mice show up-regulation of cell surface markers, such as MHC class II, Fas, and B7.2, upon induction of cGVH. This suggested a general activation of B cells by allostimulation (4). We asked whether such phenotypic changes also occurred when 3H9 tg B cells were transferred to JHT recipients and were provided with an allostimulus. The JHT recipients were sacrificed 1 wk after adoptive cell transfer and induction of cGVH, and their splenic B cells of 3H9 origin were analyzed. As seen in Fig. 2A, following induction of cGVH, adoptively transferred 3H9 B cells were activated as reflected by up-regulation of surface MHC II, B7.2, and Fas, whereas CD21 was down-regulated. This difference in CD21 expression is consistent with our previous findings in cGVH model (4). Similar changes in CD21 levels have also been observed in other murine models (15) and in SLE patients (16). The increase in CD23 levels has also been observed in other models of cGVH such as in B6.56R (5).

**Figure 2.** Phenotypic changes of B cells after induction of cGVH. Spleen cells from JHT mice were analyzed by flow cytometry a week after transfer of B cells from 3H9 tg mice and induction of cGVH. Histograms represent B cells gated by scatter on the lymphocyte region and CD19 and IgM positivity. A, Expression of activation markers MHC II, B7.2, and Fas, and developmental markers, CD23 and CD21, in JHT recipients after cGVH (heavy line) and JHT recipients with only donor 3H9 B cells (thin line) and isotype control (filled histograms). The y-axis represents the percentage of B cells gated on CD19 and IgM positivity. B, MFI of B cells from experimental group (■) and the control recipients (□). Four to five mice per group were analyzed. The p values of MHC II (p = 0.010), B7.2 (p = 0.004), Fas (p = 0.002), CD23 (p = 0.003), and CD21 (p = 0.005) give the statistical significance between control JHT recipients and those with cGVH.
activated after cGVH in JHT recipients, and the cell surface marker profiles are consistent with our previous observations of activation of B cells in 3H9 mice following induction of cGVH (4).

Mature B cells are more susceptible to activation and loss of tolerance

After ascertaining that adoptively transferred spleen cells from 3H9 tg mice could be activated and produced autoantibodies in JHT recipients, we dissected the particular B cell subsets that were affected by the allograft rejection. We separated mature B cells from immature B cells in the adult spleen based on the expression of AA4.1 on the latter. Surprisingly, JHT recipients of mature B cells produced quantitatively more autoantibodies than those with immature B cells (Fig. 3). It is possible that the significantly lower response in immature B cells could be attributed to their more rapid turnover rate (17).

MZ B cells initially respond to allostimulation

Once it became apparent that the cGVH stimulus preferentially activated mature B cells, we further assessed the proclivity to lose tolerance in different populations of mature B cells. Based on their phenotype, location, and functional characteristics, mature B cells have been subdivided as recirculating FO B cells (CD21<sup>hi</sup>CD23<sup>hi</sup>CD1<sup>hi</sup>), and resident MZ B cells (CD21<sup>+</sup>CD23<sup>−</sup>CD1<sup>hi</sup>). Because MZ B cells have been found in some systems to play a role in the spontaneous development of autoantibodies (5, 18), we hypothesized that they might show a special susceptibility to allo-T help in the cGVH. Our data show that the JHT recipients of MZ B cells developed high titers of both anti-dsDNA Abs (Fig. 4A) and anti-chromatin Abs (Fig. 4B). The autoantibody titers in JHT recipients of FO B cells progressed more slowly and peaked at around 4 wk, when the autoantibodies in the MZ group were already declining (Fig. 4). As seen in Fig. 4C, total IgM titers also followed a similar pattern. These data illustrate that the initial autoantibody production in cGVH probably comes mainly from autoreactive B cells residing in the MZ. This interpretation of our data would apparently seem inconsistent with our flow cytometry data in Fig. 2 where it is shown that CD21 expression is down-regulated upon induction of cGVH, and this is similar to our previous findings in cGVH models (4). Also studies from Weigert’s group showed a decrease in MZ B cells following induction of cGVH in 56R strain of mice (5). However, we hypothesize that MZ B cells in situ are initially activated by cGVH and change their phenotype and/or their anatomical location. This would explain the down-regulation of CD21 expression as seen upon induction of cGVH in intact animals and why adaptively transferred MZ B cells mount the initial response to cGVH compared with FO B cells. At this point, we do not have data to show that adaptively transferred MZ B cells also populate the MZ area. Further elucidation of the involvement of MZ B cells in the SLE autoantibody response will be the subject of future studies.

In subsequent experiments, we examined B cells from another anti-DNA knockin transgenic mouse, 56R. 56R is similar to 3H9, except for an additional arginine in the CDR2 of the 3H9 H chain, which enhances the antibodies’ affinity for dsDNA (8). JHT recipients of MZ B cells from 56R mice showed higher titers of anti-dsDNA Abs (Fig. 5A) and anti-chromatin Abs (Fig. 5B), compared with other B cell fractions. These data reinforce our findings that...
loss of B cell tolerance leads to production of typical SLE autoantibodies. The induced nature of this model permitted us to investigate the stage of ontogeny at which autoreactive B cells can be influenced by abnormal T cell help. Normally, autoreactivity is controlled at various checkpoints through mechanisms of deletion, anergy, and receptor editing (20, 21). By isolating B cells through the expression of characteristic surface markers, we could determine when in development they could lose self-tolerance. Somewhat surprisingly, we found that mature B cells were most amenable to loss of tolerance, particularly the MZ subset.

Our previous studies with 3H9KI mice showed that allo-T help triggered specific activation of anti-dsDNA B cells. In our present studies, we wanted to utilize this cGVH system in an adoptive transfer model to determine which particular B cell populations are activated by the allograft. A successful adoptive transfer model is greatly dependent on the choice of the host recipient. Our initial studies were conducted using slgM knockout mice (μMT) as recipients. These mice lack only the IgM transmembrane exons, so their B cell development is blocked at the pro-B cell stage (11). Our data (Fig. 1), showed that adoptively transferred 3H9 spleen cells were activated by allograft and could secrete autoantibodies. However, the strong allograft stimulus also elicited endogenous Ig (but not autoantibody) production from the host recipient as seen in Fig. 1A. This implied leakiness in our recipient and suggested that allograft stimulus could circumvent the block in B cell development as seen in other studies (12). This finding led us to use JHT knockout mice in subsequent experiments. Unlike μMT mice where it is possible to have Ig expression by isotype class switch-primary to secondary on polyclonal activation of MZ B cells and FO B cells. However, in allo-T help, the MZ B cells were activated by allostimulus and could secrete autoantibodies. This implied leakiness in our recipient and suggested that allograft stimulus could circumvent the block in B cell development as seen in other studies (12). This finding led us to use JHT knockout mice in subsequent experiments. Unlike μMT mice where it is possible to have Ig expression by isotype class switch-primary to secondary at 106 bm12 cells. Anti-dsDNA Abs (p = 0.001) (A) and anti-chromatin Abs (p = 0.003) (B) with adoptively transferred 56R B cells following induction of cGVH. Anti-dsDNA (p = 0.007) (C) and anti-chromatin Abs (p = 0.006) (D) secreted by B6 B cells. Error bars, SEM samples. The p values refer to the difference in autoantibody titers secreted by JHT recipients with MZ B cells and those with FO B cells.

Discussion
The autoimmune syndrome of cGVH results from cognate recognition of host B cells by alloreactive donor T cells. The subsequent

FIGURE 5. MZ B cells isolated from wild-type B6 or 56R mice show similar trends of initially losing tolerance under cGVH. MZ B cells and FO B cells were sorted by expression of CD23 on mature splenic B cells by using magnetic beads. CD23− fraction was considered as MZ B cells, whereas FO B cells were CD23+ The FO fraction also had immature (AA4.1+) cells. A total of 10 × 106 of MZ B cells (●) and FO B cells (○) were adoptively transferred into lightly irradiated (3 Gy) JHT recipients (n = 5), and cGVH was induced by transferring 1 × 106 bm12 cells. Anti-dsDNA Abs (p = 0.001) (A) and anti-chromatin Abs (p = 0.003) (B) with adoptively transferred 56R B cells following induction of cGVH. Anti-dsDNA (p = 0.007) (C) and anti-chromatin Abs (p = 0.006) (D) secreted by B6 B cells. Error bars, SEM samples. The p values refer to the difference in autoantibody titers secreted by JHT recipients with MZ B cells and those with FO B cells.
Ig gene rearrangement (25). These observations might have predicted that under allostimulus, immature B cells would preferentially become autoreactive, but this was contrary to our present data. In future work, it will be important to study the extent of receptor editing and secondary rearrangements that occur in the transferred populations of anti-dsDNA B cells upon stimulation with allo-T cells in the secondary JHT host.

Our studies further suggest that MZ B cells played an important role in the pathogenesis of the disease. We found that transferred MZ B cells responded more strongly to allostimulation than did FO B cells. Although, the FO B cell population also contained immature B cells (AA1.4+), but it is only a small fraction and we have shown that immature B cells are less responsive to cGVH stimulation. Therefore, any contribution from immature B cells would not dilute the response of FO B cells significantly (data not shown). MZ B cells occupy a unique niche in the splenic architecture and have been under scrutiny because of their possible role in autoimmunity. It has been shown that MZ area is enriched in autoreactive B cells in certain transgenic models and autoimmune-prone mice. CD1<sup>high</sup>B cells have been reported to produce large amounts of IgM anti-dsDNA Abs in lupus-prone (New Zealand Black × New Zealand White)F<sub>1</sub> mice, (26), which have an increased number of MZ B cells (27). It has also been suggested that defective removal of apoptotic cells causes activation of autoreactive anti-Sm MZ B cells in anti-Sm tg mice (28). Using the K/BxN mouse model of rheumatoid arthritis, Mandlik-Nayak et al. (18) have shown that autoreactive MZ B cells are spontaneously activated. These studies collectively implicate a role for MZ B cells in autoimmunity.

Studies with VH3H9 Ig transgenic mice have shown that anti-dsDNA B cells are excluded from the follicles (4, 29). We hypothesize that these anti-dsDNA B cells probably accumulate in the MZ area. Recent work from Weigert’s laboratory using the 56R model also indicated that dual receptor expressing autoreactive B cells home to the MZ area, and these B cells may contribute to the autoimmune response generated in cGVH (5). Autoreactive B cells tend to concentrate in MZ region, and MZ B cells can undergo class-switching and somatic hypermutation in response to certain Ags. In fact, for some Ags, MZ B cells may be the most important source of somatically mutated post germinal center B cells (30). It has also been shown that MZ B cells, not FO B cells, are most potent activators of CD4<sup>T</sup> cells (31). In another recent study, it was suggested that autoreactive B cells are retained for a prolonged period in the MZ area because they over-express BAFF, and BAFF-tg mice have an expanded MZ B cell pool and develop lupus-like disease (32). Based on these studies and our own data, we therefore propose two possible reasons why the onset of the disease is initiated from the MZ region. First, it is possible that most anti-dsDNA-specific B cells that are barred from entering the follicles “home” to the MZ area where they may persist for a long time. Second, the MZ B cells that arise from precursor transitional cells probably have an intrinsic potential to become autoreactive when they encounter suitable Ag and T cell-derived signals.

Our previous studies in 3H9 and 56R mice showed that upon induction of cGVH, CD23 expression level was significantly reduced in activated B cells, especially in λ<sup>+</sup>dsDNA B cell population, and the MZ B cell population was reduced (4, 5). We postulate that the MZ cells in situ were preferentially affected by the cGVH, and changed their phenotype and/or their anatomical location. Our adoptive transfer studies also indicate that MZ B cells indeed have autoreactive potential and are preferably activated by alloreactive T cells.

In our study, the production of autoantibodies by adoptively transferred FO B cells followed a slower time course than did MZ cells in the cGVH. Previous reports have shown that adoptive transfer of highly purified FO B cells in lymphopenic hosts, such as RAG<sup>−/−</sup> mice, led to their differentiation into MZ B cells (33). It is possible that the autoantibodies in our model may be the product of newly differentiated MZ B cells that arise from the FO B cells. Alternatively, the FO B cells could be activated by a mechanism that differs from that operating on MZ cells. We cannot rule out a role for the contaminating immature B cells in our FO population, but we doubt that they were important, because their numbers were relatively few, and our data had already indicated that they were even less susceptible to autoreactivity in this model.

In conclusion, we have established that cGVH reactions could be successfully initiated in adoptive transfer studies. We have also shown that mature B cells from 3H9 mice are more predisposed to loss of tolerance than immature B cells. Furthermore, we show that MZ cells are mostly readily activated by allo-T help and differentiate to become autoantibody-producing B cells. Because loss of tolerance and autoantibody production may commence from MZ B cells, it is possible that they could represent a useful target for therapeutic interventions.

Disclosures

The authors have no financial conflict of interest.

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