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Transgenic Expression of a Human Polyreactive Ig Expressed in Chronic Lymphocytic Leukemia Generates Memory-Type B Cells That Respond to Nonspecific Immune Activation

George F. Widhopf II,†* Diana C. Brinson, † Thomas J. Kipps,* and Helen Tighe†‡

We generated transgenic mice, designated SMI, expressing unmutated H and L chain Ig genes encoding a low-affinity, polyreactive human (h)IgM/κ rheumatoid factor. These animals were compared with control AB29 transgenic mice expressing a hlgM/κ rheumatoid factor specific for human IgG, with no detectable reactivity with mouse proteins. SMI B cells expressed significantly lower levels of surface hlgM/κ than did the B cells of AB29 mice, but still could be induced to proliferate by surface Ig cross-linking in vitro and could be deleted with anti-Id mAb in vivo. Transgene-expressing B cells of AB29 mice had a B-2 phenotype and were located in the primary follicle. In contrast, a relatively high proportion of hlgM-expressing B cells of SMI mice had the phenotype of B-1 B cells in the peritoneum or marginal zone B cells in the spleen, where they were located in the periaortolar sheath, marginal zone, and interfollicular areas that typically are populated by memory-type B cells. Although the relative proportions of transgene-expressing B cells in both types of transgenic mice declined with aging, SMI mice experienced progressive increases in the serum levels of IgM transgene protein over time. Finally, SMI transgene-expressing B cells, but not AB29 transgene-expressing B cells, were induced to secrete Ab when cultured with alloreactive T cells. These results indicate that expression of polyreactive autoantibodies can allow for development of B cells that are neither deleted nor rendered anergic, but instead have a phenotype of memory-type or Ag-experienced B cells that respond to nonspecific immune activation.


The elimination of B cells expressing Ig with self-reactivity is critical for establishing and maintaining immunological self-tolerance. Most autoreactive B cells are deleted or rendered anergic at the immature stages in the marrow, as has been clearly demonstrated in studies of Ig transgenic mice (1–5). Autoreactive, nonanergic B cells that escape the marrow generally are eliminated upon encounter with autoantigen in the periphery (6–8).

Although anergy is the primary mechanism of tolerance to soluble self-Ags in the periphery, a notable exception is tolerance to self-IgG. High-affinity rheumatoid factor (RF)3-expressing B cells can be deleted by activation-induced apoptosis upon encounter with soluble human (h)IgG (9, 10). In contrast, B cells expressing Ig with low affinity for self-IgG are apparently unaffected by the presence of autoantigen (11). Many such Abs are also polyreactive, because they bind with low affinity to two or more distinct self-Ags. Unlike the pathologic high-affinity RF found in patients with rheumatoid arthritis, these natural autoantibodies are present in the circulation of many normal healthy individuals where they have no apparent pathological significance. However, similar Abs are also frequently associated with graft-vs-host disease (GVHD), mixed cryoglobulinemia, or Waldenström macroglobulinemia, and are expressed by the malignant B cells of patients with various lymphoproliferative diseases, such as chronic lymphocytic leukemia (CLL) (12). The link between Ab specificity and these disease processes is unclear.

Polyreactive autoantibodies are naturally occurring Ig, primarily of the IgM isotype, that bind with low affinity to two or more distinct self- or nonself-Ags, such as hlgM, ssDNA, dsDNA, histones, cardioliopin, actin, and/or cytoskeletal components (13, 14). The autoreactive B cells that produce these Ig are not rendered anergic, because the autoantibodies they produce constitute a large fraction of total serum Ig. Such Abs additionally account for a large proportion of the early human B cell repertoire and are speculated to contribute to homeostasis and/or competence of the primary humoral immune response. Polyreactive autoantibodies generally are encoded by unmutated germline V region genes (V genes). In both humans and mice, the cells that frequently produce these autoantibodies are B-1 B cells that coexpress B cell surface Ags and the CD5 molecule. In mice, these cells are commonly found in the peritoneum (15, 16).

Mounting evidence exists from several recent studies that engagement of the B cell receptor (BCR) with self- or autoantigen can influence the fate and differentiation of B cells in lymphoid tissues (17–22). Antigenic selection also has been suggested for the development and maintenance of natural autoantibodies (13, 14, 20, 23). This is supported by studies showing that such polyreactive Ig are encoded by a limited and conserved set of Ig VH genes (24). Also, structure-function analyses of Ig H chains have demonstrated that the somatically generated third complementarity determining region contributes significantly to the polyreactive
binding activity of natural autoantibodies (25–27). Several of the latter studies were conducted with polyreactive Ig isolated from leukemic B cells of patients with CLL, a disease in which there is frequent neoplastic B cell expression of polyreactive IgM autoantibodies, such as those that bind IgG, cardioliopin, ssDNA, actin, and/or thyroglobulin (12, 28). How cells expressing polyreactive or natural autoantibodies are regulated or are able to evade immune tolerance is not known. Neither is it clear why such Abs are disproportionately represented in CLL, mixed cryoglobulinemia, Waldenström’s macroglobulinemia, or during GVHD following allogenic stem cell transplantation.

To study this, we generated transgenic mice with B cells that express a hlgM/k Ab with low-affinity RF activity (23). This Ab was derived from a patient with CLL (designated SMI), whose H and L chain genes were encoded by 51p1 and A27, respectively. This Ab also binds myoglobin, thyroglobulin, actin, and ssDNA. The SMI mice were compared with transgenic mice, designated AB29, that have B cells that express a hlgM/k RF with high affinity for hlgG, but without reactivity with other known Ags, and in this case, represent a negative control (29). We demonstrated that SMI hlgM/k-expressing B cells were neither deleted nor rendered anergic, but populated distinct lymphoid compartments, displayed the phenotype of Ag-experienced, or memory-type B cells.

Materials and Methods

Transgenic mice

Transgenic mice were generated that expressed the genes encoding a polyreactive hlgM/k RF isolated from the leukemia B cells of a patient with CLL. The V regions of the H and L chains of SMI, respectively, are encoded by an unmutated allele of the V H 51p1 gene, designated 51p1, and an unmutated V L 51p1 gene, designated A27 (23). The vectors containing human Ig constant regions are those previously described for the generation of the AB29 mice that express the rearranged Ig H and L chains encoding the LES hlgM/k RF, and include the murine H chain enhancer for B cell-specific expression (29). The S12 and S6 transgenic lines were generated from individual founder mice expressing identical H and L chain genes encoding the SMI polyreactive IgM Ab. Data from experiments using S12 mice are shown, although numerous experiments have been repeated using both strains. All transgenic lines were maintained by backcrossing heterozygous males with C57BL/6 females (The Jackson Laboratory, Bar Harbor, ME). Positive progeny are identified at 4–5 wk of age by testing sera for the presence of hlgM and human k L chains, as previously described (29). All mice were housed in the animal facility of the University of California, San Diego.

Flow cytometry analyses

Harvested mouse spleens were teased into single-cell suspensions using RP-10 medium (RPMI 1640 medium supplemented with penicillin, streptomycin, 2 mM t-glutamine, 5 × 10–3 M 2-ME, and 10% heat-inactivated FBS). Peritoneal cells were harvested before spleenectomy by injecting the peritoneum of each mouse with 3 ml of RP-10 medium followed by withdrawal of the peritoneal exudate. Single-cell suspensions were purified of RBCs by hypotonic lysis with ACK lysis solution (BioWhittaker, Walkersville, MD). Washed spleen cells were incubated for 10 min at 4°C with anti-CD16/32 (2.4G2) to block FcR-mediated cytophilic binding (BD Pharmingen, San Diego, CA). Cells were stained on ice for 20 min with optimized amounts of the appropriate mAbs conjugated to FITC, PE, PerCP, allophycocyanin, or biotin; washed; and, when necessary, stained for an additional 20 min with streptavidin-allophycocyanin (BD Pharmingen). Cells were examined by four-color, multiparameter flow cytometry using a dual-laser FACSCalibur (BD Biosciences, San Jose, CA). Data were analyzed using FlowJo analysis software (Tree Star, San Carlos, CA). Viable lymphocytes were defined by exclusion of propidium iodide and c protein-binding sites.

mAbs used included B220 (RA3-6B2), CD23 (B384), CD43 (S7), CD21 (7G6), CD5 (53-7.3), heat-stable Ag (HSA; M1/69), CD3 (17A2), hlgM (G20-127), hκ (G20-193), and mouse (mlgM) (R6-60.2). These mAbs were purchased from BD Pharmingen. The G6 anti-idiotypic mAb was as described (30).

Immunohistochemistry

Spleens were snap frozen in optimal temperature medium (OCT; Miles Laboratories, Naperville, IL). Five-micrometer sections were prepared from tissue blocks. After air-drying and acetone fixation, endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide. Slides were blocked with 10% goat serum/1% BSA in PBS (pH 7.4) and then stained first for mouse T cells using biotinylated anti-CD4 (BD PharMingen), followed by HRP-streptavidin (Jackson ImmunoResearch, West Grove, PA), and diaminobenzidine substrate (Dako, Carpinteria, CA). Human IgM-expressing cells were detected by sequential addition of alkaline phosphatase-conjugated anti-hlgM (Southern Biotechnology Associates, Birmingham, AL), followed by Vector Blue (Vector Laboratories, Burlingame, CA).

B cell proliferation assays

Freshly harvested splenocytes were washed, counted, and then suspended at 3.5 × 105 cells/ml in RP-10 medium. We added 100 μl of this cell suspension to each well of a 96-well round-bottom plate. To each well, we added another 100 μl of RP-10 medium containing either FlAb1, a goat anti-hlgM (Jackson ImmunoResearch), aggregated hlgG, or LPS (Salmonella minnesota; Sigma-Aldrich, St. Louis, MO), as indicated in the text. Aggregated hlgG was prepared by incubating a solution of IgG at 10–20 mg/ml in PBS (pH 7.4) at 63°C for 1 h followed by cooling on ice for 2 h. All tests were performed in triplicate. Proliferation was measured on day 2 or 3 of culture by adding 1 μCi of [3H]thymidine to each well. The assay was terminated 18 h later by harvesting plates using an automated cell harvester.

Injection of deaggregated hlgG or G6 anti-Ig mAb

Human IgG (Miles Laboratories) was purified using a protein A-Sepharose column, as described (29). The G6 mAb reactive with Ig encoded by the 51p1 V H gene was prepared from the ascites fluid of SCID mice (The Jackson Laboratory) injected with the G6 hybridoma. G6 mAb was partially purified by a 50% ammonium sulfate precipitation, followed by protein A purification and repeated dialyzes into PBS. All preparations of hlgG and G6 mAb were tested by the Limulus amebocyte lysate assay (Associates of Cape Cod, Falmouth, MA) and found to contain <0.2 ng of endotoxin for each milligram of protein. Aggregated hlgG or G6 were removed from the preparations immediately before injection by ultracentrifugation at 40,000 rpm for 150 min at 4°C using an SW60 rotor. The top 25% of the preparation was removed gently and then diluted in RPMI/10% FCS/PBS to achieve a final Ig protein concentration of 3–4 mg/ml. Immediately thereafter, 2 mg of the deaggregated hlgG or G6 was injected into the peritoneum of each recipient mouse. All mice were sacrificed 7 days later, and the spleen cells were removed for immune phenotypic analyses.

Induction of hlgM secretion in vitro

Spleen cells were added to 96-well round-bottom plates at 3.5 × 105 cells/well in 100-μl volume. We then added either medium or hlgG or aggregated hlgG to a final concentration of 10 or 0.5 μg/ml, respectively. To determine whether T cell help alone was sufficient to stimulate secretion of hlgM, C57BL/6mig212 allogeneic spleen cells were used as a source of T cells, as described previously (9). Briefly, 5 × 105 SMI, AB29, or nontransgenic littermate spleen cells were added to 96-well flat-bottom plates followed by 5 × 104 C57BL/6mig212 allogeneic spleen cells or 5 × 105 C57BL/6 control filler spleen cells per well in a total volume of 200 μl. All cultures were performed in duplicate wells and using duplicate plates, and incubated at 37°C in 5% CO2. One set of plates was used for measuring hlgM-secreting cells by ELISPOT after 4 days of culture. Supernatants were harvested from the other plate after 7 days of culture to determine levels of hlgM in the culture supernatant by ELISA (29).

Measurement of hlgM-secreting cells by ELISPOT and ELISA

The numbers of hlgM-secreting cells were measured by ELISPOT. Briefly, 96-well flat-bottom microtiter plates were incubated overnight at 4°C with goat anti-hlgM at 10 μg/ml in PBS. After washing, the plates were washed with PBS containing 1% BSA to block nonspecific protein-binding sites. Cultured spleen cells were washed to remove secreted hlgM, suspended in fresh RP-10, and then added at various concentrations to the ELISPOT plates. After incubation at 37°C for 4 h, Ab-producing cells were detected using sequential addition of goat anti-hlgM-biotin (Accurate Chemical, Westbury, NY) and streptavidin–alkaline phosphatase (Kirkegaard & Perry Laboratories, Gaithersburg, MD) diluted in PBS followed by 5-bromo-4-chloro-3-indolyl phosphate substrate (Sigma-Aldrich). After developing
the plates, the wells containing 5–100 spots were counted. The mean numbers of spots per 10^6 hlgM^+ cells were calculated from the percentage of hlgM^+ cells at the initiation of the spleen cell cultures.

Results
Generation and characterization of SMI transgenic mice

We generated transgenic mice (designated SMI) that expressed a hlgM/k polyreactive, low-affinity RF derived from the leukemic B cells of a patient with CLL (23). These animals were compared with control transgenic mice, designated AB29, that expressed a hlgM/k high-affinity RF that reacts with hlgG, but not with other known Ags, including mouse IgG (data not shown) (9, 23, 29, 31). We found that expression of a low-affinity polyreactive Ab does not lead to cell deletion. Approximately 12% (mean ± SD, 12.0 ± 1.9%; n = 4) of the splenic mononuclear cells of SMI transgenic mice expressed surface (s)hlgM/k at 8–10 wk of age (Fig. 1A). The H and L chain Ig transgenes were coordinately expressed on the surface of 32% (31.7 ± 6.0%; n = 4) of the splenic B cells. Furthermore, hlgM/k expression was restricted to B cells (Table I). Allelic exclusion of the H chain was nearly complete, because cells expressing shlgM did not coexpress hlgM. In some experiments, a small proportion (<5%) of B cells expressed hlgM in the absence of human k, suggesting that L chain allelic exclusion was not absolute. Lastly, we found that SMI splenic B cells expressed levels of sIgM (mean fluorescence intensity ratio, 41.1 ± 3.4) that were significantly lower than that found on AB29 splenic B cells (mean fluorescence intensity ratio, 67.0 ± 3.2; n = 4; p < 0.0001, Student’s t test), consistent with the phenotype of anergic B cells (2).

To examine whether the hlgM/k^+ B cells of SMI were resistant to deletion, we injected a high-affinity ligand (G6) into the peritoneum of SMI mice. G6, a mouse mAb specific for the cross-reactive Id present on the SMI hlgM H chain, does not compete with anti-hlgM mAb for binding to SMI-expressing B cells. Injection of G6 reduced the mean proportion of hlgM/k-expressing SMI splenic B cells from 48 to <3% (Table II). The reduction of shlgM/k-expressing cells was not due to BCR down-modulation, because SMI mice that received deaggregated G6 had a concomitant and corresponding loss of splenic B220^+ cells (Table II). This depletion of hlgM/k^+ B cells was similar to that noted for AB29 mice injected with deaggregated hlgG. However, SMI animals injected with deaggregated hlgG had negligible loss of transgene-expressing splenocytes, as did either mouse strain after injection with saline alone (Table II), demonstrating the significance of the affinity of the Ag-Ab interaction in B cell clearance.

SMI B cells are responsive to ligation of their transgenic BCR

To examine whether SMI hlgM/k B cells were anergic, we examined whether we could induce proliferation of splenic hlgM/k-expressing B cells through ligation of the BCR complex. For this, we cultured splenocytes of SMI or AB29 transgenic mice with either F(ab')_2 anti-hlgM or aggregated hlgG. F(ab')_2 anti-hlgM induced significant proliferation of the hlgM^+ splenocytes from either SMI (mean ± SD, 90 ± 12 × 10^3 cpm; n = 4) or AB29 (136 ± 45 × 10^3 cpm) (Fig. 2). However, 10 μg/ml aggregated hlgG did not induce significant proliferation of splenic hlgM^+ cells (1.4 ± 0.5 × 10^3 cpm), as compared with medium alone (1.2 ± 0.2 × 10^3 cpm). In contrast, the same amount of aggregated hlgG induced proliferation of AB29 splenocytes (26 ± 6 × 10^3 cpm). LPS induced proliferation of splenocytes from SMI, AB29, or nontransgenic mice (data not shown). We conclude that SMI B cells are neither deleted nor rendered anergic in vivo.

SMI splenic B cells are enriched in T1 and marginal zone (MZ) B cell subsets

Human Ig-expressing splenic B cells from SMI mice were examined by flow cytometry for expression of surface Ags that distinguish various defined B cell subsets. We found that the proportion of the hlgM^+ B cells from 8- to 10-wk-old SMI mice that expressed CD23 (78%) (Fig. 1B, Table III) was significantly lower than that of hlgM^+ B cells of age-matched AB29 control mice (97%; n = 4; p < 0.0001, Student’s t test). The subset of B cells
that express CD23 is comprised of both mature B-2 cells (CD23+CD21lowHSAint) and T2 transitional B cells (CD23+CD21highHSAhigh) (32). In SMI mice, 57 ± 3.9% of the splenic hlgM+ B cells were B-2 type cells and 19 ± 2.1% were T2 B cells. In contrast, 82 ± 4.4% of the splenic hlgM+ B cells of AB29 mice were B-2-type B cells and 14 ± 4.4% were T2-type B cells. As such, the proportion of the hlgM transgene-expressing B cells of SMI mice that were mature B-2 type B cells was significantly lower than that found in AB29 mice (p < 0.0001; n = 4; Student’s t test).

The population of splenic B cells that do not express CD23 is comprised of both immature T1 (CD23+CD21highHSAhigh) and MZ B cells (CD23−CD21highHSAhigh) (Table III). Eight- to 10-wk-old SMI mice had significantly higher proportions of transgene-expressing splenic T1 B cells (15.3 ± 2.2%) and MZ B cells (6.8 ± 1.8%) than did age-matched AB29 mice, which have only 1.5 ± 0.2% (p < 0.0001; n = 4; Student’s t test) and 1.3 ± 0.5% (p < 0.0002; n = 4; Student’s t test) T1-type and MZ-type transgene-expressing splenic B cells, respectively.

**SMI peritoneal B cells are enriched in both B-1a and B-1b subsets**

We also examined the peritoneal lymphocytes of SMI mice for coexpression of the hlgM transgenes and surface Ags that distinguish various defined B cell subsets. As noted for other mouse strains, the peritoneal B cells of SMI and control AB29 transgenic mice had higher proportions of B-1-type B cells than did the B cells in the spleen (data not shown) (33). B cells that express hlgM/hlgK constituted 7.2 ± 1.9% or 20 ± 2.0% of the peritoneal lymphocytes from 8- to 10-wk-old SMI or AB29 mice, respectively (p < 0.0001; n = 4; Student’s t test). However, SMI mice exhibited significantly higher proportions of hlgM/hlgK B-1 B cells in their peritoneum, because 21 ± 4.4% (n = 4) of the peritoneal transgene-expressing B cells from SMI mice were B-1a B cells (CD5+CD23−CD11b+) (Fig. 3) and 4.4 ± 1.2% (n = 4) were B-1b cells (CD5+CD23+CD11b+). In contrast, in AB29 mice, only 2.8 ± 1.9% (n = 4) or <1% (0.7 ± 0.4%; n = 4) of transgene-expressing peritoneal B cells were B-1a- or B-1b-type B cells, respectively. The remaining human transgene-expressing peritoneal B cells were B-2 B cells, as indicated by their coexpression of the CD23 (Fig. 3).

**SMI B cells localize within the perivascular sheath (PALS), MZ, and interfollicular space, as well as in the primary follicle**

Fresh-frozen sections of spleens from SMI or AB29 control mice were examined to study the tissue distribution of hlgM/hlgK BCR. Splenocytes from SMI, AB29, or nontransgenic littermates were stimulated in vitro with 10 µg/ml F(ab’)2 anti-hlgM or cultured in medium alone. Proliferation was measured on day 2 or 3 of culture by addition 1 µCi of [3H]thymidine per well. Cells were harvested 18 h later using an automated cell harvester, and the incorporated [3H]thymidine was assessed by scintillation counting. A. Graphs depict data expressed as the mean counts per minute incorporated ± SD per 3.5 × 10⁶ input spleen cells. Data represent groups of four mice, with all tests performed in triplicate. B. Graphs depict the same data normalized for equal input of 3.5 × 10⁶ hlgM+/hlgK+ B cells, calculated based on the percentage of spleen cells expressing hlgM at the start of the culture. Background proliferation in the presence of medium alone has been subtracted from all values.

### Table I. **Phenotype of splenic lymphocyte populations of SMI and AB29 hlg-transgenic mice**

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<tr>
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<th>SMI</th>
<th>AB29</th>
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<tr>
<td></td>
<td>Of total</td>
<td>Of B cells</td>
</tr>
<tr>
<td></td>
<td>Of total</td>
<td>Of B cells</td>
</tr>
<tr>
<td>B220</td>
<td>39.2 ± 2.0</td>
<td>47.0 ± 1.6</td>
</tr>
<tr>
<td>hlgM/</td>
<td>12.0 ± 1.9</td>
<td>32.3 ± 6.3</td>
</tr>
<tr>
<td>hlgK</td>
<td>15.1 ± 1.8</td>
<td>6.8 ± 0.4</td>
</tr>
<tr>
<td>mlgM</td>
<td>50.4 ± 4.0</td>
<td>16.8 ± 1.8</td>
</tr>
</tbody>
</table>

Values shown represent the mean percentage ± SD of viable splenic mononuclear cells (of total) or splenic B220+ cells (of B cells) that express each surface Ag(s). Values are the average of four 8- to 10-wk-old mice per group and are representative of multiple experiments.

<table>
<thead>
<tr>
<th></th>
<th>SMI</th>
<th>AB29</th>
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<tr>
<td></td>
<td>Of total</td>
<td>Of B cells</td>
</tr>
<tr>
<td></td>
<td>Of total</td>
<td>Of B cells</td>
</tr>
<tr>
<td>B220</td>
<td>18.8 ± 4.3</td>
<td>46.1 ± 5.5</td>
</tr>
<tr>
<td>hlgG</td>
<td>4.6 ± 0.3</td>
<td>40.5 ± 4.6</td>
</tr>
<tr>
<td>mlgK</td>
<td>1.8 ± 0.4</td>
<td>30.5 ± 5.7</td>
</tr>
<tr>
<td>mlgG</td>
<td>1.9 ± 0.4</td>
<td>48.2 ± 8.6</td>
</tr>
</tbody>
</table>

Values shown represent the mean percentage ± SD of viable splenic mononuclear cells (of total) or B220+ cells (of B cells) that express each surface Ag(s). Values are the average of four 8- to 10-wk-old mice per group and are representative of multiple experiments.

*SMI and AB29 mice were injected in the peritoneum with 2 mg of G6 mAb, 2 mg of deaggregated hlgG (hlgG), or PBS alone (none), and sacrificed 7 days later for immune phenotypic analysis. Splenic lymphoid cells were stained for expression of hlgM or B220, and analyzed by flow cytometry. Data are expressed as the mean percentage of positive cells ± SD of either total viable lymphocytes (of total) or B220+ cells (of B cells). The data represent the average of four mice per treatment group and are representative of duplicate experiments.

### Table II. **Deletion of SMI hlgM/hlgK B cells upon introduction of high-affinity ligand in vivo**

<table>
<thead>
<tr>
<th></th>
<th>SMI</th>
<th>AB29</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>% hlgM of Total</td>
<td>% hlgM of B Cells</td>
</tr>
<tr>
<td>SMI</td>
<td>None</td>
<td>19.3 ± 7.7</td>
</tr>
<tr>
<td>SMI</td>
<td>hlgG</td>
<td>18.8 ± 4.3</td>
</tr>
<tr>
<td>SMI</td>
<td>G6 mAb</td>
<td>0.8 ± 0.4</td>
</tr>
<tr>
<td>AB29</td>
<td>None</td>
<td>32.8 ± 6.2</td>
</tr>
<tr>
<td>AB29</td>
<td>hlgG</td>
<td>5.0 ± 0.3</td>
</tr>
</tbody>
</table>
We examined for age-related changes in the proportion of transgene-expressing cells were found to reside within the MZ. In addition, SMI hIgM-κ-expressing cells also were found in the interfollicular space and PALS, areas typically inhabited by B cells that have encountered Ag. In contrast, AB29 mice had few transgene-expressing B cells in these subnecrotic sites.

Age-related changes in transgene expression

We examined for age-related changes in the proportion of transgene-expressing B cells and serum hIgM/κ over time. We found the relative proportions of B cells that expressed hIgM/κ declined as the mice aged (Table IV). In addition, the total number of hIgM/κ-expressing splenic B cells decreased upon aging from 9 to 45 wk, from 10.8 × 10^6 to 9.7 × 10^6 in SMI mice, and from 14.2 × 10^6 to 9.7 × 10^6 in AB29 mice. In contrast, SMI mice had significantly higher levels of serum hIgM/κ than did AB29 mice at any age (p < 0.001; Student’s t test). For example, at 4–6 wk of age, the mean serum concentration of hIgM/κ was 33 μg/ml (32.5 ± 9 μg/ml; n = 7) in SMI mice, but only 12 μg/ml (12.4 ± 3.1 μg/ml; n = 5) in AB29 mice. The differences in the serum concentrations of the transgene IgM/κ between the two types of transgenic mice increased as the animals got older. Whereas SMI mice at 30–32 wk of age had an elevated mean serum concentration of hIgM/κ of 55 μg/ml (54.7 ± 21.3 μg/ml; n = 7), comparably aged AB29 mice showed a decline in mean serum concentration to only 4 μg/ml (4.4 ± 1.4 μg/ml; n = 5).

SMI B cells secrete Ab in response to T cell help alone

Given the indications that a proportion of the SMI B cells had an Ag-experienced phenotype and the well-documented association of polyreactive autoantibodies with GVHD, we attempted to determine whether T cell help alone is sufficient to stimulate secretion of SMI hIgM, by culturing splenocytes from SMI mice with C57BL/6^bm12^-alloreactive T cells. Addition of C57BL/6^bm12^-alloreactive T cells to SMI splenocytes induced differentiation of IgM/κ-secreting cells, as assessed by ELISPOT assay on day 4 of culture (2.7 ± 0.7 × 10^3 spots per million B cells; n = 4)) (Fig. 5). We also noted increased hIgM in the culture supernatants of wells containing the C57BL/6^bm12^-alloreactive T cells by ELISA (data not shown). In contrast, addition of C57BL/6^bm12^-alloreactive T cells to AB29 splenocytes generated significantly fewer IgM/κ-secreting cells per million B cells (173 ± 50; n = 4; p < 0.001, Student’s t test). All animals had negligible responses to either medium alone or to control autologous C57BL/6 splenocytes (Fig. 5). AB29 splenocytes, but not SMI splenocytes, also responded to aggregated hIgG (4.8 ± 0.3 × 10^3 vs 48 ± 55; n = 4).

**Discussion**

In this study, we found that expression of human low-affinity, polyreactive Ig, encoded by unmutated Ig V genes that are used frequently by CLL B cells, could induce mouse B cells to differentiate into nonnaive, memory-type B cells that are hypersensitive to nonspecific T cell help. The human Ig-expressing B cells from SMI mice were neither deleted nor rendered anergic, because they were found in the peripheral lymphoid compartments and responded well to stimulation via their transgenic Ag receptors, respectively. Importantly, we found SMI mice, but not control AB29 mice, developed some hIgM/κ-secreting cells per million B cells (173 ± 50; n = 4; p < 0.001, Student’s t test). All animals had negligible responses to either medium alone or to control autologous C57BL/6 splenocytes (Fig. 5). AB29 splenocytes, but not SMI splenocytes, also responded to aggregated hIgG (4.8 ± 0.3 × 10^3 vs 48 ± 55; n = 4).

**FIGURE 3.** Phenotypic characterization of SMI hlgM/κ peritoneal B cells. Peritoneal B cells of 8-wk-old SMI and AB29 mice were analyzed by flow cytometry for expression of human Ig transgenes and other cell surface markers that define B cell subsets. Contour plots depict staining of live lymphocytes, as determined by propidium iodide exclusion and light scatter characteristics, with fluoro-chrome-conjugated mAbs specific for hlgM and either murine CD5 or CD23 (upper panel). The bottom panels depict the logarithmic fluorescence intensity (x-axis) of CD5 (lower left panel) or CD23 (lower right panel) of gated hlgM+ SMI (shaded histogram) or AB29 (open histograms) peritoneal B cells.
are 42–45 wk old. Values represent the average of four to six mice per group and are representative of multiple experiments.

The Ig molecule is derived from the B cells of a patient with CD5 B cell populations of SMI vs control AB29 mice. Whereas the SMI ranged human Ig transgene, as noted by the differences in splenic differentiation is not solely a consequence of the expression of a rear-
tabled by injection of deaggregated high-affinity anti-idiotypic G6 mAb, indicating that SMI transgenic B cells are not protected from clearance by high-affinity Ag.

A number of other Ig transgenic mice have been used to exam-
ined the development and differentiation of B cells, particularly those that express Ag receptors that are reactive against self-Ags. They have demonstrated that differentiation of these B cells is influenced by a combination of mechanisms operating in both the central and peripheral lymphoid compartments (34–36). Although BCR expression is critical for B cell differentiation (37, 38), engagement of the BCR with self-Ag can profoundly influence the fate of B cell development. The ultimate fate of an autoreactive B cell depends upon several factors, such as the form and location of Ag, Ag concentration, structure, the affinity of the expressed BCR, and the availability of T cell helper activity (34, 36). Mice that are made transgenic for both Ig with high Ag-binding affinity and specific Ag, experience either anergy or deletion of B cells that expressed the Ig transgenes, unless they undergo Ig receptor editing to express BCR that no longer react with the transgenic Ag (39). Unlike transgenics expressing higher affinity receptors for soluble IgG (29) or reacting to membrane-bound hen egg lysozyme (HEL) (3) or H-2 Ags (4), SMI B cells are not deleted and are found in peripheral lymphoid compartments. Additionally, they are not ren-
dered anergic as a consequence of maturational arrest or exclusion.

The influence that expression of hlgM/κ has on B cell differen-
tiation is not solely a consequence of the expression of a rear-
ranged human Ig transgene, as noted by the differences in splenic B cell populations of SMI vs control AB29 mice. Whereas the SMI Ig molecule is derived from the B cells of a patient with CD5⁺ CLL and is a low-affinity RF that also binds to a variety of self-Ags (23), the mutated high-affinity hlgM/κ RF of AB29 reacts only with hlgG. Unlike AB29 mice, SMI mice do not experience de-
letion of transgene-expressing B cells upon treatment with deaggregated hlgG. Nevertheless, SMI hlgM/κ B cells can be de-
icted anergic as a consequence of maturational arrest or exclusion.
from the B cell follicles of the spleen, as are those of other mice such as those specific for dsDNA (40, 41) or SHEL (2, 6, 42).

The SMI mice share some, but not all, characteristics with other transgenic mice that express Ig with low affinity for self-Ag, such as anti-Sm or anti-ssDNA. The B cells of 2–12H anti-Sm mice, which express a low-affinity Ig specific for an Ag associated with systemic lupus erythematosus, are anergic as a consequence of maturational arrest at the T1 transitional stage of development, and have low levels of transgene in the sera (43–45). However, an interesting similarity with SMI is that ~30% of the peritoneal B cells of anti-Sm mice are B-1 B cells (44). B cells expressed by the anti-ssDNA mice were phenotypically mature B-2 cells and were not excluded from B cell follicles, but in contrast to SMI, they were anergic (41, 46).

Other Ig transgenic mice also have been noted to have an expanded number of transgene-expressing B cells in the MZ. Examples include mice that are transgenic for Vγ81x-neonatal-derived IgV (47, 48), or mice made transgenic for certain autoantibodies (e.g., Ig directed against DNA (1, 49, 50), phosphorylcholine (51), or nucleoprotein (52)). These findings indicate that transgenic lines with Ig genes derived from diverse sources can have larger MZ B cell compartments, suggesting that this feature is not the result of BCR specificity per se. However, unlike the transgene-expressing B cells of SMI mice, most of these other transgenic mice have loss of allelic exclusion, consistent with the notion that the driving force of MZ B cell development in these animals results from rearrangement and expression of endogenous Ig genes (49, 53). However, the MZ B cells of SMI mice maintain allelic exclusion, suggesting that the expression of the SMi transgenes is sufficient for MZ B cell development.

Several studies have provided evidence that engagement of the BCR results in positive selection of B cells (17–22). Engagement of the BCR is believed to influence the differentiation of a B cell into a particular B cell subset, as well as the peripheral lymphoid compartment in which it resides, and is best demonstrated for the B-1 (18, 54) and MZ (17, 48) B cell lineages. SMI mice have significantly higher percentages of human Ig-expressing peritoneal B cells that express CD5 than do AB29 mice. CD5 is a molecule that modulates BCR signaling, because it acts as a negative regulator in association with Src homology protein-1 phoshptase (55, 56). It is expressed on the surface of both human and murine B-1 B cells, and on the leukemic B cells of virtually all patients with CLL (57). Transgenic mice with BCR characteristics of CD5+ B cells typically develop increased numbers of B-1 B cells, demonstrating the importance of a particular BCR specificity in generating these cells (reviewed in Refs. 58 and 59). If B-1 B cell development requires BCR specificity, then the lack of specific Ag should result in diminished numbers of B-1 B cells in transgenic animals that lack the relevant Ag. Such is the case for transgenic mice that express irrelevant H-2 (54). CD5 can have a direct effect on tolerance induction, because expression of CD5 by anti-HEL B cells was found directly responsible for the reduction or absence of circulating anti-HEL Ab as compared with the large amounts produced by cells lacking CD5 (60). Therefore, it is possible that, by decreasing the sensitivity of the BCR to activation by Ag, low-affinity polyreactive B cells such as SMI might avoid tolerance by differentiation to B-1 cells.

CLL B cells have been shown to express IgM Abs that display reactivity to self-proteins (27, 61, 62). In addition to SMI, several other 51p1-encoded Ig expressed in CLL are polyreactive, having reactivity to IgG, cardiolipin, DNA, actin, and thyroglobulin (23, 63–67). Several studies have postulated that CLL may arise from a clonal outgrowth of B cells (68, 69), based on identification of the restricted use of certain D segments and JH genes in 51p1-encoded Ig expressed by CLL B cells encode a third complementarity-determining region with conserved amino acid motifs (69). More recently, a study using microarray analysis revealed that the gene expression pattern of CLL B is distinct from that of other B cell lymphomas or of adult or cord blood B cells (70). Moreover, this CLL gene expression profile is similar to that of nonnaive, or memory-type, B cells that frequently reside in the MZ of secondary lymphoid tissue. In addition, CLL B cells that have unmaturated Ig genes express several genes that are up-regulated during BCR ligation. As such, these leukemia cells in particular appear to have genetic features in common with B cells that have encountered Ag. Although expression of such Ig by normal B cells does not by itself induce the development of CLL, low-level chronic stimulation may result in prolonging the life of an autoreactive B cell and thus provides an expanded window of time in which the requisite events for leukemogenesis can occur. Additionally, because B cells expressing SMI IgMκ are susceptible to nonspecific immune activation by the provision of T cell help alone, such encounter could render them more susceptible than naive or primary B cells to incurring somatic mutations and cytogenetic changes that result in CLL. Conceivably, these cells could be predisposed to leukemogenesis by virtue of their expressed Ig. As such, the Ig expressed by such B cells might be relevant to the etiopathogenesis of CLL, independent of specific human or environmental Ags.

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**References**


