Maintenance of Tolerance by Regulation of Anti-myeloperoxidase B Cells


*UNC Kidney Center, Department of Medicine, Division of Nephrology and Hypertension, and †Department of Microbiology and Immunology, University of North Carolina, Chapel Hill, North Carolina

ABSTRACT

Anti-neutrophil cytoplasmic autoantibodies directed toward myeloperoxidase or proteinase 3 are detected in sera of patients with small vessel vasculitis and participate in the pathogenesis of this disease. Autoantibodies develop when self-reactive B cells escape the regulation that ensures self-tolerance. In this study, regulation of anti-myeloperoxidase B cells was examined in mice that express an anti-myeloperoxidase V/H92601C-J/H92605 light-chain transgene, which confers anti-myeloperoxidase specificity when combined with a variety of heavy chains. V/H92601C-J/H92605 transgenic mice have splenic anti-myeloperoxidase B cells but do not produce circulating anti-myeloperoxidase antibodies. Two groups of transgenic mice that differed by their relative dosage of the transgene were compared; high-copy mice had a mean relative transgene dosage of 1.92 compared with 1.02 in the low-copy mice. These mice exhibited a 90 and 60% decrease in mature follicular B cells, respectively. High-copy mice were characterized by a large population of anti-myeloperoxidase B cells, a preponderance of B-1 cells, and an increased percentage of apoptotic myeloperoxidase-binding B cells. Low-copy mice had similar changes in B cell phenotype with the exception of an expanded marginal zone population. B cells from low-copy mice but not high-copy mice produced anti-myeloperoxidase antibodies after stimulation with lipopolysaccharide. These results indicate that tolerance to myeloperoxidase is maintained by central and peripheral deletion and that some myeloperoxidase-binding B cells are positively selected into the marginal zone and B-1 B cell subsets. A defect in these regulatory pathways could result in autoimmune disease.


Anti-neutrophil cytoplasmic autoantibodies (ANCA), directed against myeloperoxidase (MPO) and proteinase 3 (PR3), are detected in 90% of patients with small vessel vasculitis, including Wegener's granulomatosis, microscopic polyangiitis, and pauci-immune necrotizing crescentic glomerulonephritis.1,2 Many in vitro2,3 and in vivo4–8 studies demonstrated that ANCA participate in the pathogenesis of small vessel vasculitis. Less work has focused on understanding the immunopathogenesis of the ANCA autoimmune response. Previous studies suggested that the human MPO-ANCA response is highly restricted.9 To investigate this response further, we used an anti-MPO antibody derived from SCG/Kj mice, a recombinant inbred strain that spontaneously develops vasculitis and crescentic glomerulonephritis.

Although SCG/Kj mice may not be a good model for pauci-immune glomerulonephritis, 30% of these mice spontaneously develop anti-MPO anti-

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Correspondence: Dr. Donna Bunch, UNC Kidney Center, Department of Medicine, Division of Nephrology and Hypertension, University of North Carolina, 5005 Burnett-Womack, Campus Box #7155, Chapel Hill, NC 27599. Phone: 919-966-2561, ext. 283; Fax: 919-966-4251; E-mail: donna_bunch@med.unc.edu

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bodies. These anti-MPO antibodies are useful in studying the derivation of this autoimmune response. Previously, we demonstrated a restriction in Vk gene use in anti-MPO-producing hybridomas derived from SCG/Kj mice. This restriction suggests an important role of the Ig light chain in determining specificity to MPO.

The aims of this study were to determine the role of the Ig light chain in determining specificity of anti-MPO antibodies and the mechanism of anti-MPO B cell regulation in a nonautoimmune animal model. Using mice transgenic (Tg) for the Vk1C-Jk5 light chain derived from an anti-MPO hybridoma from SCG/Kj mice, we investigated several questions. Does expression of the Vk1C-Jk5 light chain lead to the generation of anti-MPO B cells? Do anti-MPO B cells produce circulating anti-MPO antibodies? If not, then at which point in their maturation are the B cells regulated to maintain a state of immune tolerance to MPO? The answers to these questions will contribute to our understanding of how tolerance is maintained in normal individuals and how a breakdown in these mechanisms can lead to the generation of anti-MPO antibodies and autoimmune disease.

RESULTS

Vk1C-Jk5 Light-Chain Tg Mice

To study the regulation of anti-MPO B cells, we generated mice transgenic for the rearranged Vk1C-Jk5 gene derived from an anti-MPO hybridoma (Figure 1A). Interbreeding produced two groups of Vk1C-Jk5 Tg mice that differed in their relative number of copies of the transgene (Figure 1B). Mice with the larger number of transgene copies are referred to as “high copy” (HC) mice and the other group as “low copy” (LC) mice. Quantitative PCR analysis revealed that HC Tg mice had a mean \(2^{(−DD_{CT})}\) of 1.92 ± 0.7 of measured transgene in their genomic DNA compared with the LC Tg mice mean \(2^{(−DD_{CT})}\) of 1.02 ± 0.2 \((P = 0.0018)\).

Tg Mice Have MPO-Binding B Cells but no Circulating Anti-MPO Antibodies

To test whether the Vk1C-Jk5 light-chain transgene was sufficient to produce anti-MPO B cells, we searched for MPO-binding cells by FACS analysis. Splenocytes of LC (3.0 ± 0.7%) and HC mice (9.7 ± 8.1%) had a significantly increased percentage of MPO-binding B cells when compared with control mice (1.5 ± 0.6%; \(P < 0.0001\); Figure 1, C and 1D). Likewise, the percentage of MPO-binding cells in the bone marrow of LC and HC mice (6.3 ± 2.3 and 13.7 ± 4.8%, respectively) was significantly greater than in controls (2.1 ± 0.9%; \(P < 0.0001\); Table 1). In contrast, no PR3-binding B cells could be detected in Tg mice, indicating that binding of MPO is specific. Despite the documented presence of anti-MPO B cells, no Tg mice were found to have circulating anti-MPO antibodies as determined by ELISA against either native human MPO or recombinant mouse MPO when compared with non-Tg mice.

Vk1C-Jk5 Splenocytes Can Produce Anti-MPO Antibodies

To verify that the Tg Vk1C/Jk5 light chain participates in the production of secreted anti-MPO antibodies, we gener-
Table 1. Comparison of B cell populations in LC Tg and HC Tg mice to non-Tg control mice

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>B Cell Population</th>
<th>Control [Cell Number (n)]</th>
<th>LC Tg [Cell Number (n)]</th>
<th>HC Tg [Cell Number (n)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow (all B cells)</td>
<td>Total B</td>
<td>50.2 ± 19.8 (7) [202.4 ± 209.5 × 10⁶ (5)]</td>
<td>11.8 ± 3.5 (4)c [31.3 ± 15.4 × 10⁶ (4)]</td>
<td>7.9 ± 8.2 (11)c [14.6 ± 16.0 × 10⁶ (7)]</td>
</tr>
<tr>
<td></td>
<td>Pro-/pre-B</td>
<td>22.2 ± 13.0 (4) [117.2 ± 155.1 × 10⁶ (4)]</td>
<td>6.6 ± 0.4 (3) [45.5 ± 26.4 × 10⁶ (3)]</td>
<td>5.6 ± 2.3 (4) [10.8 ± 7.0 × 10⁶ (4)]</td>
</tr>
<tr>
<td></td>
<td>Immature B</td>
<td>5.4 ± 2.4 (4) [21.7 ± 15.6 × 10⁶ (4)]</td>
<td>1.1 ± 0.2 (3) [7.4 ± 4.5 × 10⁶ (3)]</td>
<td>2.3 ± 0.9 (4) [4.5 ± 3.2 × 10⁶ (4)]</td>
</tr>
<tr>
<td></td>
<td>Recirculating B</td>
<td>24.4 ± 6.9 (4) [91.4 ± 51.7 × 10⁶ (4)]</td>
<td>4.1 ± 2.2 (3) [27.8 ± 18.7 × 10⁶ (3)]</td>
<td>2.3 ± 1.2 (4) [4.2 ± 2.9 × 10⁶ (4)]</td>
</tr>
<tr>
<td>Bone marrow (MPO binders)</td>
<td>Pro-/pre-B</td>
<td>0.9 ± 0.7 (4) [3.7 ± 3.1 × 10⁶ (5)]</td>
<td>7.8 ± 4.4 (3) [1.9 ± 0.6 × 10⁶ (4)]</td>
<td>7.8 ± 11.9 (4) [1.4 ± 2.1 × 10⁶ (7)]</td>
</tr>
<tr>
<td></td>
<td>Immature B</td>
<td>3.2 ± 1.3 (4) [0.7 ± 0.6 × 10⁶ (4)]</td>
<td>3.7 ± 1.5 (3) [0.3 ± 0.2 × 10⁶ (3)]</td>
<td>15.2 ± 13.7 (4) [0.7 ± 0.8 × 10⁶ (4)]</td>
</tr>
<tr>
<td></td>
<td>Recirculating B</td>
<td>1.0 ± 0.3 (4) [3.7 ± 3.1 × 10⁶ (5)]</td>
<td>4.0 ± 0.4 (3) [1.0 ± 0.5 × 10⁶ (4)]</td>
<td>2.9 ± 2.2 (4) [0.2 ± 0.2 × 10⁶ (4)]</td>
</tr>
<tr>
<td>Spleen (all B cells)</td>
<td>Total B</td>
<td>65.6 ± 7.1 (14) [48.8 ± 15.5 × 10⁶ (14)]</td>
<td>48.1 ± 9.9c [32.6 ± 28.9 × 10⁶ (8)]</td>
<td>22.4 ± 17.9 (13)c [20.2 ± 29.3 × 10⁶ (13)]</td>
</tr>
<tr>
<td></td>
<td>Follicular</td>
<td>73.6 ± 7.7 (14) [38.0 ± 13.4 × 10⁶ (14)]</td>
<td>23.1 ± 9.0c [8.2 ± 9.2 × 10⁶ (8)c]</td>
<td>5.2 ± 4.4 (13)c [0.5 ± 0.5 × 10⁶ (13)c]</td>
</tr>
<tr>
<td></td>
<td>Marginal zone</td>
<td>9.2 ± 4.0 (14) [4.6 ± 2.5 × 10⁶ (14)]</td>
<td>39.5 ± 10.2c [13.6 ± 13.5 × 10⁶ (8)c]</td>
<td>8.3 ± 8.0 (13) [0.8 ± 0.8 × 10⁶ (13)]</td>
</tr>
<tr>
<td></td>
<td>B-1</td>
<td>7.5 ± 4.3 (14) [3.5 ± 2.1 × 10⁶ (14)]</td>
<td>25.4 ± 11.2c [6.8 ± 4.6 × 10⁶ (8)]</td>
<td>50.6 ± 21.5 (13)c [13.0 ± 25.6 × 10⁶ (13)]</td>
</tr>
<tr>
<td></td>
<td>MPO binders</td>
<td>1.5 ± 0.6 (14) [0.7 ± 0.3 × 10⁶ (14)]</td>
<td>3.0 ± 0.7c [1.1 ± 1.0 × 10⁶ (8)]</td>
<td>9.7 ± 8.1 (13)c [0.7 ± 0.7 × 10⁶ (13)]</td>
</tr>
<tr>
<td>Spleen (MPO binders)</td>
<td>Follicular</td>
<td>50.5 ± 12.3 (14) [33.0 ± 14.0 × 10⁶ (14)]</td>
<td>18.1 ± 10.1c [14.1 ± 10.7 × 10⁶ (8)]</td>
<td>21.0 ± 16.9 (13)c [9.9 ± 8.7 × 10⁶ (13)]</td>
</tr>
<tr>
<td></td>
<td>Marginal zone</td>
<td>12.0 ± 9.8 (14) [10.8 ± 17.2 × 10⁶ (14)]</td>
<td>36.2 ± 15.6c [48.0 ± 57.4 × 10⁶ (8)c]</td>
<td>5.4 ± 6.1 (13) [3.2 ± 4.4 × 10⁶ (13)]</td>
</tr>
<tr>
<td></td>
<td>B-1</td>
<td>38.9 ± 12.5 (14) [24.7 ± 13.5 × 10⁶ (14)]</td>
<td>51.8 ± 15.1 (8) [49.7 ± 41.4 × 10⁶ (8)]</td>
<td>66.1 ± 18.1 (13)c [52.4 ± 45.9 × 10⁶ (13)]</td>
</tr>
<tr>
<td></td>
<td>Apo MPO binders</td>
<td>14.4 ± 4.8 (14) [1.2 ± 0.5 × 10⁶ (14)]</td>
<td>29.9 ± 10.2c [1.9 ± 1.6 × 10⁶ (8)]</td>
<td>52.5 ± 15.1 (13)c [1.7 ± 2.4 × 10⁶ (13)]</td>
</tr>
<tr>
<td>Peritoneal cavity</td>
<td>Total B</td>
<td>70.4 ± 14.5 (6) [3.3 ± 4.0 × 10⁶ (6)]</td>
<td>70.4 ± 5.1 (3) [9.7 ± 11.8 × 10⁶ (3)]</td>
<td>73.2 ± 12.0 (3) [0.4 ± 0.2 × 10⁶ (3)]</td>
</tr>
<tr>
<td></td>
<td>B-1</td>
<td>40.0 ± 18.6 (6) [1.5 ± 2.4 × 10⁶ (6)]</td>
<td>50.6 ± 7.8 (3) [4.8 ± 6.0 × 10⁶ (3)]</td>
<td>64.7 ± 13.2 (3) [0.2 ± 0.0 × 10⁶ (3)]</td>
</tr>
<tr>
<td></td>
<td>MPO binders</td>
<td>2.3 ± 0.9 (6) [0.1 ± 0.1 × 10⁶ (6)]</td>
<td>1.5 ± 0.1 (3) [0.1 ± 0.2 × 10⁶ (3)]</td>
<td>6.7 ± 6.4 (3) [0.0 ± 0.0 × 10⁶ (3)]</td>
</tr>
</tbody>
</table>

B cell populations were evaluated in the bone marrow, spleen, and peritoneal cavity of Tg and non-Tg control mice. Values are presented for all B cells and MPO-binding B cells in the spleen and bone marrow. In addition, values are given for apoptotic (Apo) MPO binders in the spleen. Values listed are means ± SD. Cell number is given as × 10⁶ for bone marrow and MPO binders as × 10⁶ for all B cells in the spleen and peritoneal cavity. The number of mice examined for each tissue is indicated by (n). Total B cells in the bone marrow are defined as B220⁺ cells; total B cells in the spleen and peritoneal cavity are defined as IgM⁺.

aStatistically different from control with alpha level ≤0.003.
bStatistically different from control with alpha level <0.0001.

determined 20 anti-MPO hybridomas from four LC Tg mice. Iso-
type analysis indicated antibodies produced were IgM/k. Speci-
city to MPO was demonstrated by lack of binding to
dNA and PR3 by ELISA. Generation of these hybridomas
confirms the presence of functional splenic anti-MPO B
cells in Vκ1C/Jκ5 Tg mice.

To verify expression of the Vκ1C/Jκ5 light-chain transgene
and determine whether the Tg light-chain conferred allelic ex-
clusion, we analyzed seven anti-MPO hybridomas by 5’ RACE
using primers in the Cκ region. Sequencing revealed that the
light-chain transgene alone was expressed in four of seven hy-
bridomas examined (Table 2). Two hybridomas expressed
Vκ12-41 in addition to the Vκ1C Tg, and one hybridoma ex-
pressed Vκ21-4 alone (Table 2), indicating that the transgene
had not conferred allelic exclusion in all cells. We also determined that four different heavy chains paired with the Vk1C/Jk5 light-chain transgene to create anti-MPO antibodies (Table 2) in addition to the V\_H\_5069 heavy chain expressed by the original anti-MPO hybridoma (Table 2). These results demonstrate that the Vk1C/Jk5 light chain can confer anti-MPO specificity when combined with a variety of heavy chains.

For determination of whether anti-MPO splenocytes were anergic, total splenocytes and B cells were cultured in the presence or absence of MPO, LPS, or anti-IgM. Anti-MPO antibodies were produced in splenocytes and B cells from LC Tg but not HC Tg stimulated with LPS (Supplemental Figure 1). Likewise, MPO-binding B cells from LC Tg but not HC Tg proliferated after LPS stimulation as measured by carboxyfluorescein diacetate, succinimidyl ester (Supplemental Figure 2). These data indicate that anti-MPO B cells in LC Tg mice are not anergic. In contrast, our data would be consistent with anergy of anti-MPO B cells from HC Tg mice.

### Bone Marrow and Splenic B Cells Are Decreased in Vk1C-Jk5 Tg Mice

To investigate why expression of the Vk1C/Jk5 light chain produced anti-MPO B cells but not circulating anti-MPO antibodies, we looked for alterations in B cell differentiation. In the bone marrow, the percentage of B220\(^+\) B cells was markedly reduced in both LC (11.8 ± 3.5%) and HC Tg mice (7.8 ± 8.7%) compared with control mice (50.2 ± 19.8%; \(P = 0.0002\); Table 1), corresponding to a decrease in the total number of B220\(^+\) B cells of 85 and 93%, respectively. To determine whether B cell development was altered in the bone marrow, pro-/pre-B cells, immature B cells, and recirculating mature B cells were distinguished by staining with B220 and IgM. The number of IgM\(^+\)/B220\(^hi\) recirculating B cells was reduced by 70% in LC mice and 95% in HC mice (Table 1). Similarly, the number of pro-/pre-B cells was decreased by 90% in HC mice and by 60% in LC mice. The number of IgM\(^+\)/B220\(^hi\) immature B cells was reduced by 66% in LC mice and 79% in HC mice (Table 1). The ratio of pro-/pre-B cells to immature B cells is not higher in LC or HC Tg (4.6:1 and 2.6:1, respectively) compared with control mice (6.4:1), suggesting that there is no block in development of pro-/pre-B cells to immature B cells in Tg mice.

### In the spleen, the percentage of total IgM\(^+\) and/or B220\(^+\) splenic B cells was decreased significantly in both LC (48.1 ± 9.9%) and HC (22.4 ± 17.9%) Tg mice compared with control mice (65.6 ± 7.1%; \(P = 0.0001\). Similarly, the absolute number of splenic B cells is reduced by 30% in LC mice and 60% in HC mice. In the peritoneal cavity, we found no significant differences in the absolute number or the percentage of IgM\(^+\) B cells in Tg as compared with control mice (Table 1).

### Tg Mice Exhibit a Decrease in Mature Follicular Cells

Tg mice demonstrated altered splenic B cell subpopulations when compared with non-Tg mice. Antibodies to CD23, CD21, and IgM were used to distinguish mature follicular (IgM\(^+\)CD21\(^+\)CD23\(^+\)) and marginal zone (IgM\(^+\)CD21\(^+\)CD23\(^-\)) B cells. Cells that are IgM\(^+\)CD21\(^+\)CD23\(^-\) could be either B-1 or transitional 1 (T1) B cells and are referred to as T1/B1 cells. Representative CD21/CD23 histograms are presented in Figure 2. LC mice had increased percentages of marginal zone and T1/B-1 cells and a decreased percentage of mature follicular cells (Figure 2C). In HC mice, the majority of B cells were T1/B-1 with few marginal zone or follicular cells (Figure 2D). Differences among the three groups are summarized in Figure 2E. Compared with controls (73.6 ± 7.7%), LC mice had a 70% (23.1 ± 9.0%; \(P < 0.0001\) and HC mice a 93% (5.2 ± 4.4%; \(P < 0.0001\)) decrease in the percentage of mature splenic follicular cells. LC mice had an increased percentage of marginal zone (39.5 ± 10.2%; \(P < 0.0001\); confirmed by staining with an antibody to the marginal zone-specific marker CD9) and T1/B-1 cells (31.5 ± 13.4%; \(P < 0.0001\)). HC mice had a preponderance of T1/B-1 cells (79.1 ± 16.7%) greater than that found in either LC or control mice (\(P < 0.0001\); however, the percentage of marginal zone cells was no different from controls. When absolute cell numbers were considered, these alterations in splenic follicular and marginal zone B cell subpopulations were confirmed (Table 1).

To distinguish B-1 cells from T1 cells, we analyzed splenic B cells with antibodies to CD5 and CD43. The majority of cells classified as T1/B-1 on the basis of CD21/CD23 staining were in fact B-1 cells. The percentages of CD5\(^+\)/CD43\(^-\) B-1 cells increased in LC mice (25.4 ± 11.2%; Figure 3, C and E) and in HC mice (50.6 ± 21.5%; Figure 3, D and E) compared with

### Table 2. Vk1C/Jk5 Light Chain Transgenic Hybridoma Sequences

<table>
<thead>
<tr>
<th>Fusion</th>
<th>Mouse</th>
<th>Clone</th>
<th>Light Chain</th>
<th>Heavy chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>479</td>
<td>F1</td>
<td>Vk1C/Vk12–41(^+)</td>
<td>VGk2/VGAM3.8</td>
</tr>
<tr>
<td>1</td>
<td>479</td>
<td>H4</td>
<td>Vk1C/Vk12–41(^+)</td>
<td>VH7183.32b</td>
</tr>
<tr>
<td>2</td>
<td>161</td>
<td>2-A4</td>
<td>Vk1C</td>
<td>VH7183.32b</td>
</tr>
<tr>
<td>2</td>
<td>161</td>
<td>6-C6</td>
<td>Vk21-4</td>
<td>VH36–60</td>
</tr>
<tr>
<td>2</td>
<td>161</td>
<td>12-B9</td>
<td>Vk1C</td>
<td>J558.47</td>
</tr>
<tr>
<td>3</td>
<td>162</td>
<td>14-C8</td>
<td>Vk1C</td>
<td>J558.45</td>
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<tr>
<td>4</td>
<td>1382</td>
<td>AE6 D4/C2</td>
<td>Vk1C</td>
<td>3609</td>
</tr>
</tbody>
</table>

\(^{a}\)Two bp differences with NZB × NZW anti-MPO IgM.

\(^{b}\)Original hybridoma used to generate Tg mice.
control mice (7.5 ± 4.3%; P < 0.0001; Figure 3, A, B, and E). Although we noted a trend toward increased numbers of B-1 cells in LC and HC mice, the absolute number of B-1 cells did not differ significantly. We observed no statistically significant differences in either the number or the percentage of B-1 cells in the peritoneal cavity of Tg mice compared with control mice (Table 1).

MPO-Binding Cells Are Marginal Zone and B-1 B Cells

We observed a significant percentage of MPO-binding B cells in spleens of Tg mice. Although MPO-binding splenic B cells are rare in non-Tg control mice, half of these MPO binders have a follicular cell phenotype (Table 1), whereas the percentage of MPO binders that progress to the follicular stage is reduced in both LC and HC Tg mice (18.1 ± 10.1 and 21.0 ± 16.9%, respectively; P < 0.0001). Most MPO-binding B cells are B-1 cells in both LC (51.8 ± 15.1%) and HC mice (66.1 ± 18.1%) compared with control mice (38.9 ± 12.5%; P ≤ 0.002; Figure 4). Both the percentage (36.2 ± 15.6%; P < 0.0001) and absolute number (48.0 ± 57.4 × 10^3; P ≤ 0.002) of MPO-binding cells that are marginal zone cells in LC mice are significantly elevated when compared with non-Tg and HC Tg mice. Control and HC mice have a similar percentage and number of MPO-binding marginal zone cells (Table 1).
Apoptotic MPO-Binding B Cells Are Increased in Tg Mice

We found a significant decrease in the number of recirculating B cells in the bone marrow and follicular B cells in the spleen of Tg mice. To investigate the loss of these mature B cells, we gated on the apoptotic cell population based on forward and side scatter and found that >95% of the MPO-binding B cells (shown in blue) in the bone marrow are apoptotic as confirmed by staining with FITC-conjugated VAD-FMK (Figure 5, A through F). Furthermore, we observed a striking increase in the percentage of apoptotic MPO-binding B cells (shown in blue) in the spleen (Figure 5, G through O) in Tg mice (LC = 29.9 ± 10.2%; HC = 52.5 ± 15.1%) as compared with controls (14.4 ± 4.8%; P < 0.0001; Table 1). Essentially 100% of the VAD-FMK-positive MPO-binding B cells also stained positive with FAM-LETD-FMK, which binds activated caspase 8 (data not shown), suggesting that apoptosis of MPO-binding B cells is triggered by death receptor activation of the extrinsic pathway of apoptosis. The ratio of T1 to follicular B cells in HC (16.8:1) and LC (0.32:1) Tg mice compared with non-Tg mice (0.06:1) indicates that deletion occurs at the T1 to follicular B cell transition.

DISCUSSION

The purpose of this work was to elucidate the regulation of the anti-MPO response in nonautoimmune mice and to assess the ability of the antibody light chain to confer specificity to MPO. To this end, we used a Vx1C-Jk5 anti-MPO light chain derived from an anti-MPO hybridoma generated from SCG/Kj mice10 to produce Tg mice. We identified two groups of Tg mice that exhibit altered B cell phenotypes compared with non-Tg controls. The phenotypic differences in LC and HC mice correlated with a doubling of the relative transgene copy number in the HC mice. It is possible that the observed differences in phenotype could result from a “transgene effect” that alters another gene as a result of the site of insertion; however, we believe that this is unlikely. The B cell phenotype of mice from three additional founder lines also correlated with relative transgene copy number (data not shown). We detected anti-MPO B cells in both groups of Tg mice; however, none expressed circulating anti-MPO antibody. These results indicate that anti-MPO B cells are regulated to prevent anti-MPO antibody production and maintain tolerance in this mouse model. Much work has demonstrated that tolerance to self-antigens may be achieved by anergy, clonal deletion, or receptor editing of autoreactive B cells.14–22

An important question is whether anti-MPO B cells are functional. Generation of anti-MPO hybridomas from LC Tg mice confirmed the presence of anti-MPO B cells and demonstrated that the Vx1C-Jk5 Tg can confer anti-MPO specificity when combined with a variety of rearranged heavy chains. Although several of the heavy chains from Vx1C-Jk5 Tg hybridomas expressed the J558 variable region gene, the number of hybridomas examined was too small to ascertain whether there is a restriction in heavy-chain variable region gene use by anti-MPO B cells. Of note, one of the non-Tg light chains, Vk12-41, expressed by hybridomas from our Vx1C-Jk5 Tg mice, is also expressed by another anti-MPO antibody produced by NZB × NZW mice,23 consistent with light-chain restriction. In addition, stimulation of splenocytes and purified B cells from LC Tg mice with LPS resulted in proliferation of anti-MPO B cells and production of anti-MPO–specific antibodies. These results indicate that anti-MPO B cells in LC Tg mice are not anergic; however, anti-MPO B cells in HC mice seem nonfunctional.
We examined the B cell repertoire to elucidate further the mechanisms of maintaining tolerance to MPO in this nonautoimmune mouse model. We observed >85% fewer B cells in the bone marrow of Tg mice, in part because of reduced clonal expansion at the pre-B cell stage as a result of expression of the light-chain transgene. Our data also support the deletion of anti-MPO B cells in the bone marrow. These results are similar to those found with anti-red blood cell (RBC) and anti-DNA Tg mice. Moreover, we observed a large population of apoptotic MPO-binding B cells in the spleens of Tg mice, suggesting that deletion of anti-MPO B cells continues in the periphery, most likely via the extrinsic pathway of apoptosis because caspase 8 is activated. Peripheral deletion of self-reactive B cells by apoptosis has been demonstrated in other Tg mouse

Figure 5. Apoptotic MPO-binding B cells are increased in Vx1C/Jx5 Tg mice. Representative histograms of cells from the bone marrow of a non-Tg (A through C) and an HC Tg (D through F) are presented. A white oval depicts the population based on forward versus side scatter that is considered apoptotic (A and D). Histograms in B and E confirm that the cells in the apoptotic gate based on forward versus side scatter are VAD-FMK+. Histograms in C and F demonstrate that 96 and 98% of the B220+/IgM+ MPO-binding B cells (shown in blue) in the apoptotic gate of non-Tg (C) and HC Tg (F) mice are VAD-FMK+. (G, J, and M) Representative forward and side scatter histograms for splenocytes from a B6 wild-type control (G), an LC Tg mouse (J), and an HC Tg mouse (M) are shown. The region referred to as the apoptotic cell gate is indicated by a white oval. The apoptotic status of cells in the apoptotic cell gate was confirmed by staining with VAD-FMK. IgM/VAD-FMK histograms for B220- cells from the apoptotic gate are shown for a B6 wild-type control (H and I), an LC mouse (K and L), and an HC mouse (N and O). A majority of B cells in the apoptotic cell gate are VAD-FMK+ as depicted in H, K, and N; percentages shown are VAD-FMK+ cells. MPO binders (shown in blue in I, L, and O) are apoptotic and are found in greater abundance in Tg mice; percentages given are VAD-FMK+ MPO binders. Data demonstrating that MPO-binding B cells in the spleen are increased in the apoptotic cell gate is summarized in P. Shown are the percentages of MPO-binding B cells in the black oval normal lymphocyte gate ( ●) and in the white oval apoptotic cell gate ( △) for each group of mice. Although there are more MPO binders in the apoptotic cell gate than in the normal lymphocyte gate in each group, the increased population of apoptotic cells that bind MPO is striking in LC mice (44.9%) and in HC mice (62.5%).
models, including MT-k Tg × 3-83 μδ Tg mice and anti-RBC Tg mice.

In Tg mice, we observed almost complete elimination of mature recirculating B cells in the bone marrow and follicular cells in the spleen. This decreased progression of B cells to the follicular stage was accompanied by diversion of B cells to either a marginal zone or B-1 phenotype. These changes affect MPO-binding cells as well as cells that seem to be non-MPO-binding cells. A likely explanation is that there are more MPO-binding B cells present than we detect by FLOW cytometry using biotinylated MPO. B cells classified as "non-MPO-binding B cells" may actually include MPO binders with an affinity or avidity for MPO that is too low to sustain strong binding of the biotinylated MPO molecule. LC mice are characterized by a four-fold increase in the number of marginal zone B cells. This finding is consistent with considerable evidence that autoreactive B cells are selected into the marginal zone of the spleen. Marginal zone B cells may arise from autoreactive cells through the process of receptor editing, which can result in the expression of two receptors and effectively dilute the autoreactive B cell receptor signal strength. Such receptor editing may occur in LC Tg mice as evidenced by the fact that two anti-MPO-binding hybridomas also expressed a different light chain in addition to the Tg Vk1C-Jk5. We hypothesize that receptor editing, with a resultant lack of allelic exclusion and production of marginal zone B cells, may be one of the mechanisms used by LC Tg mice to negatively regulate autoreactive cells.

In contrast to LC mice, HC mice did not exhibit an increase in marginal zone cells but rather a marked increase in B-1 cells in the spleen. This diversion of autoreactive B cells into the B-1 compartment has been observed in other Tg mouse models. B-1 cells express high levels of CD5, a molecule known to regulate negatively B cell receptor signaling and, therefore, reduce antibody production. B cell antigen receptor specificity and surface density have been shown to drive B-1 versus follicular cell development. Although we detected an increase in splenic B-1 cells, not peritoneal B-1 cells, our results are comparable to those of the tandem anti-RBC heavy- and light-chain Tg mouse, in which homozygosity of the transgene resulted in deletion of follicular cells from both bone marrow and spleen and an increase in peritoneal B-1 cells. Our results parallel this Tg model whereby the relative copy number drove the B cell phenotype. We see an increase in marginal zone cells in our Vk1C-Jk5 Tg mice that have fewer copies of the transgene and a diversion of B cells to the B-1 subtype in mice with the larger relative transgene copy number. Gene copy number correlates with expression levels of the corresponding protein, and B cell receptor density is known to affect B cell receptor signal strength. Our results are consistent with the B cell receptor signal strength model, which proposes that B cell receptor signaling determines peripheral B cell lineage commitment. Specifically, a weak B cell receptor signal drives production of marginal zone B cells, and a strong B cell receptor signal triggers B-1 B cell production.

In conclusion, our results are consistent with a model of autoreactive B cell regulation whereby anti-MPO B cells are deleted via apoptosis in the bone marrow and in the spleen before maturation to follicular B cells. Anti-MPO B cells that survive this negative selection are diverted to a B-1 phenotype or to a marginal zone phenotype depending on B cell receptor strength, apparently determined by the relative transgene copy number. Moreover, remaining anti-MPO B cells in HC Tg mice seem to be nonfunctional.

**CONCISE METHODS**

**Generation of Vk1C-Jk5 Light-Chain Tg Mice**

A rearranged Vk1C-Jk5 light chain from an anti-MPO–specific hybridoma derived from SCG/Kj mice was used to produce Tg mice. Genomic DNA from the 5F2 hybridoma was used to amplify the light chain by PCR using 5’ TATAGAATTCAGCCAGGTAGTGTCTTTGATGACCCAAAATC and 3’ CAGTGAATTCCTCAGAAGAAGCACGGCTACTG primers. The 642-bp PCR product was subcloned into a pCR/Neo expression vector containing the endogenous Vk21C promoter and leader sequences and Ck secretion exons as similarly described for the Vκ3/1/k4 light chain. Vector DNA was linearized with Sall, injected into single-cell embryos, and transferred to pseudopregnant females (C57BL6 or C3H). Offspring carrying the 5F2 Vk1C-Jk5 transgene were identified by PCR analysis using the Vk1C forward (5’-GGCTCAGGTGTGGTTTTGAT-3’) and Vk4long reverse (5’-AGGAGCTGAACTTGGACTAC-3’) primers.

Six 5F2 transgene-positive founder mice were mated to B6C3F1 mice and backcrossed to C57BL/6 (Jackson Laboratory, Bar Harbor, ME). Mice from five of six founder lines were examined and had similar phenotypes; therefore, we focused our analysis on one of the lines. HC Tg mice resulted from interbreeding of LC Tg mice from this one founder line. Mice were maintained in a pathogen-free colony at the University of North Carolina Animal Facility. All animal protocols were approved by the Institutional Animal Care and Use Committee.

**Taqman Quantitative PCR**

Relative copy number of genomic Vk1C-Jk5 Tg mice was determined using real-time quantitative PCR quantification (Taqman) on ABI Prism 7900 HT sequence detector (Applied Biosystems [ABI], Foster City, CA). Quantitative PCR primers for Vk1C-Jk5 (accession no. AF113232) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; accession no. BC014085) were designed using Primer Express (V1.5; ABI). Forward and reverse primer pairs were GGCGATGTACCAGAGCAGAT (bp 216 to 237) and ACCGAAACGCTGAGGAAA (bp 305 to 324) for Vk1C-Jk5 and GCACCGCTTCTTACTGCACTAC and GCTCTCAGAGATTTTGATGG for GAPDH. PCR reactions were performed using Taqman Universal PCR Master Mix (ABI) with SYBR Green (Molecular Probes, Eugene, OR) for fluorescence detection of amplified product. Primer linearity and proportionality were tested by PCR amplification of increasing dilutions of mouse genomic DNA. Relative quantification was performed by the ΔΔCt method whereby the difference or fold change (FC) between samples was de-
termed using the following equation: $\text{FC} = 2^{(-\Delta\Delta CT)}$. Relative transgene copy number was measured as a ratio to input genomic DNA as measured by GAPDH.

**ELISA**

Anti-MPO antibodies were detected in ELISA using native human MPO (Calbiochem, San Diego, CA), recombinant human MPO, or recombinant mouse MPO as described previously.44,45 Mouse and human recombinant MPO were expressed in HEK293 cells.46 Calf thymus DNA (Sigma, St. Louis, MO) and native human PR3 (Elastin Products Company, Owensville, MO) served as control antigens. IgM production was measured by ELISA using goat anti-mouse Ig (SouthernBiotech, Birmingham, AL) as the capture antibody as described previously.46 Plates were incubated with 100 µl of hybridoma supernatant, splenocyte or B cell culture supernatants, or mouse sera diluted 1:10 to 1:250. Mouse IgM (SouthernBiotech), anti-mouse MPO mAb 8F4 (Hyctul Biotechnology, Uden, Netherlands), and anti-human MPO mAb 2C7 (Abcam, Cambridge, MA) were used as positive controls as appropriate. Alkaline phosphatase–conjugated goat anti-mouse IgG and IgM (Pierce, Rockford, IL) or goat anti-mouse IgM (SouthernBiotech) were used as secondary antibodies. Reactivity was detected with an alkaline phosphatase substrate kit (Bio-Rad Laboratories, Hercules, CA). Samples giving an OD405 value at least twice that of the negative control were considered positive.

**Generation of B Cell Hybridomas**

B cell hybridomas were generated from Tg mice as described previously.12 Splenocytes from Tg mice were fused with P3X-Ag8.653 or NSOcδ-2 myeloma cells. After fusion, tissue culture supernatants were screened for MPO binding by ELISA. Hybridomas expressing anti-MPO antibodies were rendered monoclonal by limiting dilution. Antibody isotype analysis was performed using a mouse mAb isotyping kit (Amersham Biosciences, Little Chalfont Buckinghamshire, England).

**Assessment of Hybridoma Light- and Heavy-Chain V Region Genes**

Total RNA was isolated from hybridomas producing anti-MPO antibodies using RNA STAT-60 (Tel-Test, Friendswood, TX); mRNA was isolated using the Poly(A)/Tract Isolation System (Promega, Madison, WI). For confirmation that the transgene was transcribed, cDNA was generated with random hexamers and Superscript RT (Life Technologies, Gaithersburg, MD) after DNase (Life Technologies BRL) treatment of the poly (A) tail RNA template. Transgene-specific primers described previously were used to amplify the transgene. The diversity in light-chain and heavy-chain variable region gene usage was determined using the 5′ Rapid Amplification of cDNA Ends system (5′ RACE; Life Technologies) after DNase (Life Technologies BRL) treatment of the poly (A) tail RNA template. Transgene-specific primers described previously were used to amplify the transgene. The diversity in light-chain and heavy-chain variable region gene usage was determined using the 5′ Rapid Amplification of cDNA Ends system (5′ RACE; Life Technologies) as described previously.12 The GSP1 primers and nested GSP2 primers used for the kappa light chain and the Mu heavy chain were as follows: κGSP1 5′-TAACGTGCTACTGGATGGTGGGAG-A3′, µGSP1 5′-CAGATTCTTATACAGACAG-3′, κGSP2 5′-ATGGATACAGTTTGGTGCACATCAG-3′, and µGSP2 5′-GCTTCTGCAGAGGAGACAG-3′. PCR products were sequenced on an automated dye-chain termination DNA sequencer with nested constant region primers (GSP2) from the second round of PCR. Sequences were analyzed with Chromas (Technelysium, Helensvale, Australia) and Sci Ed Central (Scientific and Educational Software, Durham, NC). The Basic Local Alignment Search Tool (BLAST) was used to search the GenBank database for homology with murine V regions. IgBLAST was used to compare the sequences with the germline Ig database.

**Flow Cytometry**

Cells were isolated from the spleen, bone marrow, and peritoneum into HBSS containing 2% FBS as described previously.46 Cells were stained with antibodies specific for IgM, CD21, CD23, CD43, CD5, B220, CD9, and CD11c that were conjugated to FITC, phycoerythrin, or allophycocyanin (BD Biosciences Pharmingen, San Diego, CA). B cells were gated on IgM and/or B220. Biotinylated MPO followed by streptavidin-peridinin chlorophyll protein (BD Biosciences Pharmingen) was used to identify MPO-binding cells. Biotinylated PR3 served as an antigen control. MPO and PR3 (Calbiochem, San Diego, CA, or Elastin Products Company, Owensville, MO) were biotinylated using EZ-Link NHS-Biotin (Pierce). CaspACE FITC-VAD-FMK (Promega) and a Caspase-8 in situ assay kit using FAM-LETD-FMK (Chemicon International, Temecula, CA) were used to detect apoptotic cells. Four-color analysis of cells was performed on a FACSCalibur (Becton Dickinson, Mountain View, CA). Data were analyzed with Summit software (DakoCytomation, Fort Collins, CO).

**In Vitro Stimulation**

Splenocytes were harvested as described previously for flow cytometry. Enriched populations of B cells were isolated by negative selection using a B cell enrichment kit (StemCell Technologies, Vancouver, BC, Canada) and were >90% pure. Total splenocytes or B cells were cultured in duplicate for 3 to 5 d in DMEM-H, 10% FBS without stimulation or stimulated with native human MPO (10 to 20 µg/ml; Calbiochem), LPS (10 µg/ml; Invivogen), or anti-IgM F(ab’)2 (10 µg/ml; JacksonImmunoResearch Laboratories, West Grove, PA). Supernatants were analyzed by ELISA. Proliferation of cells was measured using a CellTrace carboxyfluorescein diacetate, succinimidyl ester cell proliferation kit (Molecular Probes). Intracellular IgM was measured by blocking surface IgM with unlabeled goat anti-mouse IgM (SouthernBiotech) before permeabilization with Perm/Wash buffer (BD Biosciences Pharmingen) and subsequent staining with allophycocyanin-labeled antibody to IgM as described previously.47

**Statistical Analysis**

Baseline characteristics were summarized with means and SD. ANOVA tests were performed to evaluate differences in B cell variables among the three groups of mice (control, LC, and HC). Using a Duncan multiple range test to control for multiple comparisons, both the LC and HC were evaluated for statistical differences from the control group. To reduce the probability of finding differences between groups across different components of the same experiment because of multiple comparisons, a Bonferroni correction was used for adjusting the α level to maintain an overall α level of 0.05. Because 17 different components of the experiments were examined, an ad-
justed α level (0.05/17 = 0.003) was used. Because of small sample sizes and skewed distribution, ranked variables were used in the analysis. All statistical programming and analyses were conducted using SAS 8.2 (SAS Institute, Cary, NC).

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DISCLOSURES

None.

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