The T Cell-Dependent B Cell Immune Response and Germinal Center Reaction Are Intact in A-myb-Deficient Mice


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Expression of the protooncogene A-myb is restricted to the developing CNS, adult testes, breasts in late pregnancy, and germinal centers of secondary B cell follicles. The functional relevance of A-myb expression at three of these sites has been demonstrated previously via the generation and analysis of A-myb-deficient mice, which display behavioral abnormalities, male sterility, and perturbed breast development during pregnancy. In contrast, here we show that the germinal center response driven by T cell-dependent Ag immunization and the associated processes of Ab V gene somatic hypermutation, affinity maturation, and heavy chain class switching are overtly normal in A-myb-deficient mice. Nonetheless, these mice display mild splenic white pulp hypoplasia and blunted primary serum Ab responses, suggesting that although A-myb is not directly involved in the regulation of the memory B cell response, it may play a role in enhancing peripheral B cell survival or proliferative capacity.

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Materials and Methods

Mice

The line of A-myb-deficient mice used in these studies has been described and was maintained by brother-sister mating of A-myb+/− mice that had been generated on a mixed C57BL6/129S/J background, and offspring were genotyped as described earlier (7). Age-matched mice (8–12 wk old) were used in all experiments.

RT-PCR analysis

Total RNA was prepared from splenocytes. After reverse transcription, cDNAs were amplified by using a c-myb-specific primer combination, run on agarose gels, blotted to nylon membranes, and hybridized with a 32P-labeled internal c-myb-specific DNA probe, all as described previously (7). The hybridized membranes were analyzed on a Fuji (Tokyo, Japan) phosphorimager to quantitate levels of cDNA.

Immunizations and serology

Mice were immunized with 100 μg of alum-precipitated (4-hydroxy-3-nitrophenoxy)acetyl chicken γ globulin (NP-CGG) i.p. for primary responses and boosted with the same amount of Ag in PBS i.p. Mice were bled via the retro-orbital sinus, and the levels of anti-nucleoprotein (NP) Abs of various isotypes were enumerated by ELISA as described previously (12). In most of the assays, monoclonal anti-NP Abs of different H chain isotypes but similar affinities for NP were used as standards, allowing the results to be presented as microgram per milliliter equivalents of these mAbs. In the IgM assay, relative levels of Ag-binding Abs in different sera were determined by using serial dilutions. The points at which the resulting OD curves were 50% maximal were then used to calculate the relative dilution factor, giving an equivalent OD for each serum sample. The data

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illustrated for different isotypes were obtained by using sera pooled from at least three different mice of each genotype. Isotype levels were determined from supernatants of in vitro-stimulated B cells by ELISA, and relative affinities of serum Abs were evaluated by using altered ligand density ELISA as described earlier (13).

**Immunohistochemistry, GC microdissection, V gene PCR amplification, and sequencing**

Processing of spleens for immunohistochemistry, sectioning, and staining for NP, \( \lambda^+ \) B cells and peanut agglutinin (PNA) \( ^+ \) GC has been described in detail previously (14). Fifty to 100 cells from Ag-specific GC were microdissected from sections, and their genomic DNA was isolated as reported earlier (15). Two rounds of PCR amplification were conducted with primers specific for the \( V_{\gamma} \) gene, and the conditions of amplification were described by Jacobs et al. (16). The amplified products were cloned into the pBluescript vector (Stratagene, La Jolla, CA), and inserts were sequenced as described (15).

**Mitogen stimulation of small resting B cells in vitro**

Small resting B cells were isolated from spleens via T cell depletion, followed by purification of high-density cells on Percoll gradients. The isolated B cells were then stimulated with various concentrations of LPS, goat anti-IgM, and goat anti-IgD, and, in some experiments, with anti-IgD. Bone marrow cells were stained with anti-IgM, anti-CD45R, and anti-IgD, and, in some experiments, with anti-CD24. Stained cells were analyzed with a Coulter Epics Elite (Coulter Pharmaceutical, Palo Alto, CA) with live lymphocyte forward and side scatter gates. The proportions of immature and mature splenic B cells were derived by determining the proportion of CD24\(^+\) and CD24\(^-\) (and in some experiments IgD high) cells, respectively, among all CD45R\(^+\) splenocytes. The proportions of cells in different stages of development in the bone marrow were determined in one experiment as follows: mature B cells were CD45R\(^+\), IgM\(^-\), and IgD\(^+\); immature B cells were CD45R\(^-\), IgM\(^-\), and IgD\(^+\); and pro- and pre-B cells were CD45R\(^-\), IgM\(^-\), and IgD\(^+\). In a second experiment, mature B cells were defined as CD45R\(^-\) and IgD\(^+\); immature and pre-B cells as CD45R\(^-\), CD24\(^+\) and IgD\(^-\); and pro-B cells as CD45R\(^-\), IgD\(^-\), and CD24\(^-\).

**Results and Discussion**

**Mild hypoplasia of the splenic white pulp in naive A-myb\(^{-/-}\) mice**

Histological studies revealed that naive A-myb\(^{-/-}\) mice have mild splenic hypoplasia (Fig. 1). This hypoplasia appeared to be confined to the B cell follicular and marginal zone regions and resulted in smaller average spleen sizes and expanded red pulp areas. The hypoplasia seemed to involve all areas of the white pulp. The in situ hybridization figures at 50 ng/ml. Cell proliferation was assayed by pulsing after 48 h with [\( ^{3}H \)]thymidine, harvesting onto glass-fiber filters, and scintillation counting. All of these procedures were performed as described before (12).

Flow cytometry

Lymphocyte suspensions were prepared as described previously (17). Marrow cells were obtained from the two hind limbs of each of two donor animals. The mice were also severalfold lower than observed in A-myb\(^{-/-}\) mice and A-myb\(^{-/-}\) littermates were processed for histology and sections stained with anti-IgM (blue) to elaborate B cell areas and PNA (red) to elaborate GC. Original magnification of images was \( \times 10 \).

**FIGURE 1.** Mild splenic white pulp hypoplasia in A-myb\(^{-/-}\) mice. Spleens from naive A-myb\(^{+/+}\) (A) and A-myb\(^{-/-}\) (B) littermates were microdissected for histology and sections stained with anti-IgM (blue) to elaborate B cell areas and PNA (red) to elaborate GC. Original magnification of images was \( \times 10 \).

does not appear to result from defects in primary B cell differentiation or selective loss of B cells at a particular stage of development. Collectively, these data suggest that A-myb is involved in regulation of peripheral B cell survival or homing to follicular areas. This result was somewhat unexpected given that previous studies have suggested that high levels of A-myb expression are only observed in GCs. Nonetheless, it is consistent with the studies of Calabretta and colleagues (11) showing that overexpression of A-myb leads to follicular hyperplasia.

**Serum Ab response to a T-dependent Ag in A-myb\(^{-/-}\) mice**

Cohorts of A-myb\(^{-/-}\) and A-myb\(^{+/+}\) mice were immunized with the T cell-dependent Ag (4-hydroxy-3-nitrophophyl)acetyl chicken \( \gamma \) globulin (NP-CGG) in alum and were bled at various times thereafter. At day 7, the levels of \( \lambda \)-bearing anti-NP Abs (characteristic of the anti-NP response) did not significantly differ in the two groups of animals, but subsequently, A-myb\(^{-/-}\) mice displayed lower primary serum Ab levels (Fig. 2). After boosting the serum anti-NP levels were comparable between the two groups of

<table>
<thead>
<tr>
<th>A-myb Genotype</th>
<th>B220(^+) Spleen Cells (%)</th>
<th>Mature B Cells (%)</th>
<th>Immature B Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(+/+-) (^a)</td>
<td>31 (29)</td>
<td>68 (66)</td>
<td>32 (34)</td>
</tr>
<tr>
<td>(+/-) (^b)</td>
<td>31 (1.3)</td>
<td>63 (6.5)</td>
<td>37 (6.5)</td>
</tr>
<tr>
<td>(-/-) (^b)</td>
<td>16 (3.3)</td>
<td>65 (11)</td>
<td>35 (11)</td>
</tr>
</tbody>
</table>

\(^a\) Values obtained from two animals are separated by commas.

\(^b\) SD of mean values obtained from four animals are shown in parentheses.
animals. Analysis of serum Ab isotypes did not reveal any major differences in the isotypes being produced in primary and secondary responses (Fig. 3). In addition, the early primary IgM response appeared to be comparable in both groups of animals. However, primary serum levels of IgG3 and IgG1 (the major isotypes in this response), as well as IgG2a, appeared uniformly lower in A-myb<sup>2</sup>/<sup>2</sup> as compared with A-myb<sup>1</sup>/<sup>2</sup> mice.

The mild follicular hypoplasia characteristic of A-myb mice may account for their blunted primary serum Ab responses. However, the anamnestic serum Ab response appears overtly normal in A-myb<sup>2</sup>/<sup>2</sup> mice, indicating that the memory B cell differentiation pathway is not perturbed by lack of A-myb.

The GC reaction is qualitatively intact in A-myb<sup>2</sup>/<sup>2</sup> mice

To characterize the GC reaction in A-myb<sup>2</sup>/<sup>2</sup> mice we used an immunohistochemical/microdissection/PCR approach. The frequency of total GCs and GCs containing λ-expressing B cells at days 9 and 12 after NP-CGG in alum immunization were evaluated in the spleens of four A-myb<sup>2</sup>/<sup>2</sup> and four A-myb<sup>1</sup>/<sup>2</sup> mice (two at each time point). A spleen section was randomly chosen from each mouse, and the number of GCs in each of three randomly chosen 10<sup>3</sup> fields that stained with either PNA-HRP or PNA-HRP and

\[ \text{Anti-NP Lambda Response} \]

FIGURE 2. Primary and secondary λ chain-bearing anti-NP serum Ab responses in A-myb<sup>2</sup>/<sup>2</sup> mice. Cohorts of three age-matched A-myb<sup>2</sup>/<sup>2</sup> and A-myb<sup>1</sup>/<sup>2</sup> mice were immunized with NP-CGG and bled at various times thereafter and their sera analyzed for anti-NP λ Ab titers by ELISA as described in Materials and Methods. The values shown are represented a microgram per milliliter equivalents of the monoclonal anti-NP Ab BBE6-12H3.

\[ \text{H chain isotype levels in the primary anti-NP response of A-myb<sup>2</sup>/<sup>2</sup> mice} \]

FIGURE 3. H chain isotype levels in the primary anti-NP response of A-myb<sup>2</sup>/<sup>2</sup> mice. Cohorts of at least three aged-matched A-myb<sup>2</sup>/<sup>2</sup> and A-myb<sup>1</sup>/<sup>2</sup> mice were immunized with NP-CGG, bled at the days indicated during the primary response, sera pooled, and levels of the various anti-NP H chain isotypes indicated measured by ELISA as described in Materials and Methods. Note that the scales on the y-axes differ for the different isotypes.
anti-λ-biotin (bio) were counted. This analysis revealed 5.7 ± 0.7 and 5.3 ± 0.5 PNA⁺ GCs per field in A-myb⁺/⁻ and A-myb⁻/⁻ spleen sections, respectively; and 4.5 ± 0.7 and 4.1 ± 1.0 PNA⁺, λ⁺ GCs per field in A-myb⁻/⁻ and A-myb⁺/⁻ spleen sections, respectively. Thus, neither the number of total or λ⁺ GCs were significantly different in A-myb⁻/⁻ and A-myb⁺/⁻ mice (total GC numbers were similar in both types of mice at the day 9 and 12 time points, but day 9 GCs were uniformly smaller). Although the numbers were similar in both types of mice at the day 9 and 12 time points, these minor differences were variable from mouse to mouse (data not shown).

Adjacent spleen sections obtained from A-myb⁺/⁺ and A-myb⁻/⁻ animals at day 12 after immunization with NP-CGG in alum were stained with NP-CGG-bio/PNA-HRP and anti-λ-bio/PNA-HRP to identify NP⁺, λ⁺ GCs. Individual GCs of this type were microdissected from the sections and genomic DNA PCR-amplified with V1 gene-specific primers, and the PCR products were cloned and sequenced. In five NP⁺, λ⁺ GCs from three A-myb⁻/⁻ mice, an average V1 mutation frequency of 0.54% was observed (Table III). Analysis of seven NP⁺, λ⁺ GC from two A-myb⁻/⁻ mice yielded a mutation frequency of 0.52%. The mutation frequencies observed in these two samples were not significantly different as evaluated by a Student’s t test (90% confidence level). In addition, we did not detect any obvious differences in the locations or chemical nature of mutations in V1 genes recovered from A-myb⁺/⁺ and A-myb⁻/⁻ GCs.

To evaluate whether the selection of high-affinity Ab variants generated by V gene hypermutation resulting in serum Ab affinity maturation was altered in A-myb⁻/⁻ mice, we assayed the relative affinities of serum Abs produced during the anti-NP response by altered ligand-density ELISA. Affinity-matured serum anti-NP Abs were detected at later time points during primary (data not shown) and early secondary (Table IV) immune responses in both A-myb⁻/⁻ and A-myb⁺/⁺ mice. Thus, two processes fundamental to the development of B cell memory, V gene somatic hypermutation and Ag affinity-based positive selection, seem normal in A-myb⁻/⁻ mice. These data are concordant with those discussed above in indicating that the GC reaction and memory B cell pathways can operate efficiently in the absence of A-myb.

**In vitro proliferative and isotype switching responses of A-myb⁻/⁻ B cells**

The mild B cell hypoplasia and lower primary serum IgG Ab levels observed in A-myb⁻/⁻ mice prompted us to evaluate the proliferative and H chain class switching potential of A-myb⁻/⁻ B cells. Pooled anti-NP mAb or sera NA 1.2

<table>
<thead>
<tr>
<th>Mice and Genotypes</th>
<th>GC Number</th>
<th>Number of Clones</th>
<th>Bases Sequenced</th>
<th>Number of Mutations</th>
<th>Average Mutation Frequency</th>
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</thead>
<tbody>
<tr>
<td>1⁺/⁺</td>
<td>1</td>
<td>6</td>
<td>1,800</td>
<td>25</td>
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</tr>
<tr>
<td></td>
<td>2</td>
<td>6</td>
<td>1,800</td>
<td>6</td>
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<tr>
<td>2⁻/⁻</td>
<td>3</td>
<td>5</td>
<td>1,500</td>
<td>6</td>
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</tr>
<tr>
<td></td>
<td>4</td>
<td>6</td>
<td>1,800</td>
<td>3</td>
<td></td>
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<tr>
<td>3⁻/⁻</td>
<td>5</td>
<td>6</td>
<td>1,800</td>
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<tr>
<td>Total</td>
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<td>8,700</td>
<td>47</td>
<td>0.54%</td>
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<table>
<thead>
<tr>
<th>Mice and Genotypes</th>
<th>GC Number</th>
<th>Number of Clones</th>
<th>Bases Sequenced</th>
<th>Number of Mutations</th>
<th>Average Mutation Frequency</th>
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<td>4</td>
<td>1,960</td>
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<td>4</td>
<td>1,120</td>
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<tr>
<td>2⁺/⁺</td>
<td>3</td>
<td>6</td>
<td>1,800</td>
<td>23</td>
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<tr>
<td></td>
<td>7</td>
<td>5</td>
<td>1,500</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>11,180</td>
<td>58</td>
<td>0.52%</td>
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</tbody>
</table>

* The mutation frequencies were determined to not be significantly different using a Students t test.

**Table IV.** Unaltered serum Ab affinity maturation in A-myb⁻/⁻ mice

<table>
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<tr>
<th>Anti-NP mAb or Sera</th>
<th>Intrinsic Affinity (K_a(M^{-1}))</th>
<th>Ratio NP12:NP3 Binding</th>
</tr>
</thead>
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<tr>
<td>BBE6-12H3</td>
<td>(9.6 \times 10^4)</td>
<td>42.8</td>
</tr>
<tr>
<td>Unmutated primary mAb</td>
<td>2.9 \times 10^6</td>
<td>3.5</td>
</tr>
<tr>
<td>Bg 110-2</td>
<td></td>
<td></td>
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<tr>
<td>Hypermutated secondary mAb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pooled +/⁻ secondary sera</td>
<td>NA</td>
<td>1.2</td>
</tr>
<tr>
<td>Pooled −/⁻ secondary sera</td>
<td>NA</td>
<td>1.9</td>
</tr>
</tbody>
</table>

**FIGURE 4.** In vitro proliferative responses of A-myb⁻/⁻ B cells. Purified small resting B cells were prepared and stimulated in vitro with the indicated concentrations of anti-IgM, LPS, and mitogenic anti-CD40 mAb, and proliferation was assessed by [³H]thymidine incorporation after 48 h. Assays were performed in triplicate and error bars are shown.
cells in vitro. Small resting B cells were purified from A-myb+/−, A-myb++/+, and A-myb−/− mice and stimulated with LPS, anti-CD40, or anti-IgM in vitro. B cells from A-myb−/− mice proliferated as well (data not shown) or better (Fig. 4) than A-myb+−/− and A-myb+−/+ B cells when stimulated with high concentrations of LPS, but no differences were observed at low LPS concentrations and in response to any concentration of anti-CD40 and anti-IgM (Fig. 4). Therefore, A-myb is not required to achieve normal rates of B cell proliferation in vitro.

To induce H chain class switching in vitro, A-myb+−/+ and A-myb−/− B cells were stimulated with high concentrations of LPS or LPS and IL-4. After 5 days, supernatants were assayed for levels of IgG3 and IgG1 Abs. As shown in Materials and Methods, purified small resting B cells were prepared and stimulated in vitro with LPS or LPS and IL-4 for 5 days and supernatants then collected and assayed for levels of IgG3 and IgG1 Abs.

A-myb is dispensable for the GC reaction and maturation of the B cell response

A-myb expression and functional importance are strongly correlated in tests and in the breast in pregnancy (7). In addition, A-myb−/− mice display behavioral abnormalities, suggesting that A-myb expression in the CNS is functionally relevant (K.A.V. and E.P.R., unpublished observations). In contrast, whereas expression of A-myb in the immune system is highly restricted to GC B cells, our data show that its absence does not overtly perturb the GC reaction and related processes necessary for memory B cell development. It will be important to determine whether this is due to redundancy of function of other myb family members and A-myb during the GC reaction. Preliminary analyses of c-myb mRNA levels in the splenocytes of A-myb−/−, A-myb+−/−, and A-myb+−/+ mice revealed no significant differences (data not shown), but detailed studies of the expression of c-myb and other myb family members in GC B cells at various stages of the immune response will be required to appropriately address this issue. Moreover, further studies will be required to determine whether GC formation or pathways are altered by A-myb deficiency under conditions of less robust antigenic stimulation and whether the memory B cells produced in A-myb−/− mice are phenotypically and functionally identical with those that develop in normal mice. Nonetheless, previous hypotheses that ascribe a crucial role for A-myb in the GC reaction need to be revised given the data presented here.

Acknowledgments

We thank David Dicker (Kimmel Cancer Institute flow cytometry facility) and Kate Dugan for technical assistance, and all members of the Manser laboratory for their indirect contributions to this work. This manuscript is dedicated to the memory of Dr. Antonio Toscani, who constructed and initially characterized the A-myb null line of mice in the laboratory of E.P.R.

References