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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 hyperplasia 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. The Journal of Immunology, 2001, 166: 3226–3230.

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 C57BL6/129SJ 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) phosphoimager to quantitate levels of cDNA.

Immunizations and serology

Mice were immunized with 100 μg of alum-precipitated (4-hydroxy-3-nitrophenyl)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 isoforms 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...
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, \( \alpha \) \( \lambda \) B cells and peanut agglutinin (PNA) \( \gamma \) 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\( \alpha \) 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 F(ab\(^9\)) \(_2\), or anti-CD40 mAb (FGK45). In some experiments, recombinant IL-4 (PeproTech, Princeton, NJ) was included in culture. The frequency of "spontaneous" GCs in naive spleens of A-myb \(^{-/}\) mice was also severalfold lower than observed in A-myb \(^{+/}\) mice (Table I), in agreement with the immunohistochemical results. Five A-myb \(^{+/}\) and A-myb \(^{-/}\) animals had an average splenic B cell percentage of \( \sim \)2-fold lower than in A-myb \(^{+/}\) and A-myb \(^{+/}\) littermates (Table I), in agreement with the immunohistochemical results. Five A-myb \(^{+/}\) and A-myb \(^{-/}\) animals had an average splenic B cell percentage of \( \sim \)16. Despite the reduced numbers of splenic B cells, the proportions of splenic immature and mature B cells were comparable in all groups of animals. Analysis of bone marrow cells in two separate experiments showed no reproducible abnormalities in the numbers of pro-B, pre-B, immature, and mature B cell subpopulations (Table II). Therefore, the splenic white pulp hypoplasia 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.

**Results and Discussion**

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

Histological studies revealed that naive A-myb \(^{-/}\) mice have mild splenoplasia (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 frequency of “spontaneous” GCs in naive spleens of A-myb \(^{-/}\) mice were also severalfold lower than observed in A-myb \(^{+/}\) animals.

Flow cytometric analysis revealed that splenic B cell percentages in A-myb \(^{-/}\) mice were \( \sim \)2-fold lower than in A-myb \(^{+/}\) and A-myb \(^{+/}\) littermates (Table I), in agreement with the immunohistochemical results. Five A-myb \(^{+/}\) and A-myb \(^{-/}\) animals had an average splenic B cell percentage of \( \sim \)30, whereas three A-myb \(^{-/}\) animals had an average splenic B cell percentage of \( \sim \)16. Despite the reduced numbers of splenic B cells, the proportions of splenic immature and mature B cells were comparable in all groups of animals. Analysis of bone marrow cells in two separate experiments showed no reproducible abnormalities in the numbers of pro-B, pre-B, immature, and mature B cell subpopulations (Table II). Therefore, the splenic white pulp hypoplasia

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

<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 $A_{\text{myb}}^{2/2}$ as compared with $A_{\text{myb}}^{1/2}$ mice. The mild follicular hypoplasia characteristic of $A_{\text{myb}}$ mice may account for their blunted primary serum Ab responses. However, the anamnestic serum Ab response appears overtly normal in $A_{\text{myb}}^{2/2}$ mice, indicating that the memory B cell differentiation pathway is not perturbed by lack of $A_{\text{myb}}$.

The GC reaction is qualitatively intact in $A_{\text{myb}}^{2/2}$ mice

To characterize the GC reaction in $A_{\text{myb}}^{2/2}$ mice we used an immunohistochemical/microdissection/PCR approach. The frequency of total GCs and GCs containing $\lambda$-expressing B cells at days 9 and 12 after NP-CGG in alum immunization were evaluated in the spleens of four $A_{\text{myb}}^{1/2}$ and four $A_{\text{myb}}^{2/2}$ 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 $10^3$ fields that stained with either PNA-HRP or PNA-HRP and PNA-HRP/anti-$\lambda$ was counted. The values shown are the mean number of GCs per field in each group.

**FIGURE 2.** Primary and secondary $\lambda$ chain-bearing anti-NP serum Ab responses in $A_{\text{myb}}^{-/-}$ mice. Cohorts of three age-matched $A_{\text{myb}}^{+/-}$ and $A_{\text{myb}}^{-/-}$ mice were immunized with NP-CGG and bled at various times thereafter and their sera analyzed for anti-NP Ab titers by ELISA as described in Materials and Methods. The values shown are the microgram per milliliter equivalents of the monoclonal anti-NP Ab BBE6-12H3.

**FIGURE 3.** H chain isotype levels in the primary anti-NP response of $A_{\text{myb}}^{-/-}$ mice. Cohorts of at least three aged-matched $A_{\text{myb}}^{+/-}$ and $A_{\text{myb}}^{-/-}$ 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.

**Table II.** Normal B cell development in the bone marrow of $A_{\text{myb}}^{-/-}$ mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Mature B Cells (%)</th>
<th>Immature and Pre-B Cells (%)</th>
<th>Pro-B Cells (%)</th>
<th>Other Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt. 1 (B220, sIgD, CD24)</td>
<td>6.6</td>
<td>26, 22</td>
<td>2, 1</td>
<td>66, 71</td>
</tr>
<tr>
<td>+/+</td>
<td>4.6</td>
<td>28, 28</td>
<td>2, 2</td>
<td>66, 64</td>
</tr>
<tr>
<td>−/−</td>
<td>5.10</td>
<td>28, 12</td>
<td>1, 2</td>
<td>66, 76</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Mature B Cells (%)</th>
<th>Immature B Cells (%)</th>
<th>Pre- and Pro-B Cells (%)</th>
<th>Other Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt. 2 (B220, sIgM, CD24)</td>
<td>9, 15</td>
<td>14, 18</td>
<td>40, 14</td>
<td>37, 53</td>
</tr>
<tr>
<td>+/−</td>
<td>8, 24</td>
<td>15, 18</td>
<td>38, 12</td>
<td>39, 46</td>
</tr>
</tbody>
</table>

* Values obtained from two animals of each genotype are separated by commas in each category.
anti-\(\lambda\)-biotin (bio) were counted. This analysis revealed 5.7 \pm 0.7
and 5.3 \pm 0.5 \text{PNA}^+ \text{GCs} per field in A-myb\(^{+/+}\) and A-myb\(^{-/-}\)
spleen sections, respectively; and 4.5 \pm 0.7 and 4.1 \pm 1.0 \text{PNA}^+,
\(\lambda^+\) \text{GCs} per field in A-myb\(^{+/+}\) and A-myb\(^{-/-}\) spleen sections,
respectively. Thus, neither the number of total or \(\lambda^+\) \text{GCs} were
significantly different in A-myb\(^{-/-}\) and A-myb\(^{+/+}\) mice (total \text{GC}
numbers were similar in both types of mice at the day 9 and 12
time points, but day 9 \text{GCs} were uniformly smaller). Although the
\text{GCs} observed in the A-myb\(^{-/-}\) mice appeared slightly smaller on
average than in A-myb\(^{+/+}\) littermates at both 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-\(\mu\)-HRP
and V gene hypermutation resulting in serum Ab affinity
GCs observed in the A-myb\(^{-/-}\) mice. These data are concordant with those discussed
above in indicating that the GC reaction and memory B cell pathway
can operate efficiently in the absence of A-myb.

Table III.  
Mutation analysis of V\text{A1} gene clones obtained from microdissected NP-specific GCs

<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>
</tr>
</thead>
<tbody>
<tr>
<td>1(^{+/+})</td>
<td>1</td>
<td>6</td>
<td>1,800</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6</td>
<td>1,800</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>2(^{-/-})</td>
<td>3</td>
<td>5</td>
<td>1,500</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>6</td>
<td>1,800</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>3(^{+/+})</td>
<td>5</td>
<td>6</td>
<td>1,800</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>8,700</td>
<td>47</td>
<td>0.54%</td>
</tr>
<tr>
<td>1(^{-/-})</td>
<td>1</td>
<td>4</td>
<td>1,960</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4</td>
<td>1,120</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>2(^{-/-})</td>
<td>3</td>
<td>6</td>
<td>1,800</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>6</td>
<td>1,800</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5</td>
<td>1,500</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>5</td>
<td>1,500</td>
<td>8</td>
<td></td>
</tr>
<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>
</tr>
</tbody>
</table>

*The mutation frequencies were determined to not be significantly different using a Student's \(t\) test.

In vitro proliferative 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

Table IV.  Unaltered serum Ab affinity maturation in A-myb\(^{-/-}\) mice

<table>
<thead>
<tr>
<th>Anti-NP mAb or Sera</th>
<th>Intrinsic Affinity (K_a) (M(^{-1}))</th>
<th>Ratio NP12:NP3 Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>BB6-12H3</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>Hypermutated secondary mAb</td>
<td>NA</td>
<td>1.9</td>
</tr>
<tr>
<td>Pooled +/− secondary sera</td>
<td>NA</td>
<td>1.2</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-\(\mu\)-G, LPS, and mitogenic anti-CD40 mAb, and proliferation was assessed by \(^{3}H\)-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 prolif-
erated as well (data not shown) or better (Fig. 4) than A-myb
+/− and A-myb+/+ B cells when stimulated with high concentra-
tions of LPS, but no differences were observed at low LPS concentra-
tions 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. Fig. 5 shows that A-myb
−/− B cells produced ~2-fold higher levels of IgG3 in response to LPS, per-
haps as a consequence of their sometimes slightly enhanced pro-
liferative response under these conditions. Stimulation of both
A-myb−/+ and A-myb−/− B cells with LPS and IL-4 resulted in
suppression of production of IgG3 and dramatic enhancement of
production of IgG1. These data indicate that regulation of class
switching is not altered by the A-myb deficiency.

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

A-myb expression and functional importance are strongly corre-
lated in testes 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 develop-
ment. 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 de-
tailed 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, fur-
ther 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 pro-
duced in A-myb−/− mice are phenotypically and functionally
identical with those that develop in normal mice. Nonetheless, pre-
vious 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.

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complex deposition in mice lacking the Fc receptor γ-chain does not alter mat-
lymphoid sheath-associated B cell focus response is not observed during the
behavior of a memory B cell clone during primary and secondary immune re-
of immunoglobulin genes in memory B cells of DNA-repair-deficient mice.
maturation. I. Immature peripheral B cells in adults are heat stable antigenγ and exhibit
cell maturation. II. Heat stable antigenγ splenic B cells are an immature devel-
opmental intermediate in the production of long-lived marrow-derived B cells.

FIGURE 5. H chain class switching by A-myb−/− B cells in vitro. Pu-
rified 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, all as described in Materials and Methods.