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

Kalpit A. Vora, Vicky M. Lentz,* William Monsell,* Sambasiva P. Rao,* Richard Mettus, † Antonio Toscani, † E. Premkumar Reddy, † and Tim Manser3*

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.

A-myb belongs to the myb family of transcription factors involved in the regulation of cell proliferation and differentiation (1). All members of this family (A-, B-, c-, and v-myb) share a high degree of homology within their DNA binding domains (2). A-myb, B-myb, and c-myb exhibit similar DNA binding specificities, and all three proteins were found to transactivate reporter constructs containing consensus Myb binding sequences (3). Of the three members of this family, A-myb and c-myb show a restricted pattern of expression, whereas B-myb appears to be expressed ubiquitously. The highest levels of A-myb are seen in developing CNS, adult testis, breast ductal epithelium during pregnancy, and in germinal center (GC) B lymphocytes (4–6). Male mice homozygous for a targeted germline mutation abating A-myb expression are infertile. Female A-myb+/− mice have defective development of breast tissue after pregnancy (7). These results confirm the critical role of A-myb in spermatogenesis and mammary gland development predicted from its restricted pattern of expression.

A-myb expression in human GCs has been sublocalized to the dark zone resident centroblast population (8). In this population, its expression has been further linked to the S and G2/M phases of the cell cycle (8, 9). Moreover, A-myb up-regulation has not been seen in in vitro stimulated B cells, and A-myb expression is rapidly down-regulated in GC-derived B cells that have further differentiated to plasma or memory B cell phenotypes (8). A-myb expression was also found to be characteristic of certain subsets of mature B cell neoplasias (Burkitt’s lymphoma, slg+ B cell-acute lymphocytic leukemia, subsets of chronic lymphocytic leukemia), supporting their GC origin (8, 10). Calabretta and colleagues found that ectopic expression of A-myb driven from a transgene led to follicular hyperplasia in peripheral lymphoid organs because of enhanced proliferation and accumulation of B cells bearing a GC phenotype (11). Based on these observations, it has been proposed that A-myb plays a critical role in the regulation of the GC reaction, including promoting high-rate B cell proliferation and Ab V gene somatic hypermutation (8). We have investigated these issues by using a previously described line of A-myb-deficient mice.

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 C57BL/6×129S1 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) phosphomager to quantitate levels of cDNA.

Immunizations and serology

Mice were immunized with 100 µg of alum-precipitated (4-hydroxy-3-nitropheno)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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4 Abbreviations used in this paper: GC, germinal center; PNA, peanut agglutinin; NP-CGG, (4-hydroxy-3-nitropheno)acetyl chicken γ globulin; bio, biotin.
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<sup>1</sup>, α<sup>1</sup> B cells and peanut agglutinin (PNA)<sup>1</sup> 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<sub>α</sub> 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-mouse IgM F(ab<sup>1</sup>)<sub>2</sub>, or anti-CD40 mAb (FGK45). In some experiments, recombinant IL-4 (PeproTech, Princeton, NJ) was included in cultures at 50 ng/ml. Cell proliferation was assayed by pulsing after 48 h with [3<sup>H</sup>]thymidine, harvesting onto glass-fiber filters, and scintillation counting at 50 ng/ml. Cell proliferation was assayed by pulsing after 48 h with [3<sup>H</sup>]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 of each genotype. Cell surface staining was then performed as described (17, 18). Splenocytes were stained with anti-CD45R (B220) and anti-CD24 (heat-stable Ag), 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<sup>hi</sup> and CD24<sup>lo</sup> (and in some experiments IgD<sup>hi</sup>) cells, respectively, among all CD45R<sup>+</sup> 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<sup>lo</sup>, IgM<sup>lo</sup>, and IgD<sup>hi</sup>; immature B cells were CD45R<sup>hi</sup>, IgM<sup>lo</sup>, and IgD<sup>lo</sup>; and pro- and pre-B cells were CD45R<sup>lo</sup>, IgM<sup>lo</sup>, and IgD<sup>lo</sup>. In a second experiment, mature B cells were defined as CD45R<sup>lo</sup> and IgD<sup>lo</sup>; immature and pre-B cells as CD45R<sup>hi</sup>, IgM<sup>lo</sup>, and IgD<sup>lo</sup>; and pro-B cells as CD45R<sup>lo</sup>, IgM<sup>lo</sup>, and CD24<sup>lo</sup>.

**Results and Discussion**

**Mild splenoplasia of the splenic white pulp in naive A-myb<sup>−/−</sup> mice**

Histological studies revealed that naive A-myb<sup>−/−</sup> mice have mild splenoplasia (Fig. 1). This hypoplasia appeared to involve all areas of the white pulp. The frequency of “spontaneous” GCs in naive spleens of A-myb<sup>−/−</sup> mice was also severalfold lower than observed in A-myb<sup>+/+</sup> animals.

Flow cytometric analysis revealed that splenic B cell percentages in A-myb<sup>−/−</sup> mice were ~2-fold lower than in A-myb<sup>+/+</sup> and A-myb<sup>+/−</sup> littersmates (Table I), in agreement with the immunohistochemical results. Five A-myb<sup>+/+</sup> and A-myb<sup>+/−</sup> animals had an average splenic B cell percentage of ~30, whereas three A-myb<sup>−/−</sup> animals had an average splenic B cell percentage of ~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.

**Serum Ab response to a T-dependent Ag in A-myb<sup>−/−</sup> mice**

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

**Table I. Mild splenic B cell hypoplasia in A-myb<sup>−/−</sup> mice**

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

<sup>a</sup> Values obtained from two animals are separated by commas.

<sup>b</sup> SD of mean values obtained from four animals are shown in parentheses.
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>−/−</sup> as compared with A-myb<sup>+/−</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>−/−</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>−/−</sup> mice

To characterize the GC reaction in A-myb<sup>−/−</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>+/−</sup> and four A-myb<sup>−/−</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× fields that stained with either PNA-HRP or PNA-HRP and

![Anti-NP Lambda Response](image1)

**FIGURE 2.** Primary and secondary λ chain-bearing anti-NP serum Ab responses in A-myb<sup>−/−</sup> mice. Cohorts of three age-matched A-myb<sup>+/−</sup> and A-myb<sup>−/−</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.

![IgM, IgG3, IgG1](image2)

**FIGURE 3.** H chain isotype levels in the primary anti-NP response of A-myb<sup>−/−</sup> mice. Cohorts of at least three aged-matched A-myb<sup>+/−</sup> and A-myb<sup>−/−</sup> 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>
<thead>
<tr>
<th>Table II. Normal B cell development in the bone marrow of A-myb&lt;sup&gt;−/−&lt;/sup&gt; mice&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-myb Genotype</td>
</tr>
<tr>
<td>Expt. 1 (B220, slgD, CD24)</td>
</tr>
<tr>
<td>+/+</td>
</tr>
<tr>
<td>+/-</td>
</tr>
<tr>
<td>−/−</td>
</tr>
<tr>
<td>Expt. 2 (B220, slgM, CD24)</td>
</tr>
<tr>
<td>+/+</td>
</tr>
<tr>
<td>−/−</td>
</tr>
</tbody>
</table>

* Values obtained from two animals of each genotype are separated by commas in each category.
anti-λ-biotin (bio) were counted. This analysis revealed 5.7 ± 0.7 and 5.3 ± 0.5 PNA<sup>+</sup> GCs per field in A-myb<sup>+/+</sup> and A-myb<sup>-/-</sup> spleen sections, respectively; and 4.5 ± 0.7 and 4.1 ± 1.0 PNA<sup>+</sup>, λ<sup>+</sup> GCs per field in A-myb<sup>+/+</sup> and A-myb<sup>-/-</sup> spleen sections, respectively. Thus, neither the number of total or λ<sup>+</sup> GCs were significantly different in A-myb<sup>-/-</sup> and A-myb<sup>+/+</sup> 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<sup>+/+</sup> and A-myb<sup>-/-</sup> 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<sup>+</sup>, λ<sup>+</sup> GCs. Individual GCs of this type were microdissected from the sections and genomic DNA PCR-amplified with V<sub>1</sub> gene-specific primers, and the PCR products were cloned and sequenced. In five NP<sup>+</sup>, λ<sup>+</sup> GCs from three A-myb<sup>-/-</sup> mice, an average V<sub>1</sub> mutation frequency of 0.54% was observed (Table III). Analysis of seven NP<sup>+</sup>, λ<sup>+</sup> GCs from two A-myb<sup>-/-</sup> mice yielded a mutation frequency of 0.52%. The mutation frequencies were determined to not be significantly different using a Student's t-test.

Table III. Mutation analysis of VA1 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&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1&lt;sup&gt;+/+&lt;/sup&gt;</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&lt;sup&gt;-/-&lt;/sup&gt;</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></td>
<td>5</td>
<td>6</td>
<td>1,800</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>8,700</td>
<td>47</td>
<td>0.54%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1&lt;sup&gt;-/-&lt;/sup&gt;</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&lt;sup&gt;-/-&lt;/sup&gt;</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>11,180</td>
<td>58</td>
<td>0.52%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>The mutation frequencies were determined to not be significantly different using a Students t-test.

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<sup>-/-</sup> 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<sup>-/-</sup> and A-myb<sup>+/+</sup> 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<sup>-/-</sup> 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.

In vitro proliferative and isotype switching responses of A-myb<sup>-/-</sup> B cells

The mild B cell hypoplasia and lower primary serum IgG Ab levels observed in A-myb<sup>-/-</sup> mice prompted us to evaluate the proliferative and H chain class switching potential of A-myb<sup>-/-</sup> B

Table IV. Unaltered serum Ab affinity maturation in A-myb<sup>-/-</sup> mice

<table>
<thead>
<tr>
<th>Anti-NP mAb or Sera</th>
<th>Intrinsic Affinity K&lt;sup&gt;a&lt;/sup&gt;(M&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Ratio NP12:NP3 Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>BB6-12H3</td>
<td>9.6 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>42.8</td>
</tr>
<tr>
<td>Unmutated primary mAb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bg 110-2</td>
<td>2.9 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>3.5</td>
</tr>
<tr>
<td>Hypermutated secondary mAb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pooled +/+ secondary sera</td>
<td></td>
<td>NA</td>
</tr>
<tr>
<td>Pooled –/- secondary sera</td>
<td></td>
<td>NA</td>
</tr>
</tbody>
</table>

FIGURE 4. In vitro proliferative responses of A-myb<sup>-/-</sup> 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 prolifer-
erated 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 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
with LPS and IL-4 resulted in
levels of IgG3 and IgG1 Abs, all as described in Materials and Methods.

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.

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 de-
velopment. 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.

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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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