T and B Cell Development in BP-1/6C3/Aminopeptidase A-Deficient Mice

Qun Lin, Ichiro Taniuchi, Daisuke Kitamura, Jiyang Wang, John F. Kearney, Takeshi Watanabe and Max D. Cooper

J Immunol 1998; 160:4681-4687;
http://www.jimmunol.org/content/160/10/4681

References  This article cites 48 articles, 19 of which you can access for free at:
http://www.jimmunol.org/content/160/10/4681.full#ref-list-1

Subscription  Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions  Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts  Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
T and B Cell Development in BP-1/6C3/Aminopeptidase A-Deficient Mice

Qun Lin,* Ichiro Taniuchi,‡ Daisuke Kitamura,‡ Jiyang Wang,‡ John F. Kearney,* Takeshi Watanabe,‡ and Max D. Cooper2*†

Stage-restricted cell surface molecules serve to delineate B lineage cells during their progressive differentiation within the bone marrow. The BP-1/6C3 Ag, aminopeptidase A (APA), is selectively expressed by the pre-B and immature B cells. This ectoenzyme, which is also present on bone marrow-derived stromal cells, thymic cortical epithelial cells, renal proximal tubular cells, intestinal enterocytes, and endothelial cells, cleaves acidic glutamyl and aspartyl residues from the N-terminus of angiotensin and other biologically active peptides to quench their functional activity. BP-1/6C3/APA expression by early B lineage cells is up-regulated by IL-7, an important growth factor for pre-B cells and T cells. To explore the physiologic role of this peptidase, we generated a mouse model of BP-1 deficiency by gene targeting in embryonal stem cells. While mice homozygous for the BP-1 mutation did not express detectable BP-1 protein or enzyme activity, they developed normally, generated normal numbers of T and B cells, exhibited integrity of Ab responses to both thymus-dependent and -independent Ags, and produced normal serum Ig levels. Phenotypic analysis of bone marrow and thymic lymphocytes indicated a normal pattern of B and T lineage differentiation. B lymphopoiesis in fetal liver cultures and the proliferative responses of bone marrow cells to IL-7 and LPS were also unimpaired. These findings indicate that BP-1 ectoenzyme activity is not essential for normal B and T cell development.


BP-1 expression is down-regulated in mature B cells and remains extinguished throughout plasma cellular differentiation.

Recognition of the APA ectoenzyme as a differentiation Ag on normal and transformed murine pre-B and immature B lymphocytes was achieved by using the BP-1 and 6C3 mAbs (4, 12–14). The BP-1/6C3 Ag was characterized as a homodimeric, phosphorylated cell surface glycoprotein comprising two disulfide-linked, 140-kDa subunits (4, 15). The deduced amino acid sequence of BP-1 cDNA predicted a type II membrane protein with a zinc-binding motif that characterizes members of the zinc-dependent metalloprotease family (15, 16). Enzymatic specificity analysis identified BP-1 as aminopeptidase A (APA), an ectopeptidase that selectively cleaves glutamyl and aspartyl residues from the N-terminus of peptides such as angiotensin (17–21). In addition to its developmentally regulated expression on the B lineage cells, BP-1 is found on stromal cells in the bone marrow and the thymic cortex and on subpopulations of cells in the ovary and placenta (22, 23). Along with several other ectoenzymes, BP-1 is abundantly expressed on the brush border of the small intestine. It is also expressed by renal glomeruli, proximal renal tubules, and vascular endothelium in many organs (22–28).

Although widely distributed, BP-1 expression on hemopoietic cells is restricted to the early stages in B lineage differentiation. Enhanced BP-1 expression is found on pre-B and immature B cells that are virus transformed, generated in long term bone marrow culture, or stimulated with IL-7, a well-known facilitator of early B cell development in mice (4, 29–32). These observations suggested that BP-1 could serve a regulatory role in pre-B cell growth or differentiation, perhaps by cleaving an inhibitory peptide to facilitate pre-B cell growth (33).

To test this hypothesis, we generated a mouse model of BP-1 deficiency. Surprisingly, mice homozygous for the BP-1 null mutation exhibit normal B and T cell development, indicating that...
BP-1 activity is nonessential for these lymphoid differentiation pathways.

Materials and Methods

Antibodies

Cy-Chrome-labeled B220, FITC-labeled 57/CD43, FITC-labeled anti-CD3, phycoerythrin (PE)-labeled anti-β2, PE-labeled anti-CD8, and biotin-labeled anti-CD4 Abs were obtained from PharMingen (San Diego, CA). PE-labeled BP-1, FITC-labeled anti-μ, streptavidin (SA)-Cy-Chrome, and SA-PE were obtained from Southern Biotechnology Associates (Birmingham, AL).

BP-1 gene targeting by homologous recombination

A 9.5-kb Sac-SacI genomic DNA fragment containing the first exon of the BP-1 gene (Enpep) was isolated from a genomic library in bacteriophage EMBL 3 (Clontech, Palo Alto, CA), and subcloned into the pUC19 vector. A 1.8-kb BstPI fragment containing part of exon 1 (34) was replaced with a 1.3-kb neomycin resistance gene, the PSV2-neo fragment. The construct, flanked by the herpes simplex virus thymidine kinase gene outside of the homology region (Fig. 1B), was linearized and electroporated into E14 ES cells. Transfected clones were selected with G418 and gancyclovir, with resistant colonies isolated for further analysis. The BP-1 mutation was confirmed by Southern blot analysis using the probe illustrated in Figure 1. ES cells colonized with the mutated allele were injected into blastocysts of C57BL/6 mice, which were transferred to pseudopregnant foster mothers. The resulting male chimeras were mated to C57BL/6 female mice for germline transmission of the BP-1 mutation. Germline transmission was determined by the coat color of offspring mice. Mice heterozygous or homozygous for the BP-1 mutation were screened by Southern blot analysis of BglII-digested genomic tail DNA or PCR analysis. PCR was performed at 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min for 30 cycles using four primers. Two primers specific for the region deleted in the targeted mutants (P1, 5′-GACAGTGAAGATGAAAGCCG-3′; and P2, 5′-ATCACCAGCTACTCTGCCTT-3′; Fig. 1) amplified a 274-bp product from the wild-type allele only, whereas additional primers specific for the neomycin resistant gene (P3, 5′-GAGGCTATTCGCTGCTATGACT-3′; P4, 5′-ATCCCTGCTTTGCGCAATATC-3′) amplified a 538-bp product in the mutated allele only.

APA enzyme assay

To measure cell surface-associated and soluble APA activity, washed cells and culture supernatants were incubated with 200 μl of PBS containing 1 mM CaCl₂ and 3 mM oไกล-γ-glutamyl-β-nitroanilide (Merck, Darmstadt, Germany) as a substrate (35, 36). Hydrolysis was performed for 2 h at 37°C. The supernatant was then transferred to 96-well plates, and the optical density determined at 405 nm.

Histochemistry

Freshly dissected kidneys were covered with OCT compound, snap frozen in liquid nitrogen, and stored at −80°C until use. Frozen sections were cut using a −30°C cryostat, dried for 5 min, overlaid with 100 μl of PBS containing 10% heat-inactivated horse serum and 0.01% azide in a humidified chamber for 20 min, and then incubated with FITC-conjugated BP-1 or control Abs for 30 min before examination by fluorescence microscopy.

Flow cytometry

Single-cell suspensions were incubated with FITC-, PE-, Cy-Chrome-, or biotin-conjugated mAbs on ice for 15 min, washed with 1% BSA/PBS, and counterstained with SA-Cy-Chrome or SA-PE to reveal biotin conjugates. Stained cells were analyzed with a Becton Dickinson FACS flow cytometer (Mountain View, CA). The data were analyzed with the Winlist 2.01 (Verity Software House, La Jolla, CA) and WinMDI 2.3 (Trotter@scripps.edu) software programs.

Ig isotype measurements

Serum samples from 6- to 10-wk-old mice were assayed for Ig isotype levels by ELISA. Mouse Ig standards and goat Abs specific for mouse Ig isotypes were purchased from Southern Biotechnology Associates.

Measurement of Abs to T-independent and T-dependent Ags

Dextran B1355S (100 μg), a type II T-independent Ag was injected i.p. into 8- to 10-wk-old mice. Serum samples were collected 7 days later, and the levels of IgM dextran-specific Abs measured by ELISA. Alum-purified 4-hydroxy-3-nitrophenylacetyl coupled to chicken γ-globulin (NP-CyG; 100 μg), was injected i.p. into 8- to 10-wk-old mice; serum samples were collected 7, 14, and 21 days later to measure IgM and IgG anti-NP Abs by ELISA.

Fetal liver culture

The in vitro B lymphopoiensis assay employed fetal liver cells obtained on day 15 of gestation. Dispersed cells were washed with PBS before resuspension in IL-7-conditioned medium, consisting of 20% supernatant from IL-7-transfected T220 fibroblasts (37) and 80% fresh RPMI 1640 medium with 5% FCS. The cells (10⁶) were then cultured in plastic wells seeded with the T220 transfectants.

Proliferation assay

IL-7 was produced by transfecting a murine IL-7 expression vector (kindly provided by Dr. Linda Park, Immunex, Seattle, WA) into COS cells. COS cell supernatant was collected 5 days after transfection and the IL-7 activity determined by ELISA.
was determined using an IL-7-dependent cell line, Scid 7, and rIL-7 (Genzyme, Boston, MA) as a standard. Fresh bone marrow cells from 6 to 8-wk-old mice were cultured in 96-well flat-bottom plates (10^5 cells/ml) for 72 h in the presence or absence of IL-7 (0.1–100 ng/ml). Splenic cells were cultured with or without LPS (20 μg/ml) (Sigma, St. Louis, MO). Cells were pulsed with 1 μCi of [3H]thymidine for the last 8 h, and the incorporated [3H]thymidine radioactivity was determined with a liquid scintillation counter for quadruplicate cultures.

Results

Generation of BP-1 deficient mice

Embryonic stem (ES) cell clones containing a mutant BP-1 gene were generated by homologous recombination of a targeting vector into the germline, employing a positive/negative selection strategy (38). The targeting vector was constructed by replacing part of exon 1 and intron 1 with the neomycin resistance gene (Fig. 1B). ES cell colonies with the mutated allele were injected into blastocysts of C57BL/6 mice. Chimera mice were mated with C57BL/6 mice, and offspring determined to be heterozygous for the disrupted BP-1 gene by Southern blot or four-primer PCR analysis were crossbred to obtain homozygotes. The genotypes of wild-type (+/+), heterozygous (+/−), and homozygous (−/−) mice were identified by Southern blot (Fig. 1E) and PCR analyses (Fig. 1D). In Southern blot analyses, the radiolabeled probe hybridized to a 2.7-kb BglII-digested band in wild-type genomes, and a 2.2-kb band in mutant genomes (Fig. 1E). In four-primer PCR analyses, two primers (P1 and P2) defining a 274-bp product within the deleted region amplified only the wild-type allele, whereas the other two primers (P3 and P4) specific for the neomycin resistance gene amplified a 538-bp product only from the mutant allele (Fig. 1D).

Analysis of BP-1 expression in BP-1-deficient and control mice

In contrast to bone marrow cells from the heterozygous and wild-type mice, cells from homozygous BP-1-mutant mice did not express the BP-1 Ag (Fig. 2A). Since BP-1 expression is relatively low on fresh bone marrow cells and is up-regulated by IL-7, bone marrow cells from the three types of mice were cultured in the presence of IL-7 (10 ng/ml) and then stained with the BP-1 Ab. An increase in BP-1 expression by early B lineage cells was observed in the wild-type and heterozygous mice but not in the homozygous mice (Fig. 2B). The level of IL-7-enhanced BP-1 expression in heterozygous mice was approximately half that seen in the wild-type mice.

When BP-1 expression was examined in kidney sections from normal mice, the glomeruli and proximal tubules were stained intensely by the FITC-labeled BP-1 Ab, whereas BP-1 staining was not observed in sections from homozygous BP-1 knockout mice (not shown). To verify that the BP-1 mutation abolishes APA function, APA activity was examined in renal cells, small intestinal enterocytes, and bone marrow cells cultured with IL-7. As shown in Figure 2C, wild-type mice exhibited high levels of APA enzyme activity, whereas background levels were seen in homozygous −/− mice. To exclude the possibility that the BP-1 targeting construct might be expressed as a secreted protein, APA activity was measured in supernatants from bone marrow cells cultured with IL-7 for 5 days. APA activity was detected in the supernatants of bone marrow culture from wild-type mice, but not in BP-1−/− bone marrow cultures.
Analysis of T and B cell development in wild-type and BP-1 knockout mice

Mice homozygous for the BP-1 mutation grew and bred normally in comparison with wild-type and heterozygous mice. Histologic analysis of heart, kidney, spleen, brain, ovary, and testis did not reveal morphologic differences between wild-type and mutant mice (data not shown).

To determine the effect of the BP-1 loss-of-function mutation on T and B cell development, the levels and composition of the T and B lymphocyte populations in the thymus, bone marrow, spleen, and lymph nodes were examined. The numbers of nucleated cells in bone marrow, spleen, lymph node, and thymus were found to be comparable in BP-1-deficient and littermate control mice: 0.75 ± 0.13 × 10⁹ for BP-1−/− vs 0.77 ± 0.06 × 10⁹ for BP-1+/+ bone marrow; 2.57 ± 0.60 × 10⁹ for BP-1−/− vs 2.67 ± 0.75 × 10⁹ for BP-1+/+ splenocytes; 0.10 ± 0.02 × 10⁹ for BP-1−/− vs 0.09 ± 0.02 × 10⁹ for BP-1+/+ lymph node cells; and 2.30 ± 0.57 × 10⁹ for BP-1−/− vs 2.20 ± 0.40 × 10⁹ for BP-1+/+ thymi. Control mice and BP-1-deficient mice also contained similar percentages of splenic and lymph node B cells expressing B220, IgM, and IgD, and CD3+ T cells expressing CD4 or CD8 (Fig. 3 and Table I). The thymocyte subset distribution defined by CD3, CD4, and CD8 analysis was also unaltered in BP-1 mutant mice relative to the wild-type mice (Table II).

B lineage cells in bone marrow samples from BP-1-deficient and normal mice were examined for stage-specific cell surface markers exclusive of the BP-1 Ag. Pro-B, pre-B, and B cells were thus defined as CD43+ B220+ (fraction A to C), CD43+B220+ IgM− (fraction D), and B220+ IgM+ (fraction E and fraction F) cells, respectively. The percentage of B lineage cells in each of these stages was similar in the BP-1-deficient and normal mice (Fig. 4 and Table III).

In vitro analysis of B lymphopoiesis

To further examine B lymphopoiesis in the BP-1−/− mice, progenitor cells in fetal liver samples were analyzed for their capacity to undergo B cell differentiation in culture. The genotype of donor embryos was identified by PCR analysis, and B cell development was monitored in cultures of fetal liver cell suspensions in the presence of confluent layers of IL-7-transfected fibroblasts. The B220+ cells comprised 2% of the cultured cells at the time of culture initiation. These reached a level of 80% by day 4. IgM+ cells comprised 5% of the cultured cells by day 7 and 30% of the cells by day 14 of culture (Fig. 5). Progenitor cells in the livers of

Table I. Distribution of B and T cell subpopulations in the spleen and lymph nodes of wild-type (+/+ ) and BP-1/APA-deficient (−/−) mice

<table>
<thead>
<tr>
<th>Donor Organ</th>
<th>B Cells</th>
<th>T Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B220+</td>
<td>IgM+</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+/+</td>
<td>54.8 ± 7.1</td>
<td>51.5 ± 6.8</td>
</tr>
<tr>
<td>−/−</td>
<td>56.0 ± 7.2</td>
<td>52.1 ± 7.0</td>
</tr>
<tr>
<td>Lymph node</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+/+</td>
<td>12.7 ± 0.5</td>
<td>12.1 ± 0.4</td>
</tr>
<tr>
<td>−/−</td>
<td>13.2 ± 0.5</td>
<td>11.9 ± 0.4</td>
</tr>
</tbody>
</table>

*Numbers represent the mean percentages of marker-positive cells ± 1 SE for six mice at age 6 to 8 wk.

Table II. Thymocyte development in wild-type (+/+ ) and BP-1/APA-deficient (−/−) mice

<table>
<thead>
<tr>
<th>Donor</th>
<th>CD4+CD8−</th>
<th>CD4+CD8+</th>
<th>CD4−CD8−</th>
<th>CD4−CD8+</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>5.0 ± 0.7</td>
<td>80.3 ± 1.8</td>
<td>11.5 ± 1.0</td>
<td>4.1 ± 0.0</td>
</tr>
<tr>
<td>−/−</td>
<td>4.2 ± 0.5</td>
<td>80.0 ± 1.1</td>
<td>12.0 ± 0.8</td>
<td>4.2 ± 0.6</td>
</tr>
</tbody>
</table>

*Numbers represent the mean percentages ± 1 SE for six mice at age 6 to 8 wk.
BP-1−/− embryos thus exhibited a normal pattern of B lymphopoiesis in this in vitro assay.

Integrity of humoral immunity in BP-1−/− mice

To assess the basal levels of Ab production in unimmunized control and BP-1−/− mice, serum Ig levels were measured. Comparable levels of the different Ig isotypes were observed (Fig. 6A) indicating that the BP-1−/− mice are capable of isotype switching and normal production of Ig isotypes. BP-1−/− mice were also able to mount comparable Ab responses to dextran, a thymus-independent Ag: 60 ± 76 vs 180 ± 158 μg/ml for wild-type mice (p > 0.05). The BP-1−/− mice produced significantly higher levels of NP Abs in response to immunization with the thymus-dependent Ag (NP-C3G) than did wild-type mice (Fig. 6B).

Proliferative responsiveness of B lineage cells in bone marrow and spleen

BP-1 expression is up-regulated by IL-7 (30, 32), an essential cytokine for murine B cell development in mice (29, 39 – 42). As a further test of the possibility that BP-1 might be involved in regulation of IL-7-induced proliferation, we determined the effect of the BP-1 mutation on IL-7-induced proliferation. The IL-7 response was examined by culturing bone marrow cells from BP-1-deficient mice and littermate controls with different concentrations of IL-7 for a 72-h interval. No differences were observed in proliferative responses by BP-1−/− and control cells as determined by [3H]thymidine incorporation (Fig. 7A). When the LPS response of splenic B cells was examined, BP-1−/− mice were found to have normally responsive B cells (Fig. 7B).

Discussion

One of the most interesting features of BP-1/6C3/APA is its lineage- and stage-specific pattern of expression in hemopoietic tissues, in which it is expressed exclusively on pre-B and immature B cells. This distinctive pattern of BP-1 expression makes it a valuable marker for the progression of cells along the B cell differentiation pathway (4, 6). However, the functional significance of this ectoenzyme expression pattern has remained speculative. The restricted expression of BP-1 during B cell differentiation (4, 6) and its elevated expression following IL-7 stimulation (30, 32) implied that BP-1 may serve not only as a marker of cell differentiation but also as a regulator of immune system development. Contrary to this expectation, the present study indicates normal development of B and T lymphocytes in BP-1-deficient mice.

Table III. Analysis of B lineage cells in bone marrow of wild-type (+/+) and BP-1/APA-deficient (−/−) mice

<table>
<thead>
<tr>
<th>Donor</th>
<th>CD43+ B220+ IgM+</th>
<th>CD43+ B220− IgM+</th>
<th>CD43− B220+ IgM+</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>6.1 ± 0.5</td>
<td>23.8 ± 3.4</td>
<td>15.4 ± 2.3</td>
</tr>
<tr>
<td>−/−</td>
<td>6.0 ± 0.3</td>
<td>23.3 ± 3.8</td>
<td>17.3 ± 1.4</td>
</tr>
</tbody>
</table>

* Numbers represent the mean percentages ± 1 SE of cells with lymphocyte light-scatter characteristics in bone marrow samples from six mice at age 6 to 8 wk.
neutral amino acids, this enzyme can also cleave basic and acidic residues at the N terminus of peptides (45). A compensatory increase in the activity of other aminopeptidases having secondary APA activity could thus rescue the BP-1 mutant mice from the effects of APA deficiency. However, the analysis of APA-like activity in homozygous mice indicated that this enzymatic activity was much lower than in normal mice. This result makes it unlikely that the normal phenotype in BP-1-deficient mice reflects a compensatory increase in the APA-like activity of another aminopeptidase.

It is also possible that natural substrates of BP-1/APA can be degraded or activated through other pathways that do not require APA activity. The best defined substrate of APA is angiotensin II (Ang II). Ang II is converted to Ang III by the removal of the N-terminal aspartyl residue from Ang II (18–21, 44, 46, 47) and can also be degraded to Ang IV by removal of the C-terminal phenylalanine. Although APA substrates have not yet been identified in the bone marrow, it is possible that unknown substrates of APA are processed by other enzyme systems to allow the normal B cell development observed in the APA−/− mice.

For the moment, we can only conclude that BP-1 serves as a convenient marker for early differentiation stages during B cell development, having failed to define a physiologic role for BP-1/APA on these cells. BP-1 expression could represent a secondary feature of highly regulated, lineage- and stage-specific transcriptional factors that interact with the regulatory region of the BP-1/APA (Enpep) gene (34). On the other hand, further study of BP-1-deficient mice could reveal a more subtle role for this ectoenzyme in B lineage differentiation.

Interestingly, the BP-1−/− mice mounted a more vigorous Ab response to the haptenic component of the thymus-dependent Ag, 4-hydroxy-3-nitrophenylacetetyl-chicken γ-globulin (NP-CγG), than did wild-type mice, although Ab responses to the thymus-independent dextran Ag, the LPS response, and serum Ig levels were comparable in the two groups of mice. Aminopeptidase N has been shown to be involved in the processing of antigenic peptides bound within the groove of MHC class II molecules by trimming the N-terminal peptide ends (48, 49). It is possible that APA may also exert an effect on certain T cell responses through a similar mechanism. The expression of BP-1/APA by stromal cells in the thymic cortex and by dendritic cells is compatible with this hypothesis (50), and future experiments will determine the potential of BP-1 for Ag processing.

The expression of BP-1 by nonlymphoid tissues, including the brush borders of enterocytes, renal glomeruli, proximal renal tubules, and vascular endothelium, suggests that this ectoenzyme may play an important role in the regulation of the renin-angiotensin system in addition to its function in a nutritional role. BP-1-deficient mice are currently being analyzed to explore the potential regulatory function of BP-1/APA in the renin-angiotensin system. An understanding of its role in this system could ultimately shed light on an immune system function.

Acknowledgments

We thank Ann Brookshire for help in preparing the manuscript and Drs. Peter D. Burrows and Pamela A. Welch for critical comments.