Bcmd Decreases the Life Span of B-2 But Not B-1 Cells in A/WySnJ Mice

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Peripheral B cells are divided into two subpopulations, B-1 and B-2, the relationship of which remains obscure. We recently showed that the Bcmd mutation in A/WySnJ mice reduces average B cell life span, yielding 90% fewer peripheral B cells. Despite this defect, A/WySnJ mice have an elevated proportion of peritoneal CD5+ B cells, suggesting that Bcmd may be the first B-cell-intrinsic gene to differentially affect the B-1 and B-2 subpopulations. To test this hypothesis in detail, we have used in vivo BrdU labeling and four-color cytofluorometry to examine the numbers and turnover rates of sIgM+ CD23-CD43+ (B-1) and sIgM+ CD23+CD43- (B-2) splenocytes in A/WySnJ and A/J mice. The results show the expected 90% reduction of splenic B-2 cells among A/WySnJ mice, but a normal splenic B-1 cell pool. Increased B-1 cell renewal cannot explain this undiminished pool, because BrdU labeling kinetics reveals an identical splenic B-1 subset turnover rate of ~4%/day in both A/WySnJ and A/J strains. Thus, B-1 cells are Bcmd-independent but B-2 cells are Bcmd-dependent, suggesting Bcmd functions in a positive signaling pathway that imparts longevity to quiescent B cells, but that is not required for cycling B cells. Moreover these results show that the requisites for maturation and longevity differ between the B-1 and B-2 subsets.

**Materials and Methods**

**Mice**

The A/J and A/WySnJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Experiments used adult male and female mice at 7 to 12 wk of age. All animal husbandry and procedures were done in accordance with the Animal Welfare Act.

**Reagents**

The following reagents were purchased from Pharmingen (San Diego, CA): APC and PE-labeled anti-CD5 (53–7.3); PE- and biotin-labeled anti-CD23 (IgE FcR) (B3B4); and PE-labeled anti-CD43 (leukosialin) (S7). AMCA-labeled donkey anti-mouse IgM was purchased from Jackson ImmunoResearch, (West Grove, PA). FITC-labeled anti-BrdU (B44) was purchased from Becton Dickinson (San Jose, CA).

**Lymphocyte preparation and staining**

Cell surface staining was done exactly as described (20, 21). Briefly, cells were preincubated in PBS (50 mM phosphate, 0.15 M NaCl, pH 7.2) containing 0.5% BSA, and then incubated in an optimal amount of fluorochrome-labeled or biotinylated Abs. Three washes preceded all incubations, which were for 30 min on ice. Cells were fixed in PBS containing 1% paraformaldehyde and were analyzed on a Becton Dickinson FACScan using B-D Cellquest software.

**Turnover studies**

In vivo BrdU labeling was done as previously described (20, 21). The adult A/J and A/WySnJ mice were injected i.p. with 0.6 mg BrdU (Sigma Chemicals, St. Louis, MO) in 0.2 ml of sterile PBS at 12-h intervals for the
duration of each experiment. Cells were stained for three or four surface markers (AMCA-, APC-, PE-, and biotin-conjugated Abs followed by streptavidin Red613), washed, and the incorporated BrdU analyzed according to our published procedures (20). Briefly, cells were permeabilized by dropwise addition of ice cold 95% ethanol, washed, and fixed in PBS with 1% paraformaldehyde and 0.05% Tween-20. The cells were then incubated in buffer plus 100 U/ml DNase to partially degrade and denature their chromatin. Finally, the cells were stained with FITC-labeled mAb to BrdU. Cytofluorometric analyses were performed by gating on all nucleated cells. For each mouse, the percentage of BrdU-labeled cells in each subset (B-1 or B-2) was determined cytometrically and multiplied by the total cell number in the subset to give the number of labeled cells. The means ± SD values were plotted as a function of time, and least squares regression analyses were done on the linear portion of the plot to obtain the BrdU cell accumulation rate. These linear plots were extrapolated to determine the time to label 50% of cells in the subset. Half-lives were calculated from a first order rate equation assuming a constant pool size during the analysis, as previously described (20). Significance was evaluated using the Student t test.

Results

Our previous studies showed that A/WySnJ mice harbor a single, autosomal, codominantly expressed trait, Bcmd, which results in profound B cell deficiency, reflecting severely reduced peripheral life span (18–20). The results of these studies are summarized in Table I. Surprisingly, when B cells were analyzed from different peripheral compartments, the fraction of A/WySnJ peritoneal B cells that were sIgM+CD5+ was disproportionately high when compared with A/J (18). Since the previously documented 10-fold reduction in peripheral B cell numbers clearly indicates a substantial effect on the B-2 subset, this observation suggested that Bcmd might differentially affect the B-1 and B-2 subsets. To probe this possibility, we determined the steady state numbers and turnover rates of each population in A/WySnJ and A/J spleens.

A/WySnJ mice have normal numbers of B-1 cells

The B-1 and B-2 subsets are distinguished by reciprocal patterns of CD23 and CD43 expression (22). We therefore used these markers among sIgM+ B cells to compare the splenic B-1 subpopulations of A/J and A/WySnJ mice.

This analysis (Fig. 1) showed that B-1 cells comprised a fourfold larger proportion of A/WySnJ splenocytes than A/J splenocytes. When multiplied by the splenocyte recoveries, these percentages yielded equal numbers of B-1 cells in the subset to give the number of labeled cells. The means ± SD values were plotted as a function of time, and least squares regression analyses were done on the linear portion of the plot to obtain the BrdU cell accumulation rate. These linear plots were extrapolated to determine the time to label 50% of cells in the subset. Half-lives were calculated from a first order rate equation assuming a constant pool size during the analysis, as previously described (20). Significance was evaluated using the Student t test.

The turnover rate of B-1 cells in A/WySnJ mice is normal

The normal number of B-1 cells in A/WySnJ mice suggested that Bcmd does not affect B-1 cells. However, adoptively transferred

![Figure 1](http://www.jimmunol.org/DownloadedFrom)
B-1 cells can fully repopulate irradiated hosts, indicating a capacity for self-renewal (23–25). Therefore, it was possible that Bcmd indeed affects B-1 cells but that compensatory proliferative activity offsets shortened life span, yielding an apparently normal B-1 compartment. To study this point, we measured the BrdU labeling kinetics in splenic B-1 and B-2 cells from A/WySnJ and A/J mice. We reasoned that, if B-1 cells are unaffected by Bcmd, then the labeling rates of B-1 cells in A/WySnJ and A/J should be the same; but if compensatory proliferative activity occurs, then the B-1 population should label faster in A/WySnJ.

The results (Fig. 3, Table II and Table III) showed identical B-1 cell labeling rates and life spans in A/J and A/WySnJ mice. Approximately 4% of the B-1 subpopulation is labeled per day in both strains. This corresponds to ~40,000 cells per day and yields a calculated half-life of ~17 days. These data indicate that the turnover rate of B-1 cells is similar in the two strains and is sufficient to completely replace the B-1 pool in 3 to 4 weeks.

The proportional labeling rate of the B-2 subset was about two-fold faster in A/WySnJ than in A/J mice (Fig. 3, and Table II and Table III) indicating that A/WySnJ B-2 cells have a shorter life span compared with A/J. Although not directly comparable, because different markers and labeling periods were used, these results are consistent with our previous work (Table I), which showed that most A/WySnJ peripheral B cells have an inordinately high decay rate and a shortened life span (20).

This analysis also resolved B cells with the surface phenotype attributed to marginal zone cells, sIgM+CD23−CD43− (22). These were 60% reduced in number, suggesting they are likely related to B-2 cells (data not shown). In both strains, this population labeled at about 4% per day, presumably reflecting proliferation associated with recent activation (26).

Discussion

The results presented herein show that Bcmd, an intrinsic B lineage-specific defect, reduces the life span and pool size of peripheral B-2 cells, but has no effect on peripheral B-1 cells. In conjunction with our previous findings, these observations extend our

Table II. Representation and labeling kinetics of splenic B-1 and B-2 subsets in strain A mice

<table>
<thead>
<tr>
<th>Days of BrdU Treatment</th>
<th>Total number of cells (× 10^6)</th>
<th>Proportion of cells BrdU^+</th>
<th>Number BrdU^+</th>
<th>Total number of cells (× 10^6)</th>
<th>Proportion of cells BrdU^+</th>
<th>Number BrdU^+</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/J</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.89 (0.17)</td>
<td>NA</td>
<td>NA</td>
<td>47.86 (10.2)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>3</td>
<td>0.99 (0.57)</td>
<td>0.24 (0.06)</td>
<td>0.22 (0.11)</td>
<td>39.30 (8.6)</td>
<td>0.08 (0.01)</td>
<td>2.29 (0.86)</td>
</tr>
<tr>
<td>6</td>
<td>0.89 (0.46)</td>
<td>0.34 (0.04)</td>
<td>0.18 (0.10)</td>
<td>30.67 (11.6)</td>
<td>0.14 (0.02)</td>
<td>4.39 (2.18)</td>
</tr>
<tr>
<td>9</td>
<td>0.53 (0.27)</td>
<td>0.36 (0.06)</td>
<td>0.29 (0.10)</td>
<td>38.96 (8.4)</td>
<td>0.16 (0.00)</td>
<td>6.11 (1.50)</td>
</tr>
<tr>
<td>12</td>
<td>0.79 (0.21)</td>
<td>0.56 (0.11)</td>
<td>0.29 (0.11)</td>
<td>37.14 (2.9)</td>
<td>0.24 (0.02)</td>
<td>8.85 (1.34)</td>
</tr>
<tr>
<td>A/WySnJ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.52 (0.19)</td>
<td>NA</td>
<td>NA</td>
<td>6.87 (2.7)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>3</td>
<td>1.09 (0.24)</td>
<td>0.27 (0.02)</td>
<td>0.29 (0.06)</td>
<td>7.65 (2.0)</td>
<td>0.28 (0.01)</td>
<td>1.92 (0.02)</td>
</tr>
<tr>
<td>6</td>
<td>0.53 (0.12)</td>
<td>0.35 (0.08)</td>
<td>0.18 (0.04)</td>
<td>3.92 (0.5)</td>
<td>0.37 (0.04)</td>
<td>1.44 (0.27)</td>
</tr>
<tr>
<td>9</td>
<td>0.68 (0.13)</td>
<td>0.36 (0.08)</td>
<td>0.24 (0.06)</td>
<td>3.82 (1.5)</td>
<td>0.37 (0.04)</td>
<td>1.42 (0.37)</td>
</tr>
<tr>
<td>12</td>
<td>0.54 (0.15)</td>
<td>0.50 (0.06)</td>
<td>0.27 (0.11)</td>
<td>6.38 (2.3)</td>
<td>0.47 (0.03)</td>
<td>3.03 (0.23)</td>
</tr>
</tbody>
</table>

* Eight-week-old A/J and A/WySnJ mice were given 0.6 mg of BrdU i.p. every 12 h, and the fraction of labeled cells within each was determined at the indicated times. Values are the mean from three mice per time point except for controls where n = 4. SEM are shown in parentheses. NA, not applicable. Results shown are one of two identical experiments.

† Percentage of cells in the indicated gate staining positive with FITC-anti-BrdU.

‡ Calculated for each mouse by multiplying the % events in the indicated gate times the number of cells in this gate staining with anti-BrdU times the number of splenic B cells.
understanding of the Bcmd mutation, characterize the first B cell-intrinsic mutation that differentially affects B-1 vs B-2 subsets, and provide the first estimate of peripheral B-1 subset turnover rates.

The long-lived mature B-2 cell subset is profoundly affected by Bcmd. In contrast, comparatively short-lived B cell populations, such as the B-1 subset and the immature B-2 compartment, show little or no Bcmd-mediated abnormalities (Ref. 20 and this report). This is most consistent with the suggestion that the Bcmd mutation disrupts a differentiation or life span-lengthening pathway (20). It is inconsistent with the alternative that the mutation disrupts a molecule that dampens a life span-shortening pathway, thereby increasing negative selection. For example, if Bcmd were to decrease the threshold for B cell receptor (Bcr)-mediated negative selection, then the short-lived, transitional B cell ought to be most sensitive to the effects of Bcmd, the opposite of what has been observed.

The suggestion that Bcmd disrupts a life span-lengthening pathway in B-2 but not B-1 cells is particularly striking because signaling differences associated with life span determination may indeed exist between these two cell types. Karras et al. (14) recently showed that B-1 cells have constitutively activated STAT3 homodimers whereas in B-2 cells STAT3 must be induced by treatment with IL-6; and Fukada et al. (27) showed that STAT3 mediates anti-apoptotic signals via the IL-6 receptor.

In addition to extending our understanding of the Bcmd mutation, our data reveal a distinction between the B-1 and B-2 subsets that constrains theories of their origins and relationships. The competing models differ regarding how commitment to the B-1 subset occurs. One model suggests that separate precursors exist in the fetal liver but not adult bone marrow (1, 15, 16), and that adult B-1 cells are maintained by self-renewal (27). The alternative model proposes that B-1 cells are the result of B-2 cell activation by TI-2 Ags (17, 28). The data presented here clearly show that B-1 and B-2 cells differ in their requisites for maturation and longevity, since the splenic B-1 cells do not require a normal Bcmd gene product for appropriate life span. Thus, while our data eliminate neither hypothesis, if there are indeed separate lineages, then B-1 cells must utilize other life span-determining mechanisms. Alternatively, if B-1 cells are an activation-induced subset, then early or continuous activation might circumvent the need for a normal Bcmd gene product.

The BrdU labeling studies herein provide the first measurement of splenic B-1 cell turnover. While known to be cycling and self-renewing, their life span and turnover rates were previously un-

### Table III. Numbers and turnover rates of splenic B-1 and B-2 subsets in A/J and A/WySnJ mice

<table>
<thead>
<tr>
<th>Subset</th>
<th>Strain</th>
<th>Pool Size</th>
<th>Number</th>
<th>Renewal Rate</th>
<th>Half-Life</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Percent</td>
<td>(× 10^-6)</td>
<td>%/day</td>
<td>(× 10^-6)</td>
</tr>
<tr>
<td>B-1</td>
<td>A/J</td>
<td>1.5 (0.4)</td>
<td>0.90 (0.2)</td>
<td>4.1</td>
<td>0.037</td>
</tr>
<tr>
<td></td>
<td>A/WySnJ</td>
<td>8.4 (2.2)</td>
<td>0.99 (0.6)</td>
<td>3.6</td>
<td>0.039</td>
</tr>
<tr>
<td>B-2</td>
<td>A/J</td>
<td>87.8 (5.6)</td>
<td>47.7 (10.3)</td>
<td>1.87</td>
<td>0.89*</td>
</tr>
<tr>
<td></td>
<td>A/WySnJ</td>
<td>65.8 (3.7)</td>
<td>7.7 (2.7)</td>
<td>3.43</td>
<td>0.26</td>
</tr>
</tbody>
</table>

* Adult A/J and A/WySnJ mice were analyzed as in Figure 1.  
\* Percentage of total sIgM^1^ within B-1 or B-2 gate.  
\+ Calculated total number of B-1 or B-2 splenocytes (percentage × total sIgM^1^ splenocytes).  
\- Derived from a least squares regression of the proportional BrdU labeling data shown in Figure 3 and Table II.  
\[ \frac{\ln(N)}{-\lambda} \]  
\[ \begin{align*} 
\text{Pool Size} & = \text{Number} \times \text{Percent} = \text{Percent} \times \text{Number} \\
\text{Renewal Rate} & = \frac{\text{Absolute Labeling Rate}}{\text{Number} \times \text{Percent}} \\
\text{Half-Life} & = \frac{\ln(2)}{\text{Renewal Rate}} \\
\end{align*} \]  

### Acknowledgments

We thank the Cell Sorting and Flow Cytometry Facility at the University of Pennsylvania for their help with the four- and five-color FACS analysis.

### References


