OcaB regulates transitional B cell selection

Mila Jankovic1 and Michel C. Nussenzweig1,2

1Laboratory of Molecular Immunology and 2Howard Hughes Medical Institute, The Rockefeller University, New York, NY 10021-6399, USA

Keywords: anti-self antibodies, B cell selection, Ig repertoire, OcaB, transitional B cells

Abstract

OcaB, also known as Bob-1 or Obf-1, is a transcriptional co-activator which regulates Igκ gene transcription, recombination and receptor editing; it is required for normal development of transitional B cells and for germinal center formation. Here we report that abnormal B cell development in OcaB±/± mice results in a skewed Igκ repertoire including anti-DNA antibodies, suggesting that OcaB is essential for antibody repertoire selection. To determine whether OcaB is required for BCR-mediated B cell selection, we introduced a pre-recombined α hen egg lysozyme (HEL) Ig transgene into OcaB±/± mice. We find that in OcaB±/± mice expressing transgenic αHEL Ig bone marrow B cell development is normal up to the immature B cell stage, but fails to progress to the transitional B cell stage. We conclude that OcaB is required for normal selection of the antibody repertoire in developing B cells.

Introduction

OcaB is a POU homeodomain transcriptional co-activator that was originally described as Igμ specific (1-3). This co-activator increases transcription by clamping the POU domains of Oct 1 and Oct 2 to DNA, thereby improving their DNA binding affinity (4,5). In addition OcaB possesses a C-terminal activation domain that appears to activate transcription directly (1,3,4,6-8). Although many promoters contain Oct 1- or Oct 2-binding sites, OcaB is promoter selective and only a subset of octamer-containing promoters is activated by OcaB (2,9-12). Depending on the octamer sequence, Oct 1 or Oct 2 dimerizes on the DNA in a manner which is permissive or non-permissive for OcaB binding (10,12). OcaB-permissive octamer-binding motifs are found in Igκ promoters, but not in Igμ promoters (9-15). This difference may explain why Ig heavy chain expression is normal, while transcription, V(D)J recombination and receptor editing of some Igκ genes is abnormal in OcaB±/± mice (9,13-16).

OcaB is expressed only in lymphoid cells, with low levels in developing B cells and high levels in germinal center B cells. Expression of OcaB is regulated by a number of different signaling pathways, including those downstream of the BCR, CD40 ligand (CD40L) and IL-4 (17,18). These signaling pathways are believed to induce OcaB expression through factors that bind to CREB/ATF sites in the OcaB promoter (19). BCR, CD40L and IL-4 signaling pathways are activated during the germinal center reaction, and in the absence of OcaB there is a B cell autonomous defect in germinal center formation (13,14,16).

OcaB±/± B cells express normal levels of surface IgM in the spleen, but there is a 2-fold reduction in the numbers of mature B cells in the mutant mice (11,13-15,20). In addition there are fewer transitional B cells in the spleen and bone marrow (15,20). This block in B cell development is accompanied by an increase in the number of apoptotic cells in the immature B cell compartment in the bone marrow (20). Here we show that OcaB also regulates selection from the immature to mature B cell compartments and that B cells in OcaB±/± mice display a distinct Igκ repertoire with elevated levels of auto-reactive antibodies.

Methods

Mice

OcaB±/± mice were backcrossed to C57BL/6 mice for over 10 generations. Matched littermates were used in all experiments. For experiments with α hen egg lysozyme (HEL) and HEL transgenes OcaB±/± mice were bred to C57BL/6 αHEL and HELxHEL transgenic mice. All mice were maintained under specific pathogen-free conditions and all protocols were approved by the Rockefeller University IACUC.

Flow cytometry and cell sorting

Single-cell suspensions from bone marrow and spleen were stained with mAb conjugated to FITC, phycoerythrin, allophycocyanin or biotin. Biotinylated antibodies were visualized with...
streptavidin–Red-670 (Gibco/BRL). Antibodies used were anti-B220, anti-IgM, anti-IgD, anti-CD43 and anti-HSA (all from PharMingen). Data were acquired with a FACSCalibur and analyzed with CellQuest software (Becton Dickinson). Lymphocytes were electronically gated on forward and side scatter. Stained cells were sorted using a FACS Vantage (Becton Dickinson). Each sorted population was checked for purity on the same machine. Cells for RNA isolation were sorted directly into TRIzol reagent (Gibco/BRL).

**Cell cycle analysis**

Bone marrow cells were incubated for 40 min at 37°C with Hoechst 33342 (Molecular Probes), and stained afterwards with anti-B220 and anti-CD43. Data were collected on a FACS Vantage and analyzed with CellQuest software (Becton Dickinson).

**Table 1. B cell development**

<table>
<thead>
<tr>
<th>B220+ cells (%)</th>
<th>Wild-type</th>
<th>OcaB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pro/pre-B</td>
<td>59 ± 5</td>
<td>69 ± 6</td>
</tr>
<tr>
<td>Immature</td>
<td>27 ± 3</td>
<td>22 ± 5</td>
</tr>
<tr>
<td>Transitional</td>
<td>6 ± 7</td>
<td>1 ± 0.5</td>
</tr>
<tr>
<td>Recirculating</td>
<td>9 ± 4</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>8 ± 7</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>T2</td>
<td>18 ± 0.3</td>
<td>19 ± 1</td>
</tr>
<tr>
<td>Mature</td>
<td>61 ± 2</td>
<td>60 ± 2</td>
</tr>
</tbody>
</table>

Data represent the mean from five mice ± SD.

**Fig. 1.** B cell development in OcaB±/± mice. (A) B220 versus IgM and CD21 versus IgM staining of wild-type and OcaB±/± bone marrow samples. Numbers indicate percentages of IgMhighB220+ cells, transitional B cells (T1 and T2) and mature, recirculating B cells (M). (B) IgM versus CD21 staining of spleen cells from wild-type and OcaB±/± mice. Numbers show percentage of IgM+ cells in T1, T2 and M cell compartments.

**Fig. 2.** Igk light chain repertoire in wild-type versus OcaB±/± B cells. Relative percentage of specific families of Igk cDNAs obtained from immature B cells from the bone marrow (B220+IgM+) and mature spleen B cells (B220+) from wild-type and OcaB±/± B cells. Two independently sorted sets of samples were analyzed from wild-type and OcaB±/± mice. Forty clones from sorted wild-type immature B cells and 65 clones from sorted wild-type mature B cells were sequenced. Forty clones from sorted OcaB±/± immature B cells and 75 clones from sorted OcaB±/± mature B cells were sequenced.

**RNA and RT-PCR**

Total RNA was extracted from $10^4$–$10^5$ sorted cells using TRIzol reagent. RNA was reverse transcribed with Superscript II (Gibco/BRL). Igk DNA was amplified from cDNA by PCR using HotStart Taq DNA polymerase (Qiagen). The following primers were used: $V_k$ 5’-GGGTCGGAGGTTAGTGCAGTG-3’ and $C_k$ 5’-GTCTGAGTACGTTAGGAGTT-3’. The PCR reaction conditions used were: 94°C, 30 s and 62°C, 30 s for 30 cycles.

**Cloning and sequencing**

PCR products were gel purified (Qiaquick; Qiagen) and cloned into the TA vector (Invitrogen). DNA sequences were obtained using T7 primer and Dye Terminator Cycle Sequencing (Applied Biosystems). Sequences were analyzed using IgBLAST (NCBI).

**Anti-nuclear antibodies and ELISA**

Serum from groups of three to six mice of different ages was analyzed for the presence of anti-nuclear antibodies using the ANA test (Bion). Hep-2 cells were incubated with different dilutions of serum and bound antibodies were detected by anti-IgM or anti IgG conjugated to FITC. Analysis was done using a Zeiss fluorescent microscope. Anti-DNA antibodies
were detected using an ELISA assay on plates coated with DNA and histones. Anti-Insulin, anti-myoglobin and anti-thyroglobulin antibodies were detected by ELISA on plates coated with given antigen.

Results

Abnormal B cell development in OcaB±/± mice
Immature B cells in the bone marrow express low levels of cell-surface IgM (IgM<sub>low</sub>), but acquire high levels of IgM when they mature and transit to the periphery. These newly produced IgM<sup>high</sup>IgD<sup>low</sup> cells are referred to as transitional (21), and it has been reported that OcaB±/± mice have a partial block at this stage of B cell development (20). To further characterize B cell development in the absence of OcaB we produced genetically homogeneous OcaB±/± mice by backcrossing to C57B6 mice for 10 generations. We found only minor differences in the numbers of pro-B/pre-B cells and immature B cells (B220<sup>low</sup>IgM<sup>low</sup>) in the bone marrow of OcaB±/± mice compared to littermate controls. In contrast, there was a significant reduction in the number of transitional B cells in the spleen and bone marrow of these mice (B220<sup>low</sup>IgM<sup>high</sup>) (Table 1) (20).

Two populations of transitional B cells have been described: the more immature T1 population is IgM<sup>high</sup>CD21<sup>+</sup> and in the spleen these cells give rise to T2 cells that are IgM<sup>high</sup>CD21<sup>+</sup> (22). OcaB±/± mice showed a 6-fold reduction in the percentage of B cells in the T1 compartment in the bone marrow and almost 3-fold reduction in the spleen. Despite an overall 2- to 3-fold reduction in the total numbers of spleen B cells, the relative percentages of T2 and mature B cells in the spleen was normal (13,14,16) (Fig. 1). Thus, absence of OcaB has a profound effect on T1 transitional B cells.

Abnormal antibody repertoire in OcaB±/± mice
B cell development from the immature to the mature B cell stage is accompanied by a shift in the antibody repertoire. OcaB±/± B cells show normal Ig heavy chain expression and repertoire development (13,14,16). In contrast to Ig heavy chain genes, V(D)J recombination and receptor editing of some V<sub>k</sub> genes is OcaB dependent (9). To determine whether Ig<sub>k</sub> gene repertoire selection between the immature and mature B cell compartment is OcaB dependent, we compared the Ig<sub>k</sub> gene repertoire in B cell subsets purified from OcaB±/± and control mice. The V<sub>k</sub> locus is 3 Mb long and contains 140 genes that have been classified into 18 families based on sequence homology (23). OcaB±/± immature B cells showed non-random Ig<sub>k</sub> family expression, but this was different from wild-type control B cells (Fig. 2). For example, V<sub>k</sub>8 comprised 25% of the repertoire in immature B cells in wild-type mice, but V<sub>k</sub>8 was difficult to detect in OcaB±/± mice (Fig. 2). In contrast, V<sub>k</sub>4 comprised 8% of the repertoire in wild-type immature B cells and 26% of the repertoire in OcaB±/± immature B cells (Fig. 2). These differences are consistent with altered V(D)J recombination and editing of the V<sub>k</sub>s in OcaB±/± mice (9).
Certain V\text{\kappa}s are positively selected in the transition to the mature B cell compartment, whereas others are negatively selected. For instance, in wild-type mice, V\text{\kappa}8 representation decreases from 25% in immature B cells to 3% in mature B cells, whereas V\text{\kappa}4 family representation increases from 8% in immature to 32% in mature B cells (Fig. 2). We found that V\text{\kappa} selection between the immature and mature B cell compartments differs between wild-type and OcaB±/± mice (Fig. 2). V\text{\kappa}4, V\text{\kappa}19 and V\text{\kappa}23 families were positively selected between the immature and the mature B cell compartments in wild-type mice, but they were selected against in the absence of OcaB (Fig. 2). In contrast, V\text{\kappa}2, V\text{\kappa}1 and V\text{\kappa}8 families were selected against between the immature and the mature B cell compartments in wild-type mice, but they were positively selected in the absence of OcaB (Fig. 2). Our results suggest that OcaB is required for normal antibody repertoire selection in vivo.

\textbf{Tolerance is disrupted in OcaB±/± mice}

To determine whether abnormal repertoire selection in OcaB±/± mice alters serum antibody composition, we analyzed the serum of OcaB±/± mice for the presence of anti-DNA, anti-nuclear antibodies, anti-thyroglobulin, anti-insulin and anti-myoglobin. Titers of anti-insulin and anti-myoglobin antibodies were slightly increased in the absence of OcaB, while anti-thyroglobulin antibody concentrations were comparable to wild-type controls. In contrast, OcaB±/± mice showed high titers of anti-DNA IgM as early as 6 weeks of age, whereas such antibodies were only detected in aged (12-month-old) heterozygous littermate controls (Fig. 3). These mice have low levels of serum IgG because there is no germinal center reaction in the absence of OcaB. Nevertheless, OcaB±/± IgGs were enriched for anti-DNA and anti-nuclear antibodies (Fig. 3 and not shown). We conclude that in the absence of OcaB there is an increase in anti-DNA antibodies, but the level of natural polyreactive antibody as measured by reactivity with insulin, myoglobin and thyroglobulin appears to be unaffected.

\textbf{Immature B cell selection is abnormal in OcaB±/± mice}

BCR signaling regulates receptor selection in immature B cells (24,25). B cells with auto-reactive receptors are edited, rendered anergic or deleted at the immature B cell stage (26–29,30). To determine whether regulation of the threshold for selection might require OcaB we bred OcaB±/± mice with well-described αHEL transgenic mice (27).

Despite accelerated B cell development in the bone marrow, αHEL transgenic B cells migrate normally to the periphery and establish a long-lived B cell pool (27). B cell development in the bone marrow of αHEL/OcaB±/± mice resembled B cell development in αHEL up to the transitional B cell stage, including heavy chain allelic exclusion (Fig. 4A and results not shown). However, αHEL/OcaB±/± B cells

\textbf{Fig. 4. B cell development in αHEL/OcaB±/± and HELOHEL/OcaB±/± transgenic mice.} (A) B220 versus IgM plots show staining of bone marrow cells from αHEL, αHEL/OcaB±, HELOHEL and HELOHEL/OcaB± transgenic mice. Numbers indicate percentage of cells within each quadrant. (B) The histogram shows IgM staining of αHEL and αHEL/OcaB± bone marrow B cells, gated on B220+. (C) Analysis of splenic B cells from αHEL, αHEL/OcaB±, HELOHEL and HELOHEL/OcaB± transgenic mice. Plots show B220 versus HSA and B220 versus IgM staining. Numbers indicate percentage of cells within each quadrant. (D) Analysis of cellular DNA content by Hoechst 33342 staining of B220\textsuperscript{hi}CD43\textsuperscript{+} pre-B and immature B cells from αHEL and αHEL/OcaB±. The number of cells with <2n DNA content is indicated.
differed from αHEL and OcaB⁺ B cells in that they failed to progress beyond the immature stage in the bone marrow so that B cells accounted for only 1% of the cells in the spleen of αHEL/OcaB⁻/⁻ mice (Fig. 4C). To determine whether there is increased B cell death in the bone marrow of αHEL/OcaB⁻/⁻ mice we measured the number of cells with <2n DNA content by Hoechst staining. We found that the proportion of pre-B and immature B cells in αHEL/OcaB⁻/⁻ with <2n DNA content was 4 times greater than in αHEL controls (Fig. 4D).

To determine whether the BCR cross-linking by HEL can rescue immature αHEL OcaB⁺ B cells, we bred αHEL OcaB⁻/⁻ mice to HEL transgenic mice (HELαHEL/OcaB⁻/⁻ mice). Soluble HEL failed to rescue B cell development in HELαHEL/OcaB⁻/⁻ mice (Fig. 4A and C). We conclude that B cells carrying αHEL-specific antibodies require OcaB expression to survive selection from the bone marrow to the periphery and that receptor engagement by soluble HEL is not sufficient to abrogate the requirement for OcaB.

**Discussion**

It has been estimated that 2 × 10⁷ immature B cells are produced daily and many of these cells (50–90%) are lost upon selection into the mature B cell compartment (31–34). Selection appears to vary depending on poorly defined physiologic parameters, e.g. the number of B cells selected into the mature compartment is increased when B cells are depleted from the periphery (35) and larger numbers of immature B cells transit to the mature compartment in mice that have been immunized (36). In addition, selection is antigen receptor specific, and there is a shift in the antibody repertoire between the immature and mature compartment (37–39). Although the nature of antigen receptor selection has not been entirely defined, it appears to be accomplished by both positive and negative selection (26–29,37–39).

BCR signaling is essential for B cells to transit from the immature to the mature stage of development (24,25,48) and OcaB expression is regulated in part by BCR signaling (18). B cells that lack the cytoplasmic domain of Igβ can develop to the immature stage, but fail to progress beyond this stage and a similar, but less pronounced, defect is seen in Igα mutant mice (40,41). B cells bearing mutations in components of signaling pathways downstream of the BCR such as btk⁻, protein kinase Cζ, phosphatidylinositol 3-kinase (P85α⁻) or BLNK/SIp-65 are unable to mature normally (22,47). How the BCR regulates the transition from the immature to the mature B cell stage has not been determined. One interpretation of our analysis is that OcaB is required for setting the normal threshold for immature B cell development and selection in the bone marrow. This idea is supported by the increased number of apoptotic cells in the immature B cell compartment in the bone marrow of OcaB⁻/⁻ mice (20) and αHEL/OcaB⁻/⁻ mice. An alternative interpretation of our results is that OcaB is simply required for normal levels of Igα (9) and, therefore, BCR expression (Fig. 4). In this model low-level expression of the BCR would lead to poorly signaling receptors that are abnormally selected.

OcaB⁻/⁻ mice show a receptor specific block at the T1 stage of B cell development. We would like to propose that absence of OcaB shifts B cell selection and thereby changes the antibody repertoire either indirectly by altering antibody expression or more directly by shifting BCR signaling thresholds. In either case, B cells bearing anti-self reactive receptors that are normally deleted would complete maturation, whereas B cells bearing non-self reactive receptors would fall below the selection thresholds. Such a shift might account for the decrease in the number of transitional B cells, the increase in autoantibodies, possibly the absence of germinal centers in OcaB⁻/⁻ mice and the increased sensitivity of OcaB⁻/⁻ transitional B cells to BCR cross-linking in vitro [(13–16, 20) and not shown]. OcaB is a unique example of a transcription factor required both for normal B cell selection and to maintain tolerance. More complete analysis of the target genes for OcaB should further our understanding of these complex processes.

**Abbreviations**

CD40L CD40 ligand
HEL hen egg lysozyme

**References**

OCA-B regulates transitional B cell selection

cocactivator OBF-1 (OCA-B, BOB-1) is mediated by a specific POU dimer configuration. Cell 103:853.


32 Forster, I. and Rajewsky, K. 1990. The bulk of the peripheral B-cell pool in mice is stable and not rapidly renewed from the bone marrow. Proc. Natl Acad. Sci. USA 87:4781.


