Development and Function of Murine B Cells Lacking RANK

Thomas Perlot and Josef M. Penninger

*J Immunol* 2012; 188:1201-1205; Prepublished online 4 January 2012;
doi: 10.4049/jimmunol.1102063
http://www.jimmunol.org/content/188/3/1201

---

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2012/01/04/jimmunol.1102063.DC1

**References**
This article cites 25 articles, 7 of which you can access for free at:
http://www.jimmunol.org/content/188/3/1201.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

---

*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2012 by The American Association of Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Development and Function of Murine B Cells Lacking RANK

Thomas Perlot and Josef M. Penninger

RANKL–RANK signaling regulates numerous physiologic processes such as bone remodeling, lymph node organogenesis, central thermoregulation, and formation of a lactating mammary gland in pregnancy. Recently, a receptor activator of NF-κB ligand (RANKL)–blocking Ab has been approved for human use in potentially millions of osteoporosis and cancer patients. However, germline deficiencies in RANKL or receptor activator of NF-κB (RANK) also lead to strong B cell defects in mice and human patients, suggesting that RANKL–RANK inhibition could interfere with B cell physiology and thereby trigger immunologic side-effects. To address this key question—that is, whether RANKL–RANK signaling affects B cell physiology directly or the observed defects are secondary because of the severe osteopetrosis—we generated B cell-specific RANK knockout mice. We show that B cells deficient for RANK undergo normal development and do not show any obvious defects in Ab secretion, class switch recombination, or somatic hypermutation. Our data indicate that ablation of the RANKL–RANK pathway has no direct adverse effect on B cell physiology. The Journal of Immunology, 2012, 188: 1201–1205.

Receptor activator of NF-κB ligand (RANKL, also known as ODF, TRANCE, OPG, and TNFSF11) is the activating ligand of the receptor RANK (receptor activator of NF-κB) of osteoclasts, whereas osteoprotegerin (OPG; also known as OCIF, TNFRSF11B, TR1, FDRC1) acts as a natural decoy receptor for RANK and, therefore, as a negative regulator of RANKL–RANK signaling ([1]). The RANKL/RANK/OPG system was shown to regulate a number of physiologic processes. RANKL–RANK signaling is essential for bone remodeling by regulating osteoclast development and function ([1]). Moreover, RANKL and RANK are indispensable for lymph node organogenesis ([2, 3]), for the development of a lactating mammary gland ([4]), the formation of AIRE+-medullary thymic epithelial cells ([5]), and were shown to regulate the fever response in the CNS ([6]). In addition, RANKL–RANK can promote progestin driven breast cancer ([7]), can act as a soil factor for cancer cells metastasizing to bone ([8]), or drive local invasion of experimental breast and prostate tumors ([9]).

Interestingly, it was also reported that B lymphocytes express RANK ([10]). The analysis of mice carrying a germline deletion of either RANK or RANKL revealed dramatic defects in B cell development, resulting in reduced numbers of peripheral B cells ([2, 3]). Importantly, human patients with a mutation in RANK also exhibit B cell defects such as reduced serum Ig levels, hypogammaglobulinemia, or impaired Ab responses to Ags ([11]). Denosumab is a fully human mAb against RANKL that has recently been approved as a treatment against osteoporosis of postmenopausal women, bone loss in men undergoing hormone ablation therapy against prostate cancer, and the effects of bone metastases from solid tumors ([12–19]). It has therefore been proposed that blocking RANKL might directly interfere with B cell function. However, both mice and human patients lacking functional RANK or RANKL develop osteopetrosis because of the absence of osteoclasts ([2, 3, 11, 20]). Osteopetrotic bones are denser than normal bones and exhibit a lack of bone marrow cavities, which are the regular environment for B cell development ([1]). Therefore, it is also conceivable that B cell defects in RANK-deficient mice and human patients are secondary to the absence of the natural site of B cell development—the bone marrow cavities.

To address this question, we generated mice that lack RANK specifically in B cells. In this study, we report that mice that lack RANK in B cells generate regular primary and secondary lymphoid organs, and RANK-deleted B cells undergo development normally. Furthermore, we demonstrate that basic B cell functions involved in humoral immunity such as Ab secretion, Ig class switch recombination (CSR), or somatic hypermutation (SHM) are undisturbed in the absence of RANK signaling. These data indicate that RANKL/RANK have no direct involvement in B cell physiology.

Materials and Methods

Mice

Mice carrying the conditional RANKℵ/ℵ (RANKK) allele and the RANKK allele have been described previously ([21]). The mutant RANK alleles were backcrossed to C57BL/6 more than ten times. MB1-cre mice ([22]) were obtained on a C57BL/6 background. We crossed RANKK females with mb1-cre+ male to obtain mb1-cre+ RANKK/+ experimental mice.

Fluorescence activated cell sorting

The following biotin-, FITC-, PE-, CyChrome-, PE Cy5-, or allophycocyanin-coupled Abs were used for flow cytometry: anti-CD20 (RA3-6B2), CD3ε (145-2C11), CD5 (53-7.3), CD6a (53-6.7), CD11b/Mac-1 (M1/70), CD11c (HL3), CD21 (7G6), CD23 (B3B4), CD25 (PC6), CD28 (37.51), CD95/Fas (Jo2), CD117/c-Kit (2B8), CD138 (281-2), IgD (11-26c.2a), IgGl (x.56), IgG3 (R40-82), Igk (187.1), Igλ (R26-46) (all Abs from BD), anti-CD4 (RM4-5), CD93 (AA4.1), IgM (II/41; Abs from eBioscience), and RANK F/+ control animals. All mice were maintained at the animal colony of Institute of Molecular Biotechnology according to institutional guidelines.

Received for publication July 15, 2011. Accepted for publication November 28, 2011.

J.M.P. is supported by grants from the Institute of Molecular Biotechnology of the Austrian Academy of Sciences, the Austrian Academy of Sciences, Genome Research in Austria (AustroMouse), and a European Union European Research Council Advanced Grant. T.P. is supported by a Marie Curie International Incoming Fellowship. The research leading to these results has received funding from the European Community’s Seventh Framework Programme (FP7/2007-2013) under Grant Agreement 252210.

Address correspondence and reprint requests to Prof. Josef M. Penninger, Institute of Molecular Biotechnology of the Austrian Academy of Sciences, Dr. Bohrgasse 3, 1030 Vienna, Austria. E-mail address: josef.penninger@imba.oeaw.ac.at

The online version of this article contains supplemental material.

Abbreviations used in this article: CSR, class switch recombination; OPG, osteoprotegerin; PNA, peanut agglutinin; RANK, receptor activator of NF-κB; RANKL, receptor activator of NF-κB ligand; SHM, somatic hypermutation; SRBC, sheep RBC.

Institute of Molecular Biotechnology of the Austrian Academy of Sciences, 1030 Vienna, Austria

Copyright © 2012 by The American Association of Immunologists, Inc. 0022-1767/12/$16.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1102063

The Journal of Immunology
FIGURE 1. B cell development. A, FACS analysis of pro-B cells (IgM<sup>+</sup>CD19<sup>-</sup>c-kit<sup>+</sup>), pre-B cells (IgM<sup>+</sup>CD19<sup>+</sup>CD25<sup>+</sup>), and immature B cells (IgM<sup>+</sup>CD20<sup>+</sup>) in bone marrow. B, FACS analysis of immature (B220<sup>+</sup>CD93<sup>+</sup>) and mature B cells (B220<sup>+</sup>CD93<sup>-</sup>; left panels); T1 (IgM<sup>+</sup>CD23<sup>-</sup>), T2 (IgM<sup>+</sup>CD23<sup>+</sup>), and T3 (IgM<sup>+</sup>CD23<sup>+</sup>) transitional B cells (middle panels), and follicular B cells (B220<sup>+</sup>CD21<sup>+</sup>CD23<sup>-</sup>) and marginal zone B cells (CD11b<sup>+</sup>CD21<sup>+</sup>CD23<sup>+</sup>CD5<sup>+</sup>) isolated from the peritoneal cavity. D, FACS analysis of B-1B cells (B220<sup>+</sup>CD11b<sup>+</sup>IgM<sup>+</sup>CD5<sup>+</sup>) isolated from bone marrow (left panels) and spleen (right panels). FACS blots are representative of at least three independent experiments and at least three mice per group, 6–12 wk of age.

anti-CD19 (6D5; from BioLegend), and fluorescein-coupled peanut agglutinin (PNA) from Vector Laboratories. Single-cell suspensions were preincubated with CD16/CD32 Ab (BD) and stained with the respective Abs. Plasma cells were identified as CD138<sup>hi</sup>CD28<sup>+</sup>Lin<sup>-</sup> (CD4<sup>+</sup>CD8<sup>+</sup>CD11b<sup>+</sup>CD19<sup>-</sup>) where lineage depletion was performed by MACS sorting (Miltenyi Biotec). FACS analysis was performed on a FACSCalibur (BD Biosciences), a FACSCanto (BD Biosciences), and on an LSRFortessa (BD Biosciences) apparatus.

Table I. Spleen cellularity

<table>
<thead>
<tr>
<th>Cell Number (×10&lt;sup&gt;6&lt;/sup&gt;)</th>
<th>Total Splenocytes</th>
<th>CD3&lt;sup&gt;e+&lt;/sup&gt; Splenic T Cells</th>
<th>CD19&lt;sup&gt;+&lt;/sup&gt; Splenic B Cells</th>
<th>IgM&lt;sup&gt;+&lt;/sup&gt; Splenic B Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>RANK&lt;sup&gt;B&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>40.6 ± 10.4</td>
<td>12.6 ± 4.1</td>
<td>20.8 ± 6.3</td>
<td>20.5 ± 6.1</td>
</tr>
<tr>
<td>36.9 ± 9.5</td>
<td>9.7 ± 2.5</td>
<td>19.8 ± 4.3</td>
<td>19.4 ± 4.3</td>
<td></td>
</tr>
</tbody>
</table>

Total spleenocyte numbers, as well as CD3<sup>e+</sup> splenic T cell, CD19<sup>+</sup>, or IgM<sup>+</sup> splenic B cell numbers are shown. Each value represents the average ± SD from four RANK<sup>B</sup> and seven control mice at 5–8 wk of age. Numbers from RANK<sup>B</sup> and control animals do not show statistical significant differences (two-tailed t test).

**Somatic hypermutation assay**

Efficiency of SHM was assessed as described previously (23). B220<sup>+</sup>PNA<sup>hi</sup> CD19<sup>+</sup>Cd23<sup>+</sup> follicular B cell clones were sorted from sheep RBC (SRBC)-immunized mice. Upon DNA extraction, the V<sub>γ</sub>J<sub>558</sub>–Ig<sub>δ</sub> intracisternal region was PCR amplified with Pfu DNA polymerase (Stratagene), cloned into Zero Blunt-TOP vector (Invitrogen), sequenced, and analyzed in comparison with nonimmunized splenocytes.

**Class switch recombination**

CD4<sup>+</sup>B cells were isolated from spleens by MACS (Miltenyi Biotec) and stimulated for 5 d with LPS (20 μg/ml) or IL-4 (50 ng/ml) plus oCD40 (1 μg/ml) to induce switching to IgG3 or IgG1, respectively. Percentages of switched B lymphocytes were assessed by flow cytometry (24).

**PCR analyses**

RANK wt (256 bp), floxed (396 bp), and Δ (566 bp) alleles were identified using PCR. The following primers and amplification conditions were used: 5<sup>′</sup>–GGAGAATTCTCCGACAGGAGATATAAGG–3<sup>′</sup> and 5<sup>′</sup>–TCGTGGTGGTGCTCTCCTGTTGTCAC–3<sup>′</sup> with 35 cycles of 95°C for 30 min, 60°C for 30 min, and 72°C for 30 min. For detection of RANK mRNA expression, RNA was isolated with Trizol (Invitrogen), followed by reverse transcription with SuperscriptII (Invitrogen) and random hexamers (Roche). Real time PCR was performed using the oligonucleotides 5<sup>′</sup>–CCAGGAGGGCATTAAGG–3<sup>′</sup> and 5<sup>′</sup>–CATTCACCGTGTCAGGTTAAGG–3<sup>′</sup> (40 cycles of 95°C for 10 min, 60°C for 60 min).

**Results**

**Deletion of RANK in B cells**

To analyze a direct effect of RANK deficiency on B cells, we used the conditional RANK allele in which exons 2 and 3 are flanked by

---

*Image* from: http://www.jimmunol.org/ Downloaded by guest on July 29, 2017
shown as mean
resent values from individual animals. Data are
6–14 wk of age, were used per group.

It has been reported that absence of RANKL–RANK signaling in mice can lead to a block in B cell development (2, 3). We
in all the experiments described below, we analyzed mb1-cre + the absence of RANK mRNA expression (Supplemental Fig. 1

Efficiency of Cre-mediated deletion of RANK exons 2 and 3 and
specific deletion in B cells, we crossed the conditional RANK
loxP sites. Cre-mediated deletion of exons 2 and 3 results in a frame shift and a RANK null allele (Supplemental Fig. 1A) (21). For a
specific deletion in B cells, we crossed the conditional RANK allele to mbl1-cre mice, which efficiently delete in the complete B cell lineage from the earliest pro B cell stage onwards (22).

In RANKL-deficient mice, it was observed that B cell zones in
lymph nodes by FACS analysis. B cells develop in the bone
marrow from hematopoietic stem cells to pro-B cells (IgM–CD19–c-kit+), pre-B cells (IgM+CD19–CD25+), and immature B cells (IgM+B220–). Mice lacking Rank in B cells display similar frequencies of pro-B cells, pre-B cells, and immature B cells in bone marrow when compared with control RANK+/+ animals (Fig. 1A). In spleens, we analyzed population frequencies of immature (B220–CD93+) and mature B cells (B220+CD93–). B220–CD93+ immature B cells can be subdivided into T1 (IgM+CD23–), T2 (IgM+CD23+), and T3 (IgM–CD23+) transitional B cell subsets. Furthermore, we analyzed the mature B cell subsets of follicular B cells (B220+CD21+CD23+) and marginal zone B cells (CD21+CD23–). All of those B cell populations were present at comparable ratios in RANK+/+ and control mice (Fig. 1B). Moreover, ratios of Igκ and Igλ expressing B cells in bone marrow and spleen were similar in RANK+/+ and control mice (Supplemental Fig. 2). We finally analyzed peritoneal B-1 B cells (B220+CD11b+IgM+CD5–; Fig. 1C) and plasma cells (Lin–CD28–CD138+) in bone marrow and spleen (Fig. 1D). Again, all those B cell populations showed similar frequencies in the presence and absence of RANK. Finally, assessment of total splenocyte numbers, as well as CD3ε+ splenic T cell, CD19+, or IgM+ splenic B cell numbers resulted in similar values (two-tailed t test) in RANK+/+ and control animals (Table I). Therefore, we conclude that RANK deletion in B cells does not affect B cell development in bone marrow, spleen, and the peritoneal cavity.

Formation of secondary lymphoid organs
In RANKL-deficient mice, it was observed that B cell zones in
spleens were dramatically reduced in size (3) (Fig. 2A). However, it was not clear whether this phenotype could be directly attributed to the lack of RANKL–RANK signaling in B cells or whether it was secondary for example to the osteopetrosis phenotype and a resulting defect in lymphocyte development. To answer this question directly, we first analyzed spleen sections of RANK+/+ mice. Histologic stainings of B and T lymphocytes revealed normal B and T cell zones in splenic white pulp in RANK+/+ mice (Fig. 2B). Moreover, we observed normal development of splenic germinal centers upon immunization with SRBCs (Fig. 2C). This result was corroborated by quantification of B220+PNA+ germinal center B cells by FACS analysis (Supplemental Fig. 3). Therefore, splenic architecture and organization of B cell and T cell zones do not depend on RANK signaling in B lymphocytes.

It was reported that mice with a germline deletion of RANK or
RANKL do not develop any lymph nodes, but do develop Peyer’s patches at reduced numbers and size (2, 3). Moreover, human patients with germline mutations in RANK do not show palpable lymph nodes. Therefore, we examined axillary, inguinal, mesenteric, and popliteal lymph nodes as well as Peyer’s patches but did

Histologic analysis of RANKL−/− and RANK+/+ spleen sections. A, White pulp in spleens from RANK−/− and wild type mice stained for IgD (brown). Original magnification ×50. B, White pulp in spleens from RANK+/+ and control mice stained with anti-B220 (blue) and anti–TCR-β (brown) Abs to identify B cell and T cell zones. Original magnification ×20. C, White pulp in spleens from SRBC immunized RANK−/− and control mice stained with an anti-IgD (blue) Ab and PNA (brown) to identify B cell follicles and germinal centers, respectively. Original magnification ×20. Representative results are shown from at least three independent experiments and at least four mice per group, 8–12 wk of age, with similar results.

Normal B cell development
It has been reported that absence of RANK–RANK signaling in mice can lead to a block in B cell development (2, 3). We therefore analyzed B lymphocytes in bone marrow, spleen, and

FACS analysis. B cells develop in the bone marrow from hematopoietic stem cells to pro-B cells (IgM–CD19–c-kit+), pre-B cells (IgM+CD19–CD25+), and immature B cells (IgM+B220–). Mice lacking Rank in B cells display similar frequencies of pro-B cells, pre-B cells, and immature B cells in bone marrow when compared with control RANK+/+ animals (Fig. 1A). In spleens, we analyzed population frequencies of immature (B220–CD93+) and mature B cells (B220+CD93–). B220–CD93+ immature B cells can be subdivided into T1 (IgM+CD23–), T2 (IgM+CD23+), and T3 (IgM–CD23+) transitional B cell subsets. Furthermore, we analyzed the mature B cell subsets of follicular B cells (B220+CD21+CD23+) and marginal zone B cells (CD21+CD23–). All of those B cell populations were present at comparable ratios in RANK+/+ and control mice (Fig. 1B). Moreover, ratios of Igκ and Igλ expressing B cells in bone marrow and spleen were similar in RANK+/+ and control mice (Supplemental Fig. 2). We finally analyzed peritoneal B-1 B cells (B220+CD11b+IgM+CD5–; Fig. 1C) and plasma cells (Lin–CD28–CD138+) in bone marrow and spleen (Fig. 1D). Again, all those B cell populations showed similar frequencies in the presence and absence of RANK. Finally, assessment of total splenocyte numbers, as well as CD3ε+ splenic T cell, CD19+, or IgM+ splenic B cell numbers resulted in similar values (two-tailed t test) in RANK+/+ and control animals (Table I). Therefore, we conclude that RANK deletion in B cells does not affect B cell development in bone marrow, spleen, and the peritoneal cavity.

Formation of secondary lymphoid organs
In RANKL-deficient mice, it was observed that B cell zones in
spleens were dramatically reduced in size (3) (Fig. 2A). However, it was not clear whether this phenotype could be directly attributed to the lack of RANKL–RANK signaling in B cells or whether it was secondary for example to the osteopetrosis phenotype and a resulting defect in lymphocyte development. To answer this question directly, we first analyzed spleen sections of RANK+/+ mice. Histologic stainings of B and T lymphocytes revealed normal B and T cell zones in splenic white pulp in RANK+/+ mice (Fig. 2B). Moreover, we observed normal development of splenic germinal centers upon immunization with SRBCs (Fig. 2C). This result was corroborated by quantification of B220+PNA+ germinal center B cells by FACS analysis (Supplemental Fig. 3). Therefore, splenic architecture and organization of B cell and T cell zones do not depend on RANK signaling in B lymphocytes.

It was reported that mice with a germline deletion of RANK or
RANKL do not develop any lymph nodes, but do develop Peyer’s patches at reduced numbers and size (2, 3). Moreover, human patients with germline mutations in RANK do not show palpable lymph nodes. Therefore, we examined axillary, inguinal, mesenteric, and popliteal lymph nodes as well as Peyer’s patches but did

The Journal of Immunology 1203

Download from: http://www.jimmunol.org/ by guest on July 29, 2017
not find an obvious defect in morphology or in numbers of these organisms in RANK\textsuperscript{AB} mice (not shown). Ratios of B cells and T cells in Peyer's patches and lymph nodes were comparable in RANK\textsuperscript{AB} and control animals (Supplemental Fig. 4). Therefore, we conclude that RANKL–RANK signaling in B cells is dispensable for the generation of lymph nodes.

**Intact B cell functions in RANK\textsuperscript{AB} mice**

It was reported that RANK mutations in human patients can lead to reduced Ig levels in serum and to impaired Ab responses to Ags. To assess whether RANK deficiency in B cells can cause such phenotypes, we measured serum Ig levels in nonimmunized and in SRBC-immunized mice. We determined levels of IgM, IgG1, IgG2a, IgG2b, IgG3, IgA, and IgE in RANK\textsuperscript{AB} and control mice, but did not detect a significant difference in any of the Ig isotypes, neither in nonimmunized nor in SRBC-immunized mice (Fig. 3). Another way to investigate efficiency of Ig CSR is to stimulate splenic B cells in vitro. Stimulation with LPS leads to switching to IgG2a and IgG3, whereas stimulation with IL-4 and αCD40 results in switching to IgG1 and IgE. Efficiency of CSR was similar in B cells from RANK\textsuperscript{AB} and from control mice (Fig. 4). Finally, we determined SHM in RANK\textsuperscript{AB} mice. To this end, we immunized mice with SRBCs and FACS sorted B220\textsuperscript{+}PNA\textsuperscript{hi}CD95\textsuperscript{+} activated B cells that undergo SHM (23). We did not observe an SHM defect in B cells lacking RANK, as the mutation rates were comparable in RANK\textsuperscript{AB} and control animals (Table II). These data indicate that RANK signaling in B cells is dispensable for Ab production, efficient Ig CSR, and SHM.

**Discussion**

We show that B cell intrinsic expression of RANK is not required for normal development or homeostasis of B lymphocytes in bone marrow and in the peripheral lymphoid organs. RANK\textsuperscript{AB} mice exhibit normal populations of pro-B cells, pre-B cells, immature or transitional B cells, mature B cells such as marginal zone B cells and follicular B cells, and plasma cells in bone marrow and spleen as well as normal numbers of B-1 B cells in the peritoneum. Our data differ from previously published work on mice carrying a germline mutation for either RANK or RANKL (2, 3).

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** Class switch recombination. FACS analysis of splenic B cells from RANK\textsuperscript{AB} and control mice stimulated with IL-4/anti-CD40 (A) or with LPS (B) inducing class switch recombination to IgG1 (A) or IgG3 (B), respectively. FACS blots are representative of four independent experiments. Four mice per group, 7–12 wk of age, were used with similar results.

In these mice, a dramatic reduction of peripheral B cells was observed, combined with a partial developmental block from the pre-B cell stage in RANKL-deficient mice (3). Because such whole body RANKL and RANK mutant mice exhibit severe osteopetrosis, it has been difficult to ascertain whether RANKL–RANK act directly in B cells. Osteopetrotic bones of RANKL and RANK knockout mice are denser than normal bones and, importantly, exhibit an almost complete lack of bone marrow cavities. The bone marrow is the natural environment for B cell development as well as the residence of hematopoietic stem cells from which all immune cell types are derived (25). Therefore, one feasible theory has always been that the observed B cell defects are secondary to the absence of bone marrow cavities in RANKL- and RANK-deficient mice rather than being a direct effect of absent RANKL–RANK signaling on B lymphocytes. Our data strongly support an argument against a direct effect of RANKL–RANK signaling on B cell development. In RANK\textsuperscript{AB} mice, we found Peyer's patches and lymph nodes of normal morphology with normal B cell and T cell numbers. In contrast, germline mutations of RANKL or RANK result in the complete absence of lymph nodes in mice (2, 3). Similarly, human patients carrying inactivating RANKL and RANK mutations lack palpable lymph nodes (11, 20). Our data demonstrate that RANKL–RANK signaling in B cells is not a requirement for the development of lymph nodes. The exact cellular and molecular mechanisms by which RANKL and RANK control lymph node organogenesis during embryogenesis remain largely elusive (1, 26).

It was reported that human patients carrying RANK mutations can exhibit disturbed B cell functions resulting in conditions such as hypogammaglobulinemia, a lack of Ab response to Ag, or in the presence of reduced serum Ig levels (11). Because we observed high RANK expression levels in IL-4/anti-CD40 activated B cells (Supplemental Fig. 1C), we investigated various functions of activated B cells, including CSR, SHM, and Ab secretion to serum with or without immunization. Again, RANK\textsuperscript{AB} mice did not display any obvious defects, arguing against an essential role for RANKL–RANK signaling in B lymphocyte functions.

Our work examines various aspects of B cell biology in the absence of RANKL–RANK signaling, without identifying an essential function of RANK in B lymphocytes. That leaves us with the question of the actual biologic function of the observed expression of RANK in B cells. One possible explanation is a functional redundancy of RANK signaling in B cells. Alternatively, although we performed comprehensive analyses of B cell development and basic B cell functions, the potential role of RANK signaling in B cells could be hidden in more complex functions of B cells that were not addressed in this study. Overall, RANK signaling does not seem to have a major role in B cell physiology.

**Table II. Somatic hypermutation**

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Independent Sequences</th>
<th>Bp Analyzed</th>
<th>Mutations</th>
<th>Mutation Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>RANK\textsuperscript{AB} 1</td>
<td>73</td>
<td>40,953</td>
<td>490</td>
<td>1.2 × 10^{-2}</td>
</tr>
<tr>
<td>RANK\textsuperscript{AB} 2</td>
<td>82</td>
<td>44,880</td>
<td>463</td>
<td>1.0 × 10^{-2}</td>
</tr>
<tr>
<td>Control 1</td>
<td>36</td>
<td>21,318</td>
<td>132</td>
<td>6.0 × 10^{-2}</td>
</tr>
<tr>
<td>Control 2</td>
<td>45</td>
<td>25,245</td>
<td>214</td>
<td>8.0 × 10^{-2}</td>
</tr>
</tbody>
</table>

B220\textsuperscript{+}PNA\textsuperscript{+}CD95\textsuperscript{+} activated germinal center B cells from spleens of SRBC-immunized RANK\textsuperscript{AB} and control mice were sorted by FACS. After DNA isolation and amplification, the mutation rate (mutations per bp) was assessed by DNA sequencing. Only independent sequences were included in the analysis.
Acknowledgments
We thank Dr. Shane Cronin, Dr. Ulrich Elling, Dr. Reiko Hanada, Dr. Toshihatsu Hanada, Dr. Gregory Neeley, Dr. Daniel Schramek, and Dr. Gerald Wünsberger for helpful comments and discussions, Qiong Sun for help with histologic stainings, and the Institute of Molecular Biotechnology service departments, especially Gerald Schmauss and Harald Scheuch, for technical support.

Disclosures
J.M.P. owns stock of Amgen. The other author has no financial conflicts of interest.

References

Downloaded from http://www.jimmunol.org/ by guest on July 29, 2017