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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, also known as TRANCE-R and TNFRSF11A), 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 RANK–RANKL signaling (1). The RANKL/RANK/OPG system was shown to regulate a number of physiologic processes. RANKL–RANKL 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 RANK/RANKL have no direct involvement in B cell physiology.

Materials and Methods

Mice

Mice carrying the conditional RANKflox (RANKf) allele and the RANKf allele have been described previously (21). The mutant RANK alleles were backcrossed to C57BL/6 more than ten times. MB1-cre mice (22) were crossed to 129Sv/EVB (C57BL/6 × 129Sv) F1 females with mb1-cre RANKf/− males to obtain mb1-cref RANKf/− experimental mice and RANKf/+ control animals. All mice were maintained at the animal colony of Institute of Molecular Biotechnology according to institutional guidelines.

Fluorescence activated cell sorting

The following biotin-, FITC-, PE-, CyChrome-, PE Cy5-, or allophycocyanin-coupled Abs were used for flow cytometry: anti-CD4 (RM4-5), CD93 (AA4.1), IgM (II/41; Abs from eBioscience), CD95/Fas (Jo2), CD117/c-Kit (2B8), CD138 (281-2), IgD (11-26c.2a), CD11c (HL3), CD21 (7G6), CD23 (B3B4), CD25 (PC61), CD28 (37.51), CD47 (145-2C11), CD5 (53-7.3), CD66a (53-6.7), CD11b/Mac-1 (M1/70), CD11c (HL3), CD21 (7G6), CD23 (B3B4), CD25 (PC61), CD28 (37.51), CD95/Fas (Jo2), CD117/c-Kit (2B8), CD138 (281-2), IgD (11-26c.2a), IgG1 (x56), 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),
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 (CD21<sup>hi</sup>CD23<sup>lo</sup>; right panels) in spleen. C, FACS analysis of B-1B cells (B220<sup>−</sup>CD11b<sup>+</sup>IgM<sup>−</sup>CD5<sup>+</sup>) isolated from the peritoneal cavity. D, FACS analysis of immature (B220<sup>−</sup>CD220<sup>−</sup>CD138<sup>−</sup>) in 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.

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;+&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>
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<tr>
<td>RANK&lt;sup&gt;−&lt;/sup&gt;</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>Control</td>
<td>36.9 ± 9.5</td>
<td>9.7 ± 2.5</td>
<td>19.8 ± 4.5</td>
<td>19.4 ± 4.3</td>
</tr>
</tbody>
</table>

Total splenocyte numbers, as well as CD3<sup>+</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>−</sup> and seven control mice at 5–8 wk of age. Numbers from RANK<sup>−</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>−</sup>CD19<sup>−</sup>CD21<sup>−</sup>CD23<sup>−</sup>CD93<sup>−</sup> follicular germinal center B cells were sorted from sheep RBC (SRBC)-immunized mice. Upon DNA extraction, the V<sub>λ</sub>558–1 Igλ intragenic region was PCR amplified with Pfu DNA polymerase (Stratagene), cloned into Zero Blunt-TOPO 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 (390 bp), and Δ (566 bp) alleles were identified using PCR. The following primers and amplification conditions were used: 5′-CCAGGAACCTCAGATGCAGATAA-3′, 5′-AGTTGTGCTGCA-TGTGACAGCCTT-3′, 5′-CTGTGGTTGTTCCTCCTGGTGTCACT-3′ 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), following by reverse transcription with SuperscriptII (Invitrogen) and random hexamers (Roche). Real time PCR was performed using the oligonucleotides 5′-CCAGGAACCTCAGATGCAGATAA-3′ and 5′-AGTTGTGCTGCA-TGTGACAGCCTT-3′ (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 anti-CD19 (6DS; from BioLegend), and fluorescein-coupled peanut agglutinin (PNA) from Vector Laboratories. Single-cell suspensions were preincubated with CD16/CD32 Fc block (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. Cell sorts were performed using a FACSaria (BD Biosciences) apparatus.

Absolute splenocyte numbers were determined by counting total splenocytes after RBC lysis with a CASY1 counter and subsequent calculation of T cell and B cell numbers based on ratios from FACS experiments.

Histology

Cryosections from spleens were fixed with acetone and blocked in TBS, 0.1% BSA. Samples were incubated with biotin-coupled TCR-β (H57-597) or FITC-coupled anti-B220 (RA3-6B2) or IgD (11-26c.2a) Abs (BD) or with biotinylated PNA, followed by streptavidin-HRP (BD) and anti Fluorescein alkaline phosphatase Fab Fragments (Roche). Fast Diaminobenzidine tablets (Sigma) and Fast Blue RR salt (Sigma) were used for detection according to the manufacturers’ instructions.

Immunizations

Fresh sheep RBCs (Innovative Research) were washed in PBS three times; 1×10<sup>8</sup> cells per mouse were injected i.p. Analysis was done after 12 d.

Determination of Ig levels

Serum levels of IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA were assessed using the Milliplex Map Kit (Millipore) with subsequent analysis on a Luminex test instrumentation. IgE serum ELISA (BioLegend) was performed according to the manufacturer’s instructions.
shown as mean ± SEM. Data are shown from three independent experiments. Three to 11 mice, 6–14 wk of age, were used per group.

FIGURE 2. Histologic analysis of RANKL−/− and RANKΔB spleen sections. A, White pulp in spleens from RANKL−/− and wild type mice stained for IgD (brown). Original magnification ×50. B, White pulp in spleens from RANKΔB 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 RANKΔB 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.

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 mb1-cre mice, which efficiently delete in the complete B cell lineage from the earliest pro B cell stage onwards (22). Efficiency of Cre-mediated deletion of RANK exons 2 and 3 and consequent loss of RANK expression in B cells was confirmed by PCR for the deleted and floxed alleles (Supplemental Fig. 1B) and the absence of RANK mRNA expression (Supplemental Fig. 1C). In all the experiments described below, we analyzed mb1-creΔB mice (hereafter termed RANKΔB) that lack RANK in B cells, and, as controls, RANKΔF/ΔF animals. Both experimental mice and controls were obtained by intercrossing RANKΔF/ΔF females to mb1-creΔB RANKΔF/ΔF males.

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 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-CD20+). 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 RANKF/ΔF mice (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 RANKΔB and control mice (Fig. 1B). Moreover, ratios of Igκ and Igλ expressing B cells in bone marrow and spleen were similar in RANKΔB 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 RANKΔB 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 RANKΔB mice. Histologic stainings of B and T lymphocytes revealed normal B and T cell zones in splenic white pulp in RANKΔB 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

FIGURE 3. Ig levels in serum. Serum Ig levels of all Ig isotypes from nonimmunized (A) or SRBC-immunized (B) mice were assessed. Values obtained from RANKΔB animals are shown as triangles, values obtained from control animals are shown as squares and are shown for each Ig isotype for both nonimmunized (A) and SRBC-immunized (B) mice. Triangles and squares represent values from individual animals. Data are shown as mean ± SEM. Data are shown from three independent experiments. Three to 11 mice, 6–14 wk of age, were used per group.
not find an obvious defect in morphology or in numbers of these organs in RANK\textsuperscript{AB} mice (not shown). Ratios of B cells and T cells in Peyser’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 serum 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 IgG2b and IgG3, whereas stimulation with IL-4 and CD40 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{+}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.

In these mice, a dramatic reduction of peripheral B cells was observed, combined with a partial developmental block from the pro- to the pre-B cell stage in RANK-deficient mice (3). Because 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 Peyser’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.
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