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RANKL Induces Organized Lymph Node Growth by Stromal Cell Proliferation

Estelle Hess,* Vincent Duheron,* Marion Decossas,† Frédéric Lézot,‡ Ariane Berdal,† Sylvestre Chea,§ Rachel Golub,§ Matteo R. Bosiso,** S. Lori Bridal,§ Yongwon Choi,# Hideo Yagita,** and Christopher G. Mueller*

RANK and its ligand RANKL play important roles in the development and regulation of the immune system. We show that mice transgenic for Rank in hair follicles display massive postnatal growth of skin-draining lymph nodes. The proportions of hematopoietic and nonhematopoietic stromal cells and their organization are maintained, with the exception of an increase in B cell follicles. The hematopoietic cells are not activated and respond to immunization by foreign Ag and adjuvant. We demonstrate that soluble RANKL is overproduced from the transgenic hair follicles and that its neutralization normalizes lymph node size, inclusive area, and numbers of B cell follicles. Reticular fibroblastic and vascular stromal cells, important for secondary lymphoid organ formation and organization, express RANK and undergo hyperproliferation, which is abrogated by RANKL neutralization. In addition, they express higher levels of CXCL13 and CCL19 chemokines, as well as MAdCAM-1 and VCAM-1 cell-adhesion molecules. These findings highlight the importance of tissue-derived cues for secondary lymphoid organ homeostasis and identify RANKL as a key molecule for controlling the plasticity of the immune system.

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The immune system displays a great capacity to adapt to environmental changes to provide the best-tailored response to a given immune challenge. Secondary lymphoid organs (SLOs), such as lymph nodes (LNs), play an important part in this activity. They are the anatomical sites where information from tissue is integrated by the immune cells. Over the last years, it has emerged that nonhematopoietic stromal cells that support the parenchyma of SLOs play a key role in orchestrating the immune response (1, 2). Stroma comprises vascular blood endothelial cells (BECs), lymph endothelial cells (LECs), and fibroblastic reticular cells (FRCs), which are interconnected through a micro conduit system (3). The FRC population consists of reticular cells of the T cell zone (TRCs) and the sinus marginal zone (MRCs), as well as B cell follicular dendritic cells (FDCs) (4–7). Vascular cells regulate entry and exit of lymphocytes (8–11), and FRCs function in the homeostasis of T and B cells (12, 13); together, they provide the necessary structural framework for immune cell encounter and activation (2, 3, 12). During an immune response, stromal cells possess the plasticity to adapt to rapid SLO expansion with massive increases in lymphocyte numbers (7, 14). However, it is not known how SLOs accomplish size increase and whether this process involves stroma.

During embryogenesis, stroma interacts with lymphoid tissue inducer (LTI) cells and orchestrates SLO development by producing chemokines and adhesion molecules (15–19). Stroma expresses the lymphotixin (LT) b receptor, which is an important molecule in this process (20, 21). LTbR and RANK are members of the TNFR superfamily (TNFRSF11a) and signal through the classical and the alternative NF-kB pathways (22, 23). RANK is activated by RANKL (TNFSF11 or TRANCE), which is blocked by the soluble decoy receptor osteoprotegerin (OPG) (22, 24). RANK- and RANKL-deficient mice lack most LNs (25–27), which renders the study of RANK function in LN immune homeostasis difficult. LTi cells express RANK and RANKL, and their numbers and cluster formation are reduced in RANKL knockout (KO) mice (27). In addition, RANK stimulates LT b expression by LTi cells, the ligand for LTbR (28). This led to the proposal that RANK-RANKL signaling in LTi cells results in activation of LTbR on stromal cells and subsequent SLO formation. However, the rescue of LN organogenesis in Rank1/−/− mice by transgenic RANKL expression in B or T cells does not restore LTi cell numbers to normal levels, and the engagement of stromal cell LTbR by an agonistic Ab appears unable to normalize LN formation (27). Moreover, Peyer’s patches and rescued mesenteric LNs of Rank1/−/− mice are smaller (25, 27) and display disorga-
nized B cell follicles (27, 29). Mice deficient in TRAF6, an adaptor molecule of RANK signaling, lack LNs; LN formation can be rescued by exogenous IL-7 but the SLOs then lack clearly defined B cell follicles (28). These findings underscore our incomplete knowledge of the prenatal and postnatal functions of RANK in SLO formation and homeostasis.

The hair follicle (HF), one of the defining features of mammals, is a miniorgan of the skin, which periodically regenerates by repetitive cycles of growth (anagen), regression (catagen), and relative quiescence (telogen) (30). It has a specialized association with the immune system, because it enjoys a relative immune privilege (31) and shares gene products with the thymus, most commonly exemplified by the Foxn1−/− nude mice that lack hair and thymus. RANK and RANKL are expressed in the thymus and function in the formation of medullary thymic epithelial cells (32–36). RANK also plays an important role in HF biology, because RANK stimulation of HF stem cells is required for entry into anagen. Anagen HFs produce soluble RANKL (37).

In this article, we present an analysis of a mouse transgenic (Tg) for Rank under the control of the S100A8 promoter, active in HF stem cells (37) and in macrophages (38). The mouse displays a massive postnatal expansion of skin-draining LNs. The proportions and organization of hematopoietic and stromal cell populations are well maintained, with the exception of an increase in small B cell follicles. The immune cells are not activated and respond rapidly to immunization. Using bone marrow (BM) transfers, skin transplantation, and genetic rescue experiments, we showed that Tg Rank expression in HFs is responsible for LN growth. RANKL is overproduced from the Tg HFs and is responsible for LN growth, because RANKL neutralization restores normal organ size. The vascular and reticular fibroblastic cells express RANK and undergo hyperproliferation, which is abrogated by RANKL neutralization. Moreover, stromal cells show increased transcription of chemokines and cell-adhesion molecules, which would contribute to LN growth. Our data showed that RANKL functions in SLO homeostasis by stimulating an equilibrium proliferation of all stromal cell subsets concomitant with increased lymphocyte recruitment.

Materials and Methods

Mice

Animals were kept in a pathogen-free barrier facility, and experimentation was performed in accordance with institutional guidelines and its animal bioethics accord (approval no. AL/01/20/11/08). All mutants were identified by PCR (Supplemental Table I), with the exception of CD45.1 mice, which were phenotyped by flow cytometry for the CD45 isofrom. Mice aged 6–8 wk were used for all experiments, unless stated otherwise. Ultrasound microscopy was performed with 24- and 47.8-MHz ultrasound systems on anesthetized mice (39). For adoptive BM transfer, 3-wk-old mice were lethally irradiated with 10-Gy x-rays (Particle accelerator, Saturn 41; General Electric); 24 h later, they received 2 × 10^8 BM cells i.v., harvested from congenic CD45.1−/− wild-type (WT) or CD45.2+ Tg mice. Chimerism was verified every month by assessing the percentage of donor blood leukocytes.

Isolation of stromal cells and lymphocytes

Stromal cells were isolated and identified using CD31 and podoplanin as markers, as described (12). Briefly, LNs were collected, and capsules were opened and incubated for 30 min at 37°C in RPMI 1640 (Lonza) containing 2% FCS (v/v; Lonza), Dispase (1 mg/ml; Roche), Collagenase D (1 mg/ml; Roche), and DNase (40 μg/ml; Sigma-Aldrich). Medium containing enzymes was then removed and renewed for another 20 min. The digest was gently pipetted every 10 min to break aggregates, until no visible fragment remained; the final pipetting was done in the presence of 5 mM EDTA. Cells were then passed through a 40-μm mesh and washed twice in RPMI 1640 10% FCS (v/v). In some experiments, CD45+ stromal cells were isolated by negative selection using anti-CD45–coupled magnetic microbeads (Miltenyi Biotec). Dendritic cells (DCs) were enriched by an Optiprep gradient (Sigma-Aldrich) (41) after LN digestion with Collagenase D (1 mg/ml) for 45 min under agitation.

Flow cytometry and cell sorting

Single-cell suspensions obtained from different organs were stained with Abs (Supplemental Table II) and analyzed on a FACSCalibur (Becton Dickinson) using CellQuest and FlowJo software. Stromal cells were sorted on a FACSVantage SE (Becton Dickinson) by gating on CD45+ (allophycocyanin) single cells and using podoplanin+ (FITC)- and CD31 (PE)-specific Abs (purity was 95 ± 2% for BECs, 95 ± 3% for LECs, and 91 ± 2% for FRCs). Regulatory T cells were labeled for Foxp3 following the manufacturer’s recommendations (eBioscience). Adult LTi-like cells, identified as lin+ (CD3, CD8, CD11c, TCRγ, TCRδ, CD19, Ter119, NK1.1, Ly6C/G) RORγt−αββ−/−CD127+, were first incubated with an anti-CD16/CD32 Ab before being labeled for Ltaβ;β, using mouse LTβR fused to hIgG-Fc, followed by fluorochrome-conjugated goat anti-human IgG. To ensure specificity, a negative control sample was preincubated with anti-Ltα and Ltβ mAbs before addition of mouse LTβR-human IgG-Fc.

Immunofluorescence

LNs were frozen in Tissue-Tek O.C.T. Compound (Electron Microscopic Science), and sections were prepared. After fixation in cold acetone and blocking with goat serum (Sigma-Aldrich), they were incubated with mouse LTβR for 1 h at 10 g anti-LTβR/Fc, followed by fluorochrome-conjugated goat anti-mouse IgG-Fc. Immunofluorescence was performed as described (40).

RT-PCR

RNA from sorted cells was extracted with RNaseasy kits (Qiagen), and cDNA was synthesized with oligo(dT)12 primers and ImProm-II (Promega) or Superscript III (Invitrogen). RT-PCR was performed using Promega GoTaq amplification mix and Eurogentec qPCR MasterMix Plus Low ROX. The gene-specific primers are listed in Supplemental Table I. Quantitative RT-PCR (qRT-PCR) was run on a Stratagen MX4000 thermal cycler, and threshold cycle values of target genes were normalized to GAPDH, hypoxanthine-guanine phosphoribosyltransferase, and β-actin. The expression factor was calculated using the Relative Expression Software Tool (http://mmmm.gene-quantification.de/rest.html).

BrdU incorporation

Mice were injected i.p. with 0.5 mg BrdU (Sigma-Aldrich) diluted in PBS and given BrdU in drinking water (0.8 mg/ml). CD45+ and CD45− cells were isolated from LNs, and the incorporation of BrdU was measured by flow cytometry using the BrdU flow kit (BD Pharmingen). Stromal subsets were identified using CD31 and podoplanin as markers.

Immunization

Twelve-week-old mice received s.c. injections of 200 μg chicken OVA with 5 μg LPS (Sigma-Aldrich), IFA, or 25 μg CpG (InvivoGen). A boost was administrated 2 wk later. Before and every 5 d thereafter, chicken OVA-reactive serum IgG was measured by direct ELISA. For Ab-affinity measures, mice received s.c. injections of 50 μg 4-hydroxy-3-nitrophenyl-acetyl (NP3)−/−keyhole limpet hemocyanin (KLH) (Biosearch Technologies) in 5 μg LPS (Sigma-Aldrich). A boost was administrated at 10 d. Serum anti-NP IgG was determined every 5 d by direct ELISA on plates coated with NP3−/−BSA or NP3−/−BSA (Biosearch Technologies). Tiers were determined as highest serum dilution that gave a value of at least three SD above the average reading for secondary controls. The relative affinity was determined as the titer NP3/titer NP3(4).
significant. GraphPad Prism version 5 for Windows (GraphPad software) was used for statistical data analysis.

**Results**

**Rank-Tg mice display prominent postnatal LN growth**

Mice overexpressing murine Rank under control of the S100A8 promoter showed a massive hyperplasia of skin-draining LNs (inguinal, axial, brachial, and superficial parotid), whereas internal LNs (mesenteric or para-aortic), spleen, and thymus showed no such growth (Fig. 1A, 1B). Cell counting revealed an 8–10-fold increase in hematopoietic cell numbers of cLNs, whereas the counts for mesenteric LNs (mLNs), spleen, thymus, blood, and BM were unchanged or even slightly reduced (Fig. 1B, Table I). To determine whether the SLO hyperplasia was acquired postnatally, we assessed cLN cell numbers 1 wk after birth and at different time points thereafter. Growth started after week 2 and reached a plateau at week 13 (Fig. 1C). To better determine its onset, we used in vivo ultrasound imaging, which measures LN size in young mice more accurately than does cell counting or organ weighing (39). It fixed the start of inguinal LN (iLN) growth at P24 (Fig. 1D). Thus, Rank-Tg mice displayed a prominent postnatal growth of their cLNs.

**Conserved LN architecture with a notable increase in small B cell follicles**

To investigate whether cLN hyperplasia was accompanied by abnormal overrepresentation of a hematopoietic cell lineage, we determined the proportion of the CD11b+ granulo-myeloid lineage, B220+ B cells, and CD3+ T cells by flow cytometry in cLNs, and determined the proportion of the CD11b+ granulo-myeloid lineage, whereas internal LNs (mesenteric or para-aortic), spleen, and thymus showed no such growth (Fig. 1A, 1B). Cell counting revealed an 8–10-fold increase in hematopoietic cell numbers of cLNs, whereas the counts for mesenteric LNs (mLNs), spleen, thymus, blood, and BM were unchanged or even slightly reduced (Fig. 1B, Table I). To determine whether the SLO hyperplasia was acquired postnatally, we assessed cLN cell numbers 1 wk after birth and at different time points thereafter. Growth started after week 2 and reached a plateau at week 13 (Fig. 1C). To better determine its onset, we used in vivo ultrasound imaging, which measures LN size in young mice more accurately than does cell counting or organ weighing (39). It fixed the start of inguinal LN (iLN) growth at P24 (Fig. 1D). Thus, Rank-Tg mice displayed a prominent postnatal growth of their cLNs.

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To investigate whether cLN hyperplasia was accompanied by abnormal overrepresentation of a hematopoietic cell lineage, we determined the proportion of the CD11b+ granulo-myeloid lineage, B220+ B cells, and CD3+ T cells by flow cytometry in cLNs, mLNs, spleen, and thymus of Tg and control animals. There was a significant increase in CD11b+ cells in both LN types and the spleen in mutant mice (Fig. 2A). An overrepresentation of these cells had also been noted in the BM (44); therefore, it is most likely the result of transgene expression by this lineage (44, 45) (data not shown). More restrictively, there was an increase in the proportion of B cells and a reduction in T cells in cLNs but not in mLNs or spleen. B cells do not express the transgene (45) (data not shown), suggesting that the change in B/T cell proportions was a reflection of the abnormal LN size. There was little alteration in B cell organization (data not shown). In addition, there was no significant difference in the numbers of cLNs, mLNs, spleen, thymus, blood, and BM in 12-wk-old WT and Tg mice. However, no such Abs were detected against actin, rheumatoid factor, and DNA in both nonimmunized and immunized Tg mice. However, no such Abs were detected (data not shown). In addition, there was no significant difference in B cell numbers in the autoimmune-sensitive salivary glands (WT: 30 ± 12 × 10^5, Tg: 35 ± 9 × 10^5; n = 8). In accord with the expanded immune compartment is not activated and responds to immunization

To address the question of whether the hematopoietic cells of the cLNs were functional, we studied the cutaneous immune response. Skin- and blood-derived DCs, purified from brachial and axial LNs and identified on the basis of I-A/CD11c expression (41, 46), responded to immunization with OVA with LPS, CpG, or IFA (Fig. 3D). Of note, Ab-affinity maturation to NP-KLH in LPS proceeded faster in Tg versus WT animals. The expanded immune compartment is not activated and responds to immunization

To address the question of whether the hematopoietic cells of the cLNs were functional, we studied the cutaneous immune response. Skin- and blood-derived DCs, purified from brachial and axial LNs and identified on the basis of I-A/CD11c expression (41, 46), showed low CD86 levels but normally upregulated this activation marker in response to s.c. CpG injection (Fig. 3A). T cells of cLNs, mLNs, or spleen were resting but responded to CpG by CD62L downregulation and CD44 upregulation (Fig. 3B). Also, B cells showed no sign of activation in nonimmunized Tg mice (Fig. 3C) and were activated in response to s.c. immunization by OVA with LPS, CpG, or IFA (Fig. 3D). Of note, Ab-affinity maturation to NP-KLH in LPS proceeded faster in Tg versus control mice (Fig. 3E). To test for a possible rupture of self-tolerance, we assessed the presence of serum autoantibodies against actin, rheumatoid factor, and DNA in both nonimmunized and immunized Tg mice. However, no such Abs were detected (data not shown). In addition, there was no significant difference in B cell numbers in the autoimmune-sensitive salivary glands (WT: 30 ± 12 × 10^5, Tg: 35 ± 9 × 10^5; n = 8). In accord with...
these results, the proportion of Foxp3+ regulatory T cells was not markedly altered in cLNs, mLNs, or spleen (data not shown). Stromal cells express Aire, which is under RANK control in the thymus (32–35), and regulate peripheral tolerance by expression of tissue-restricted peripheral tissue Ags (PTAs) (5, 47–49). Therefore, we evaluated Aire and PTA expression in Tg mouse cLNs by qRT-PCR. No upregulation of Aire expression in CD45R0+ stromal cells was seen (relative expression level, WT; 1, Tg: 0.642 ± 0.15; p = 0.092), and the stromal subset-restricted expression of Mlana and tyrosinase PTAs (48, 49) was also conserved (data not shown). Therefore, postnatal LN growth has no discernable negative influence on its immune cells, and peripheral tolerance is maintained.

**LN growth is associated with the transgenic HF**

To elucidate the mechanisms for organized cLN expansion, we investigated the contribution of the Rank transgene in the CD11b+ granulo-myeloid cell lineage by performing adoptive BM transfers. Lethally irradiated 3-wk-old Tg mice were reconstituted with BM of congenic CD45.1+ C57BL/6 mice; the reciprocal transfer was also done. BM transfers were complete, because all hematopoietic cells, including the CD11b+ lineage, were of donor origin (Supplemental Fig. 1A,1B). WT → Tg chimeras developed LN hyperplasia to the same extent as did control Tg → Tg animals (Fig. 4A). Reciprocally, Tg BM could not transfer LN growth to WT mice. These findings demonstrated that Rank transgene expression by the granulo-myeloid cell lineage is not responsible for cLN growth. We determined whether the radioresistant stromal cells expressed the Rank transgene by performing RT-PCR on cLNs recovered from WT → Tg chimeras. The transgene was no longer expressed, demonstrating that the cLN stroma is not transgenic for Rank (Supplemental Fig. 1C). We then considered the possibility that cLN growth is conveyed by Rank transgene expression in HFs (37). To address this issue, we first transplanted Tg or WT skin from 3-wk-old donors onto the backs of age-matched nude mice. Seven weeks later, cell numbers of brachial and axial LNs versus iLNs were determined. Because iLNs are not expected to drain

### Table I. Absolute cell numbers in organs (10⁶)

<table>
<thead>
<tr>
<th>Animal</th>
<th>cLNs</th>
<th>mLNs</th>
<th>Spleen</th>
<th>Thymus</th>
<th>Blood</th>
<th>BM</th>
</tr>
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<tbody>
<tr>
<td>Rank-Tg (n = 11)</td>
<td>196 ± 39</td>
<td>6.3 ± 3.1</td>
<td>76 ± 14</td>
<td>96 ± 44</td>
<td>5.6 ± 4.8</td>
<td>5.6 ± 4.8</td>
</tr>
<tr>
<td>WT (n = 8)</td>
<td>22 ± 3</td>
<td>7.2 ± 1.6</td>
<td>105 ± 20</td>
<td>125 ± 44</td>
<td>6.2 ± 4.0</td>
<td>6.2 ± 4.0</td>
</tr>
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Cell numbers (mean ± SD) of organ and blood cell suspensions from 12-wk-old Tg and WT animals were counted in a hemacytometer.

**FIGURE 2.** The enlarged cLNs display a well-conserved cell composition and organ architecture. A, Cell lineage proportions in cLNs, mLNs, and spleen (S) of CD11b+ granulo-myeloid cells, B220+ B cells, and CD3+ T cells in Rank-Tg versus WT mice were identified by flow cytometry (n = 11/genotype). B, Representative images of T cell zone and B cell follicle organization (CD3 and B220 staining), MRCs (MadCAM-1), and FDCs (MadCAM-1, FDC-M2, CD35) in brachial LNs of Rank-Tg and WT mice. Scale bars, 100 μm; insets, 50 μm. C, Identification of the CD45R0 FRC, LEC, and BEC stromal cell subsets by flow cytometry, using podoplanin and CD31 cell surface markers. The percentage ± SD is indicated next to the cell type. Graph (lower panel) depicts proportional changes. Table (lower panel) shows their absolute numbers in pooled inguinal, axial, and brachial LNs of Rank-Tg versus WT mice. Data are from 11–14 mice, accumulated in four experiments. D, Representative images showing BEC/FRC (CD31/podoplanin), LEC/B cell (Lyve-1/B220), and LEC/BEC (CD31/peripheral LN addressin) distribution on a brachial LN section. Scale bars, 100 μm; insets, 50 μm. *p < 0.05, **p < 0.01.
from the graft, their cell counts served as internal control for natural LN size and experimental variations. We found a significant increase for Tg skin recipients compared with WT skin, indicating that LN growth is associated with Rank transgene expression in HFs (Fig. 4B). We verified this conclusion by reducing transgene expression in HFs. This was done by crossing the transgene onto Mx2-Cre/lacZ mice, which have a shortened anagen phase (50) and, therefore, are expected to express lower the transgene onto Tg mice in terms of phenotype, numbers, and frequency. Moreover, because RANK stimulates LTαβ2 expression by LTi cells (28), we measured expression of LTαβ2. The experiments were performed at P24 and P42, shortly after skin RANKL peaks and initiation of LN growth. No significant difference in cell numbers, phenotype, or LTαβ2 expression was noted (Fig. 6A, Supplemental Fig. 2F). We also measured LTαβ2 expression by B cells but did not find a significant difference (Fig. 6A). We then studied FRC and endothelial cells, in view of their role in coordinating lymphoid organ formation (2, 4–6). First, we determined Rank expression by RT-PCR on FACS-sorted FRCs, LECs, and BECs. Rank was transcriptionally active in all stromal subsets (Fig. 6B), suggesting that skin-derived RANKL could directly stimulate cLN stroma. Because chemokines and cell-adhesion molecules are expressed by stroma and play an important role in SLO growth (9, 55), we tested whether these type of molecules were upregulated. We performed qRT-PCR on sorted stromal subsets harvested from cLNs of 6-wk-old WT and Tg animals. Among the different genes tested (Supplemental Table I), we found in Tg mice that FRCs expressed more CXCL13 transcripts; LECs expressed more CCL19, MAdCAM-1, and VCAM-1 transcripts; and BECs expressed more CCL19 transcripts (Fig. 6C). This suggested that cLN growth is promoted by RANK-stimulated expression of
chemokines and adhesion molecules. In light of the almost unchanged stromal cell proportion of the enlarged cLNs, together with the finding that RANKL induced medullary thymic epithelial cell proliferation (35), we next asked whether stromal cells proliferated in response to RANKL. To this end, we measured cell division by BrdU incorporation in 6-wk-old WT mice, as well as mock- and anti-RANKL–treated Tg mice. Mice were given BrdU for 6 d, and DNA incorporation of the nucleotide analog was measured by flow cytometry. The WT CD45^2 stroma had a reasonable proliferation rate, but it was much faster in Tg animals. Strikingly, when RANKL was neutralized, the proportion of BrdU+ stroma decreased to WT levels (Fig. 6D). In contrast, the BrdU incorporation rate for hematopoietic cells was similar between WT and Tg animals, and there was no change after administration of the blocking mAb. We next analyzed FRCs, LECs, and BECs separately. All subsets proliferated faster in Tg mice, and their greater BrdU incorporation was abrogated upon RANKL neutralization (Fig. 6E). Pulse-chase experiments were performed to address stromal cell viability. Loss of the BrdU label was not delayed in Tg mice, indicating that RANKL promoted stromal cell division without altering cell viability (Supplemental Fig. 2G).

Thus, RANKL stimulates cLN growth by inducing the proliferation of its vascular and reticular fibroblastic stromal cells.

**Discussion**

In this study, we showed that RANK stimulation resulted in the proliferation of LN stromal subsets and their expression of chemokines and adhesion molecules, culminating in LN growth. LN
organization was preserved, with a notable increase in B cell follicles. RANK stimulation did not provoke an immune activation; no autoreactivity could be detected, and immune cells responded rapidly and strongly to immunization by foreign Ags.

Although recognized as an important regulator of immunity, the lack of almost all SLOs in Rank or Rankl KO mice has hampered elucidation of RANK function in the development and homeostasis of the immune system. Therefore, we studied a Rank−Tg mouse, which displayed greatly oversized skin-draining LNs, whereas other SLOs, spleen, thymus, or BM were normal. LN growth occurred postnatally and, at its plateau, the lymphocyte number surpassed that of the spleen. We could not detect other major abnormalities of the immune system, aside from a general overrepresentation of T cells, B cells, and DCs.

FIGURE 5. RANKL induces cLN growth. A, Weight (left panel) and cell numbers (right panel) of cLNs, normalized to body weight, of WT, control Ig-administered Rank−Tg mice, and Rank−Tg mice, treated with anti-RANKL blocking mAb from P10 to 6 wk of age. B, Percentage of T and B cells in cLNs of WT, control Ig-administered Rank−Tg mice, and Rank−Tg mice, treated with anti-RANKL blocking mAb. C, Left panel, Representative visualization of B cell follicles in WT mice, control Ig-administered Rank−Tg mice, and Rank−Tg mice, treated with anti-RANKL blocking mAb by immunofluorescence on axial LN sections. Scale bars, 200 μm. B cell follicles visualized on B220-labeled axial LN cross-sections were counted (middle panel) and their area was quantified (right panel). Data are mean ± SEM of nine mice/group acquired in three separate experiments. D, MAdCAM-1+ MRCs in LNs of mock-treated and anti-RANKL-treated Rank−Tg mice (mouse #1 and #2). Arrows indicate MRCs at the outer margin of the B220+ B cell follicles. FDC-M2+ FDC networks on serial sections are indicated by large arrows. Dashed lines mark the limit of the LNs. Scale bars, 100 μm. **p < 0.01, ***p < 0.001.

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mature DCs would principally lead to an expanded T cell zone. This was not seen, and the proportion of DCs and their maturation status were unchanged. Because shortening of anagen by loss of Msx-2 abrogated LN growth, we deduced that the signal arises from the conduits (3). We found that RANKL neutralization by a blocking mAb restored normal LN size, B/T cell ratio, and follicle area and numbers. We cannot formally exclude the possibility that blocking RANKL from other sources contributed to normalization; however, skin RANKL was definitely affected, because abnormal HF cycling and epidermal dysplasia were corrected (37). Loss of B cells and reduction of RANKL beyond WT levels in the treated mice may be due to blocking RANKL produced by MRCs (7). Indeed, because RANKL neutralization led to the disappearance of MRCs from the preserved B cell follicles, a role for endogenous RANKL in the maintenance of MRCs can be evoked. Among other potential sources of SLO RANKL, we can exclude T cells, which synthesize RANKL only when activated (57). Although the mAb was administrated s.c., bone homeostasis was affected, because the treated mice displayed growth retardation. However, a disturbed hematopoietic cell output by reduction of BM niches (63) was not seen, because spleen cell numbers were not diminished. In a different model of skin RANKL overexpression by RANK transgenesis under control of the keratin 14 promoter, cLN hyperplasia was not reported (64). Although this difference may suggest that factors other than RANKL are implicated in cLN growth, the cardinal difference between the two mouse models is that RANKL is not soluble in keratin 14 promoter RANK Tg mice and, therefore, cannot freely reach the draining LNs. As proposed by the investigators, it is likely that the Tg RANKL principally affects Langerhans cells.

To identify the cellular RANKL target, we considered adult LTI-like cells and stromal cells as candidates. However, the proportion of adult LTI-like cells was not altered, nor was the phenotype and expression of LToβ2 by adult LTI-like cells or B cells different. We could also not detect an increase in IL-7 synthesis by FRCs, an important SLO source of IL-7 (12) (data not shown). We could also rule out Rank transgene expression by the radioresistant stromal cells, because its mRNA was no longer detectable after WT → Tg BM transfer. However, all stromal subsets expressed endogenous RANK. It is known that endothelial cells (65–67) and mesenchymal cells (56, 68) express RANK, but whether their SLO counterpart likewise carries RANK has not been studied. The cLN stroma upregulated chemokines and cell-adhesion molecules; however, more strikingly, it showed a dramatic hyperproliferation in the Tg mice, which was fully abolished by RANKL neutralization. Because of podoplanin coexpression it was not possible to differentiate between TRCs and MRCs within the FRC population. Other attempts to identify MRCs within the FRC population were not successful because of technical challenges, likely caused by the incompatibility of enzymatic liberation of cells with preservation of cell-adhesion molecules, such as MadCAM-1. It is also possible that MRCs are concealed within the CD45+ cluster, given their close association with B cells (7). However, if MRCs were the only RANK-expressing cell type, one could expect a specific enrichment of MRCs versus TRCs. The unchanged qRT-PCR measures of Rankl mRNA, expressed by MRCs but not TRCs (7) (data not shown), do not support a scenario of MRC-restricted proliferation. Whether FDCs also express RANK is uncertain, owing to the known difficulties with obtaining a sufficiently homogeneous FDC population. RANK-mediated FDC proliferation would support the increase in B cell follicles. Indeed, the Rank gene was expressed in a transcriptome study of FDCs (69) (C. Berek, personal communication). However, in keeping with the idea that MRCs may be FDC precursors (7), it remains a possibility that RANK stimulation of MRC translates into de novo B cell follicle formation comprising FDCs. We observed that the proportion of LECs is diminished in Tg LNs, although pro-
liferation and turnover rate are similar to those of BECs/FRCs. Occasionally, older mice (>20 wk) present with lymphedema in cLNs. This suggests an impaired function in the LEC compartment, so that reduction of egress rates may contribute to LN hypoplasia (11). The reasons for the loss of LECs are unclear and necessitate further investigations.

It was found that RANK ligation on vascular cells stimulates angiogenesis and ICAM-1 and VCAM-1 expression (26, 27). An artificial SLO-like cell system, high fluid pressure activates angiogenesis and ICAM-1 and VCAM-1 expression (66, 67, 70). In this context, these results support the idea that tissue-derived RANKL contributes to the peripheral tolerance paradigm. Cytokine Growth Factor Rev. 14: 275–288.

Tumor aversion is an important function of the tumor necrosis factor family member TRANCE. Immunity 17: 823–833.

Lymph nodes and Peyer’s patches.

The Journal of Immunology


