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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,§ Mattéo R. Bosisio,†‡§ 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. The Journal of Immunology, 2012, 188: 000-000.

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The online version of this article contains supplemental material.

Abbreviations used in this article: BEC, blood endothelial cell; BM, bone marrow; cLN, cutaneous lymph node; DC, dendritic cell; FDC, B cell follicular dendritic cell; FRC, fibroblastic reticular cell; HF, hair follicle; iLN, inguinal lymph node; KLH, keyhole limpet hemocyanin; KO, knockout; LEC, lymph endothelial cell; LN, lymph node; LT, lymphotoxin; LTI, lymph node tissue inducer; mLN, mesenteric lymph node; MRC, reticular cell of sinus marginal zone; NP, 4-hydroxy-3-nitrophényl-acetyl; OPG, osteoprotegerin; P, postnatal day; PTA, peripheral tissue Ag; qRT-PCR, quantitative RT-PCR; SLO, secondary lymphoid organ; Tg, transgenic; TRC, reticular cell of T zone; WT, wild-type.

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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 Foxn-1/−/− 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 equilibrated 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 CD45.1 isoform. Mice aged 6–8 wk were used for all experiments, unless stated otherwise. Ultrasound biomicroscopy 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 × 10⁷ 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.

For skin transplantation, skin from age-matched mice was transplanted onto Msx-2lacZ/lacZ-verified every month by assessing the percentage of donor blood leukocytes. 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, TCRβ, TCRγδ, CD19, Ter119, NK1.1, Ly6C/G) RORγt−aβ7−/CD127+, were first incubated with an anti-CD16/CD32 Ab before being labeled for Ltsb β, using mouse LTβR fused to human IgG-Fc, followed by fluorochrome-conjugated goat anti-human IgG. To ensure specificity, a negative control sample was preincubated with anti-LTs and LTβs Abs before addition of mouse LTβR-human IgG-Fc.

Immunofluorescence

LNs were frozen in Tissue-Tek O.C.T. Compound (Electron Microscopy Science), and sections were prepared. After fixation in cold acetone and blocking with goat serum (Sigma-Aldrich), they were incubated with primary Abs and, if necessary, secondary detection Abs (Supplemental Table II). Images were acquired with a Zeiss Axiovert 200M microscope and AxioVision Rel.4.8 software. Quantification of area and number of B cell follicles was done on images using Imaged software. β-galactosidase activity was assessed, as described (42).

RT-PCR

RNA from sorted cells was extracted with RNeasy kits (Qiagen), and cDNA was synthesized with oligo(dT)₁₂ Primer 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 Stratagene MX4000 thermal cycler, and threshold cycle values of target genes were normalized to GAPDH, hypoxanthineguanine phosphoribosyltransferase, and β-actin. The expression factor was calculated using the Relative Expression Software Tool (http://mmb.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-Alderich), 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 (NP) keyhole limpet hemocyanin (KLH) (Biosearch Technologies) or chicken 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 NP30-BSA (Biosearch Technologies). Titers 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 NP30 (43).

Statistical analysis

An unpaired two-tailed Student t test was used to determine statistically significant differences. The p values < 0.05 were considered statistically
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). Absolute numbers demonstrated a robust increase in the abundance of all stromal subsets in the hyperplastic LNs. The subsets were normally distributed, with FRCs dispersed around cortical BECs, and LECs juxtaposed to B cell follicles (Fig. 2D). Peripheral LN addressin-expressing high endothelial venules were abundant and normally located in interfollicular regions (Fig. 2D). Thus, the cLN cell composition and its architecture showed remarkably little alteration for such a significant growth, with the exception of an increase in the number and location of B cell follicles and their associated stromal compartment.

**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 podoplanin and CD31 expression (12), were assessed for their capacity to present and stimulate T cells of WT and Tg mice. However, no such Abs were detected against actin, rheumatoid factor, and DNA in both nonimmunized control mice (Fig. 3A). 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: \(30 \pm 12 \times 10^3\); Tg: \(35 \pm 9 \times 10^3\); \(n = 8\)). In accord with

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

**FIGURE 1.** Postnatal cLN hyperplasia in Rank-Tg mice. A, Representative photographs of cLNs (left to right: inguinal, axillary, brachial, and superficial parotid LN) and an mLN from 12-wk-old WT and Rank-Tg mice. Two-fold magnification. B, Fold difference in cell numbers of cLNs, mLNs, spleen, thymus, blood, and BM in 12-wk-old Rank-Tg versus WT mice. C, cLN cell numbers from week 1 to 16 in Rank-Tg and WT mice. D, iLN surface area, measured by ultrasound, in Rank-Tg and WT mice from P4–P58. Insets show iLN ultrasound images of 12-wk-old WT and Tg mice. Data in B are from 11 mice, collected in three experiments; in C and D, data are mean ± SD from six mice/genotype for each time point. **p < 0.01, ***p < 0.001.
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 CD45^2 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. cLN cell numbers were determined 3 mo later. 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>
<thead>
<tr>
<th>Animal</th>
<th>cLNs</th>
<th>mLNs</th>
<th>Spleen</th>
<th>Thymus</th>
<th>Blood</th>
<th>BM</th>
</tr>
</thead>
<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>
</tbody>
</table>

*p < 0.001 NS

Table I. Absolute cell numbers in organs (10^6)

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 m; insets, 50 m. C, Identification of the CD45^2 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, axillary, 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 m; insets, 50 m. *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 Msx-2lacZ/lacZ mice, which have a shortened anagen phase (50) and, therefore, are expected to express lower levels of the Rank transgene (Supplemental Fig. 1D). This mutant gene combination resulted in strongly reduced cLN growth (Fig. 4C). To exclude the possibility that Msx-2 affected the organ size through local expression, we assessed β-galactosidase activity in brachial LNs of Rank Tg;Msx-2lacZ/+ animals. lacZ gene activity was not detected in the LNs, but it was found at the base of the HFs (Fig. 4D) (50). Taken together, these findings supported the conclusion that Rank transgene expression in HFs is necessary and sufficient for skin-draining LN hyperplasia.

RANKL induces cLN growth
To identify the signal that is conveyed by the anagen HF and that is responsible for cLN growth, we considered RANKL as a probable candidate, because the anagen HF is an important source of soluble RANKL (37); soluble RANKL is a small (>10 kDa) globular protein, which can be expected to have direct access to LN stroma through its conduit system (3, 51, 52); and RANKL is known to play a critical role in SLO formation (25–27). We measured soluble RANKL and OPG release from Tg and WT skin at different time points during HF morphogenesis and cycling. We confirmed that RANKL was highest at anagen, when OPG levels were low (37). Moreover, higher levels of RANKL were released from Tg skin at P14 and during anagen (Supplemental Fig. 2A, 2B). We then directly tested the role of RANKL by s.c. administration of a RANKL-blocking mAb. Tg mice received the mAb from P10 to wk 6, whereas littermates were administered control mAb. cLNs and spleen were weighed and cells counted, and B/T cell populations were assessed. The RANKL-neutralizing mAb restored cLN weight and cell numbers (Fig. 5A). The proportion of B cells was reversed (Fig. 5B). The number of B cell follicles was recovered, and their size was restored (Fig. 5C). We then determined the presence of MRCs by labeling sections for MadCAM-1 and discovered a distinct reduction (thin arrows, mouse #1) or even a complete absence of these B cell-associated stromal cells (mouse #2). In contrast, FDCs remained intact, as shown by labeling for MadCAM-1 or FDC-M2 within the follicles (Fig. 5D, large arrows). This underscores the importance of MRCs in LN homeostasis and suggests a role for RANKL in MRC maintenance. RANKL neutralization did not lead to a reduction in splenocyte numbers (Supplemental Fig. 2C) nor were B or T cells activated, as assessed by plasmocyte and memory T cell formation (Supplemental Fig. 2D, 2E). The anti-RANKL treatment provoked a diminution in body weight (WT: 17.7 ± 0.5 g, Tg: 18.7 ± 1.1 g, treated Tg: 12.1 ± 1.6 g; n = 12), most probably attributable to perturbation of bone homeostasis (25, 26). These data showed that RANKL is responsible for LN growth and formation of small B cell follicles in the Rank−/− mouse.

**RANKL activates chemokine synthesis and proliferation of SLO stroma**
To further address the mechanism of LN growth, we first considered adult LTI-like cells as a possible RANKL target. They have been shown to express RANK, and their role in stroma activation could depend on RANK−RANKL interactions (27, 53). Moreover, their implication in SLO expansion was observed in the case of enhanced survival by ectopic IL-7 expression (54). We compared adult LTI-like lin−Rorγt−CD127−nag7+ mice of WT and Rank−/− mice in terms of phenotype, numbers, and frequency. Moreover, because RANK stimulates LToβ2 activation by LTI cells (28), we measured expression of LToβ2. 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 LToβ2 expression was noted (Fig. 6A, Supplemental Fig. 2F). We also measured LToβ2 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

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### Table II. Absolute cell numbers of hematopoietic cell types (10⁶)

<table>
<thead>
<tr>
<th>Animal</th>
<th>Organ</th>
<th>CD3⁺ T Cells</th>
<th>CD11b⁺ Macrophages</th>
<th>B220⁺ B Cells</th>
<th>Mature B IgD⁺IgM⁺</th>
<th>T2 B IgD⁺IgM⁺</th>
<th>T1/MZ B IgD⁺IgM⁺</th>
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</thead>
<tbody>
<tr>
<td>Rank-Tg</td>
<td>cLNs</td>
<td>53.9 ± 17.7</td>
<td>2.7 ± 1.2</td>
<td>65.4 ± 30</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>WT (n = 8)</td>
<td></td>
<td>8.5 ± 3.1</td>
<td>0.2 ± 0.06</td>
<td>5.6 ± 2.3</td>
<td></td>
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<tr>
<td>Rank-Tg</td>
<td>mLN</td>
<td>3.3 ± 1.4</td>
<td>0.08 ± 0.07</td>
<td>2.3 ± 1.3</td>
<td></td>
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<tr>
<td>WT (n = 8)</td>
<td></td>
<td>4.3 ± 1.1</td>
<td>0.07 ± 0.06</td>
<td>2.4 ± 0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rank-Tg</td>
<td>Spleen</td>
<td>16.4 ± 4.7</td>
<td>4.4 ± 2</td>
<td>36.1 ± 9.5</td>
<td>28 ± 12</td>
<td>2.6 ± 1.6</td>
<td>8.8 ± 4.5</td>
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<tr>
<td>WT (n = 8)</td>
<td></td>
<td>25.4 ± 6.1</td>
<td>3.3 ± 0.6</td>
<td>57 ± 13</td>
<td>39 ± 9</td>
<td>3.6 ± 0.6</td>
<td>7.4 ± 3.4</td>
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<tr>
<td>Rank-Tg</td>
<td>Blood (ml)</td>
<td>2.2 ± 1.3</td>
<td>1.4 ± 1.0</td>
<td>2.9 ± 2.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT (n = 8)</td>
<td></td>
<td>1.7 ± 0.7</td>
<td>1.0 ± 0.4</td>
<td>2.7 ± 1.5</td>
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</table>

Cell numbers (mean ± SD) of organs and blood cell suspensions were counted in a hemacytometer and analyzed for T cells, macrophages, B cells, and B cell subsets by flow cytometry. The total number was determined using the percentage composition of the different cell types.

aCLNs included inguinal, axillary, brachial, and superficial parotid; for B cells and macrophages, cell numbers were determined excluding brachial LNs.

T1, transitional type 1; T2, transitional type 2; MZ, marginal zone.
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

FIGURE 3. cLN immune cells are resting but respond rapidly to immunization. A. CD86 expression levels of skin- and blood-derived DCs, purified from brachial and axial LNs in nonimmunized WT and Rank-Tg mice, as well as from Rank-Tg mice 4 d after a s.c. immunization boost with CpG. Graph (left panel) shows the mean ± SD of three animals/genotype performed in duplicates. The flow cytometry dot blot (right panel) depicts the gate setting for I-A^d CD11c^c skin-derived DCs and for I-A^d CD11c^hi blood-derived DCs. B. Percentage of CD44^+ and CD62L^+ expressing Th cells in cLNs, mLNs, or spleen in WT, Rank-Tg, and Rank-Tg mice 4 d after a s.c. immunization boost with CpG (left panel). Data are mean ± SD of six to eight animals/genotype. The flow cytometry dot plots (right panel) depict a representative analysis of CD62L and CD44 expression of nonimmunized and CpG-immunized WT and Tg Th cells in cLNs, mLNs, or spleen in WT, Rank-Tg, and Rank-Tg mice 4 d after a s.c. immunization boost with CpG (left panel). Data are mean ± SD of six to eight animals/genotype. The flow cytometry dot plots (right panel) depict a representative analysis of CD62L and CD44 expression of nonimmunized and CpG-immunized WT and Tg Th cells from brachial LNs. C. CD86 and CD69 expression levels of B220^+ B cells in cLNs, mLNs, and spleen from WT and Rank-Tg mice. Data are mean ± SEM of five animals/group. D. Serum anti-OVA IgG levels in WT and Rank-Tg mice, measured after a s.c. immunization boost of OVA with LPS, CpG, or IFA. Each symbol represents one animal, and the horizontal lines show the mean values. E. High-affinity anti-NP IgG levels after s.c. immunization of NP30 conjugated to KLH with LPS. Serum anti-NP IgG was measured by ELISA for NP3-BSA or NP30-BSA, and the relative affinity was determined as the titer NP3/titer NP30. Data are mean ± SEM of seven mice/group. **p < 0.01, ***p < 0.001.

FIGURE 4. Tg RANK overexpression in HFs induces cLN hyperplasia. A. Analysis of cell numbers in cLNs (brachial, inguinal, and axial) after adoptive BM transfer. Data are from three mice/group compiled from seven different experiments. B. Relative cLN cell number increase in brachial and axial LNs, normalized to iLNs in nude mice after skin grafting. Data are from three mice/group compiled from three different experiments. C. cLN cell numbers in WT, Msx-2^Lox/Lox, Rank-Tg, and Rank-Tg;Msx-2^Lox/Lox mice. Data are from eight mice/group from four experiments. D. Representative β-galactosidase staining in a brachial LN (left panel) and skin (right panel) of Rank-Tg;Msx-2^Lox/Co mice. Arrowheads point to β-galactosidase enzymatic reactivity of HF matrix cells. Dashed line indicates the dermo–epidermal junction. Scale bar, 100 μm. Data are mean ± SD. *p < 0.05, ***p < 0.001.
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.

organization was preserved, with a notable increase in B cell follicles. RANK stimulation did not provoke an immune activation; no autoactivity 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 the immune cells of the Tg animals. 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.

that MRCs are implicated in de novo B cell follicle formation. In support of this is the loss of MRCs, as well as B cells, after RANKL neutralization.

The greatly expanded SLO size could have been the consequence of a dysfunctional hematopoietic cell compartment. However, T cells, B cells, and DCs showed no signs of activation, and no autoantigen reactivity could be detected. Moreover, T cells did not spontaneously proliferate in culture, as described in a different model of SLO hyperplasia (60), and transfer of cLN CD4⁺ T cells into Rag-2 KO mice did not result in abnormal expansion (data not shown). The cLN immune cells of the Rank-Tg animals were not anergic, because they responded to immunization. The immune response elicited from the Tg mice was even moderately superior to that of WT mice, and a more rapid Ab-affinity maturation was seen. The increased efficiency of the humoral response may reflect increased numbers of B and T cells, as well as more B cell follicles comprising MRCs and FDCs. Despite the relationship between RANKL and thymic epithelial cells (32–35), and although LN Aire appears to contribute to peripheral T cell tolerance (49, 61), we could not detect such an association between RANKL and Aire in cLN stroma of the Tg animals.

By performing BM transfer, skin transplantation, and genetic rescue experiments, we discovered that the SLO growth signal originated in the HF. This finding provides an explanation for why only skin-draining LNs were affected and is in accord with initiation of LN growth at the start of the HF anagen phase after P24. The growth plateau is likely due to loss of transgene expression in the HF as the mouse develops alopecia (37). It is interesting to note that the postnatal period during which skin-draining LN expansion surpasses body growth occurs during HF morphogenesis and the first anagen phase (39). This is suggestive of a naturally operating link between HFs and cLNs, an association that appears to be amplified in the Tg mouse. We considered the possibility that DCs mediate the HF–LN link; however, in analogy to dermatopathic lymphadenopathy (62), more numerous, long-lived, or

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FIGURE 6. RANKL activates stromal cell proliferation and chemokine/adhesion molecule expression. A, Left panel, Adult LTi-like cells (Lin−RORγt−α4β7−CD127+) were identified in LNs of WT and Rank-Tg mice at P24 and P42, and absolute numbers were determined. Middle panel, LTαβ2 mean fluorescence intensity of adult LTi-like cells. Right panel, Relative expression levels of LTαβ2 mRNA in FACS-sorted adult LTi-like cells and CD19+ B cells at P42 (n = 6 animals). B, RT-PCR analysis of Rank and GAPDH mRNA expression by stromal subsets from cLNs of WT and Rank-Tg mice, sorted by flow cytometry on the basis of podoplanin/CD31 expression. Skin cDNA served as positive control, and water served as negative control. Image is representative of four separate experiments with 15 pooled WT mice and 5 pooled Tg mice. C, mRNA expression of chemokines and adhesion molecules in flow cytometry-sorted stromal subsets from WT and Rank-Tg mice. Measurements were done by qRT-PCR and are depicted as the fold increase with respect to WT. Data are the mean ± SEM of four independent experiments performed in duplicate with 15 pooled WT mice and 5 pooled Rank-Tg mice. Data in C are mean ± SEM of four independent experiments performed in duplicate with 15 pooled WT mice and 5 pooled Rank-Tg mice. D, BrdU incorporation by cLN stromal subsets from WT, control Ig-administered Rank-Tg mice, and Rank-Tg mice treated with anti-RANKL blocking mAb. E, BrdU incorporation by cLN stromal subsets from WT, control Ig-administered Rank-Tg mice, and Rank-Tg mice treated with anti-RANKL blocking mAb. Data in D and E are mean ± SEM of six to nine mice per group, obtained by flow cytometry using podoplanin/CD31 markers to identify the subsets. **p < 0.01, ***p < 0.001.

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 during the anagen phase of the hair cycle. High levels of soluble RANKL released from skin prompted us to propose the concept that HF RANKL conveys LN growth. In fact, the level of HF RANKL is higher in the mutant animal, and the skin undergoes additional anagen phases, leading to a continuous RANKL release. The underlying reason for the decrease in HF RANKL is unclear. One possibility is the existence of a self-amplifying RANK–RANKL loop, similar to what was described for thymic epithelial cells (36). Another argument in favor of RANKL as mediator of SLO growth is that RANKL is a small globular protein, which can be expected to have unobstructed access to all stromal cells through their 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 FDC proliferation 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 Rankl 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 Rankl 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 LTαβ2 by adult LTi-like cells or B cells different. We also could 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 Rank 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 (66, 67, 70). In an artificial SLO-like cell system, high fluid pressure activates stromal cell chemokine synthesis (71). This invites speculation on the existence of an interlinked direct RANK action on endothelial cells and reticular fibroblasts. Importantly, LTβR and TNFR signaling are mandatory for FDC formation and maintenance (72, 73), and LTβR signaling contributes to SLO growth in response to viral infection (14). Also, CXC1L1 and MAI1C1-MI expression on BECs are increased upon LTβR engagement (9). The possibility that LTβR signaling plays a part in cln growth cannot be excluded, but we did not detect an upregulation of LTβR mRNA by FRCs (data not shown), nor could we find evidence for an increase in B cells and reticular fibroblasts. Importantly, LTβR signaling in the absence of LTβR cannot be activated by the finding that LTβR stimulates RANK and RANKL expression (36, 74). It would be of interest to test the effect of RANKL neutralization in a model of viral-induced SLO growth (14). Our results, together with the current understanding of RANK and LTβR actions, raise the question of whether RANK and LTβR functions are redundant. However, specificity in biological systems may be introduced by variations in spatial-temporal ligand availabilities. The production of RANKL by activated T cells (75, 76) invites the idea that, during an immune response, T cells sustain SLO expansion and contribute to tertiary lymphoid tissue by stimulating RANK. Conversely, CD40-stimulated B cells release OPG, which would keep RANKL in check (77). The findings presented in this article should prompt novel investigations into the role of T cell RANKL in regulating the quality and quantity of the immune response through stromal cells. The question of whether RANK-directed therapy can find novel applications in boosting the immune system or, on the contrary, in the treatment of inflammatory diseases, now also merits close examination. Our results support the idea that tissue-derived RANKL contributes to lymphoid tissue formation and growth by stimulating stromal cells, as well as highlight the importance of environmental cues for immune regulation (78).

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Disclosures
The authors have no conflicts of interest.

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