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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,§ Matthéo 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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ized B cell follicles (27, 29). Mice deficient in TRAP6, 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<sup>−/−</sup> 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) transfer, 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 administrated 2 wk later. Before and every 5 d thereafter, chicken OV A-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)<sub>3</sub>-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 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 NP<sub>3</sub>/titer NP<sub>30</sub> (43).

**Statistical analysis**

An unpaired two-tailed Student test was used to determine statistically significant differences. The p values < 0.05 were considered statistically significant.
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, axillary, 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). 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<sup>+</sup> granulo-myeloid lineage, B220<sup>+</sup> B cells, and CD3<sup>+</sup> T cells by flow cytometry in cLNs, mLNs, and spleen of Tg and control animals. There was a significant increase in CD11b<sup>+</sup> 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). In addition, there was no significant difference in cell numbers in the autoimmune-sensitive salivary glands (data not shown). In addition, there was no significant difference in cell numbers in the autoimmune-sensitive salivary glands (WT: 30 ± 12 × 10<sup>3</sup>, Tg: 35 ± 9 × 10<sup>3</sup>; n = 8). In accord with

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 BM of congeneric 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.

<table>
<thead>
<tr>
<th>Table I. Absolute cell numbers in organs (10⁶)</th>
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<tbody>
<tr>
<td>Animal</td>
</tr>
<tr>
<td>Rank-Tg (n = 11)</td>
</tr>
<tr>
<td>WT (n = 8)</td>
</tr>
<tr>
<td>p &lt; 0.001</td>
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</tbody>
</table>

Cell numbers (mean ± SD) of organ and blood cell suspensions from 12-wk-old Tg and WT animals were counted in a hemacytometer.
expression in HFs (Fig. 4D). In contrast, FDCs remained intact, as shown by labeling sections for MAdCAM-1 and VCAM-1 transcripts; and BECs expressed by stroma and play an important role in SLO growth formation (2, 4–6). First, we determined expression of LTα1 gene activity by RT-PCR on FACS-sorted FRCs, LECs, and BECs. 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 proportion was 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-Tg mouse.

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 proportion was 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-Tg 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−n487+ cells of WT and Rank-Tg mice in terms of phenotype, numbers, and frequency. Moreover, because RANK stimulates LTα1β2 expression by LTI cells (28), we measured expression of LTα1β2. 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 LTα1β2 expression was noted (Fig. 6A, Supplemental Fig. 2F). We also measured LTα1β2 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

### 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>
</tr>
</thead>
<tbody>
<tr>
<td>Rank-Tg (n = 11)</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>
</tr>
<tr>
<td>WT (n = 8)</td>
<td>cLNs</td>
<td>8.5 ± 3.1</td>
<td>0.2 ± 0.06</td>
<td>5.6 ± 2.3</td>
<td></td>
<td></td>
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<tr>
<td>Rank-Tg (n = 11)</td>
<td>mLNs</td>
<td>3.3 ± 1.4</td>
<td>0.08 ± 0.07</td>
<td>2.3 ± 1.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT (n = 8)</td>
<td>mLNs</td>
<td>4.3 ± 1.1</td>
<td>0.07 ± 0.06</td>
<td>2.4 ± 0.5</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Rank-Tg (n = 11)</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>
</tr>
<tr>
<td>WT (n = 8)</td>
<td>Spleen</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>
</tr>
<tr>
<td>Rank-Tg (n = 11)</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>Blood (ml)</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 T cell subsets by flow cytometry. The total number was determined using the percentage composition of the different cell types.

### Notes
- cLNs 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 un-
changed 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 pro-
liferated 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 rea-
sonable 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. 6
D
). In contrast, the
BrdU incorporation rate for hematopoietic cells was similar be-
tween WT and Tg animals, and there was no change after ad-
ministration 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. 6
E
). 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. 2
G
).
Thus, RANKL stimulates cLN growth by inducing the prolifera-
tion 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 che-
moskines and adhesion molecules, culminating in LN growth. LN
organization was preserved, with a notable increase in B cell follicles. RANKL 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 immune cells of the lymphoid lineage. Therefore, we studied a Rank-Tg 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 LCs and RANKL-regulated LCs mediate the HF–LN link; however, in analogy to dermopathic lymphadenopathy (62), more numerous, long-lived, or

**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.
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 FRC 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 administered 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 LTiβ2 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 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-

FIGURE 6. RANKL activates stromal cell proliferation and chemokine/adhesion molecule expression. A, Left panel, Adult LTi-like cells (Lin–RORγt–αβCD127+) were identified in LNs of WT and Rank-Tg mice at P24 and P42, and absolute numbers were determined. Middle panel, LTo,β2 mean fluorescence intensity of adult LTi-like cells. Right panel, Relative expression levels of LTo,β2 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 fold increase with respect to WT. Data are the 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 cLN 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 increase 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
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, CXCL13 and MAdCAM-1 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 LTα1β2 by adult LT-like cells or B cells. Elucidation of the precise roles of RANK and LTβR in SLO homeostasis is complicated 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 necessary, so that reduction of egress rates may contribute to LN hypoplasia.

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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 LTβR. Conversely, CD40-stimulated B cells release OPG, which would keep RANKL in check (14). 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 the peripheral tolerance paradigm.

Acknowledgments

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Disclosures

The authors have no conflicts of interest.

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