

# Expression pattern changes and function of RANKL during mouse lymph node microarchitecture development

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## Abstract

Receptor activator of nuclear factor kappa-B ligand (RANKL) expression was examined during the development of mouse fetal peripheral lymphoid organs. A shift in the expression pattern was detected during the transition from lymphoid tissue inducer (LTi) cells to lymphoid tissue organizer (LTo) cells in the lymph node (LN) anlagen but not in the Peyer's patch anlagen. In order to understand the functional impact of these changes in the fetal expression of RANKL, the RANKL function was blocked by a blocking antibody. Excess anti-RANKL antibody was administered to pregnant mice between 13.5 and 16.5 dpc and was found to completely block LN anlagen development, suggesting that RANKL function during this period is critical for LN development. In addition, small amounts of anti-RANKL antibodies were injected directly into the amniotic space at 13.5 dpc, resulting in perturbed B-cell follicle formation and high endothelial venule differentiation after birth. These results suggest that RANKL expression on LTi cells during the early phase of LN development is critical for the development LN microarchitecture.

Keywords: HEV, LTi cell, LTo cell, RANK, RANKL

## Introduction

Lymph nodes (LNs), part of the peripheral lymphoid organs (PLOs) distributed throughout the mammalian body, play an important role in immune reactions against foreign particles. Previous studies of LN development in knockout (KO) mice demonstrated that a few cell populations and molecules contribute to the development of these structures (1, 2). The pivotal players in LN development are the lymphoid tissue inducer (LTi) cells, which develop in the fetal liver and migrate to the LN anlagen region (3–5), and the lymphoid tissue organizer (LTo) cells, which develop in the LN anlagen and express adhesion molecules or chemokines involved in the organization of the basic microarchitecture of the LN (6). Development of LTo cells in PLO tissue has been confirmed by the presence of several adhesion molecules expressed in conjunction with lymphotoxin alpha (LT $\alpha$ )/lymphotoxin beta receptor (LT $\beta$ R) signaling; LT $\alpha$  and LT $\beta$ R are expressed on LTi cells and LTo cells, respectively. In fact, the majority of the molecules known to play indispensable roles in PLO

development have been reported to have some relationship with these two cell types or molecules (7).

Another receptor ligand pair has also been found to be required for LN development (8–10). The ligand is a tumor necrosis factor (TNF) family ligand, named receptor activator of nuclear factor kappa-B ligand (RANKL); it is also known as tumor necrosis factor ligand superfamily member 11 (TNFSF11), TNF-related activation-induced cytokine (TRANCE), osteoprotegerin ligand, and osteoclast differentiation factor. This ligand's corresponding receptor is RANK, and the signal transduction molecule is TRAF6. LTi cell migration to the LN anlagen has been observed in the absence of specific adhesion molecules expressed on LTo cells in TRAF6-KO mice LN anlagen. This observation led to the proposal that one of the important functions of this ligand-receptor signal transduction system was the induction of LT $\alpha$  on LTi cells in the LN anlagen at an appropriate time during ontogeny (8).

Additional studies have suggested that the induction of LT $\alpha$  on LTi cells is necessary, but insufficient, for the proper development of LN microarchitecture. Another distinct function of the RANKL/RANK signal might be needed because forced expression of LT $\alpha$  on LTi cells by IL-7 in TRAF6-KO mice could induce vascular cell adhesion molecule (VCAM)-1 expression in the LN anlagen, but the microarchitecture of the LNs in those animals remained perturbed. In addition, the RANKL/RANK signals were reported to have other distinct roles in the development of various immune system cells and other organs at various time points during ontogeny (9–11).

In this study, we monitored the expression of RANKL during LN and Peyer's patch (PP) development to estimate the roles of RANKL in PLO development. The function of RANKL was examined by administration of a blocking antibody to mouse fetuses or pregnant female mice. The expression of RANKL on LTi cells, LTo cells and other mesenchymal cells spatiotemporally regulated in the LN anlagen or PP anlagen, and the important function of RANKL in development of the LN anlagen and subsequent LN microarchitecture were clarified by using blocking antibodies.

## Methods

### *Mice*

C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan) and were maintained under specific pathogen-free condition in the vivarium RIKEN Research Center for Allergy and Immunology and Yokohama City University (Yokohama, Japan) until use in experiments. Female and male mice were mated overnight, and female mice with vaginal plugs were judged pregnant. Noon of the day when the vaginal plug was identified was calculated as 0.5 days post coitus (dpc). Excised tissues were fetal and neonatal intestine and kidney.

### *Antibodies*

For immunostaining, flow cytometry analyses, the following antibodies were used: anti-VCAM-1 (429), anti-IL-7R $\alpha$  (A7R34), anti-ICAM-1 (YN1/1.7.4), anti-RANKL (IK22/5) and anti-Rat IgG2a isotype control (eBR2a), were purchased from eBioscience. And anti-LPAM-1 (DATK32) was purchased from BD Pharmingen.

For injection, the following antibodies used; anti-mTRANCE neutralizing antibody and Normal Goat IgG were purchased from R&D systems. And anti-mouse RANKL antibody (IK22/5) used *in vivo* was prepared as described previously (12). Pregnant mice were treated with 0.02–0.04 mg g<sup>-1</sup> body weight (BW).

### *Flow cytometry*

Embryonic intestines, mesenteries and para-aortic region were harvested and dissociated by Accutase (Innovative Cell Technologies, Inc. San Diego, CA, USA) or Dispase (Gibco in invitrogen, Carlsbad, CA, USA). For flow cytometry analysis, cells were incubated with blocking solution [1% crystallizable fragment of Ig (Fc)/block (Affinity Purified anti-mouse CD16/32-blocks Fc binding, clone 93 eBioscience) and 10% Normal Mouse Serum in 2% FCS/PBS] for 15 min.

They were then incubated with the primary antibodies as identified above. They were washed and then incubated with Allophycocyanin-conjugated Streptavidin (eBioscience) for 15 min. They were washed and filtered through nylon mesh to remove large clusters. These cells were analyzed by FACSCalibur or FACSria11 (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

In the case of intracellular staining, molecules on cell surface were stained as previously noted and then these cells were incubated with Fixation/Permeabilization working solution (eBioscience) >30 min. After being washed with Permeabilization Buffer (eBioscience), they were incubated with blocking solution (1% Fc/block and 10% Normal Mouse Serum in Permeabilization Buffer) for 15 min. Without washing after blocking step, they were then incubated with the above antibodies. After being washed twice with permeabilization buffer, they were suspended in buffer solution and filtered through nylon mesh to remove large clusters. These cells were analyzed by FACSCalibur.

### *Fluorescent immunostaining*

The frozen sections of each embryonic tissue were exposed microwave for 10 s. And then, they were incubated with blocking solution (2% Serum and 0.5% BSA in PBS) at room temperature (RT) for 20 min and incubated with first antibodies or biotin-conjugated antibodies at 4°C for 1 h. After being washed three times with PBS, they were incubated with streptavidin Alexa Fluor 488 conjugate (sAV-488) (Molecular Probes in invitrogen, Carlsbad, CA, USA) and Cy<sup>TM</sup> 3-conjugated affinitypure Goat Anti-Rat IgG (Anti-Rat Cy3) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 1 h in the dark at 4°C. After being washed three times with PBS, they were incubated with Alexa 647 phalloidin (Molecular Probes in invitrogen) for 20 min in the dark at 4°C. After being washed with PBS, they were mounted with Aquatex (Merck, Germany) or performed counterstaining using VECTASHILD Mounting Medium with diaminodino-2-phenylindole (VECTOR Laboratories, Burlingame, CA, USA). And then, they were observed with a confocal microscope (Leica, Wetzlar, Germany).

RANKL expression in neonatal peripheral LN was detected by augmentation with TSA Fluorescein System (PerkinElmer, Inc. Waltham, MA, USA).

### *Hematoxylin-eosin staining*

The polyester wax embedded specimens were sliced by microtome and dewaxed by ethanol, washed with distilled water and then kept in hematoxylin for 20 min. After washing with tapping water and 60% ethanol, sliced sections were incubated in eosin solution for 10 min. Specimens were gradually dehydrated with ethanol and xylene and mounted with Entellan new (Merck, Germany).

### *Whole-mount immunostaining*

Harvested tissues were fixed in ice-cooled fixing solution (2% PFA in PBS) for 30 min and then washed with PBS for 10 min at 4°C. To block endogenous peroxidase activity, the fixed specimens were incubated with 0.3% hydrogen peroxide in methanol for 15 min and then rinsed in PBS three times for

10 min each. Specimens were at first incubated with PBS-MT (1% skim milk and 0.1% Triton X-100 in PBS) for 1 h at 4°C to block non-specific staining and then incubated with various antibodies solved in PBS-MT overnight at 4°C. After being washed four times in PBS-MT for 1–2 h at 4°C, primary antibodies were detected with HRP-conjugated Goat anti-Rat Ig's (BIOSOURCE, Carlsbad, CA, USA) antisera overnight. After being washed with PBS-MT at 4°C for 1 h 4 times each, with PBS-T at RT for 20 min, specimens were soaked in PBS-T containing 0.08% NiCl<sub>2</sub> and 0.025–0.03% diaminobenzidine (DAB; DojinChemical Co., Kumamoto, Japan) for 30 min, and hydrogen peroxide was added to 0.025%. The enzyme reaction was allowed to proceed until the desired color intensity was obtained, stopped and fixed by 4% PFA/PBS.

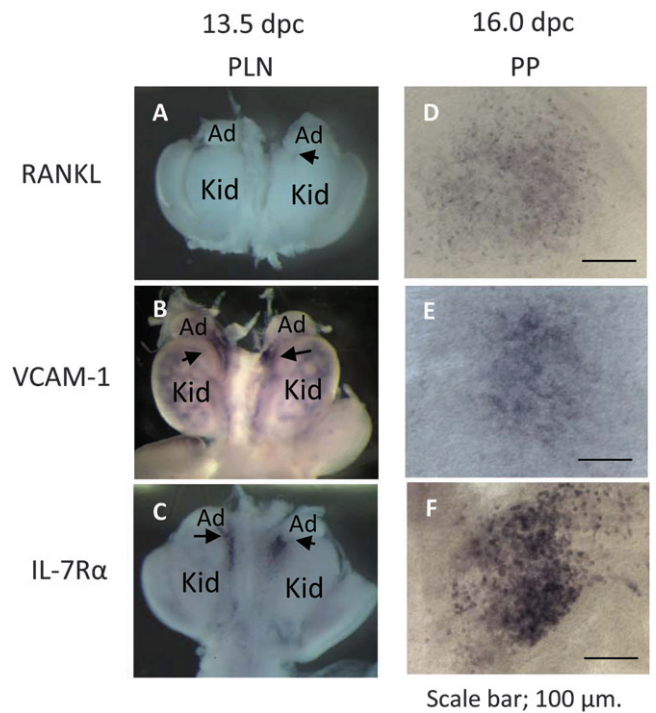
#### Real-time quantitative RT-PCR

Total RNA was purified using the RNeasy Mini kit (QIAGEN, Hilden, Germany). cDNA was synthesized from 500 ng of RNA using ReverTra Ace- $\alpha$ - (TOYOBO, Osaka, Japan). The PCR reaction consisted of 12.5  $\mu$ l of SYBR Premix Ex Taq (TAKARA, Shiga, Japan), 1  $\mu$ l of 1/3-diluted template cDNA and 10  $\mu$ M forward and reverse primers in a total volume of 25  $\mu$ l. Real-time reverse transcription (RT)-PCR analysis was performed on Thermal Cycler Dice Real-Time System TP800 (TAKARA). Cycling was performed the following: 10 s at 95.0°C, followed by 40 cycles of 5 s at 95.0°C and 30 s at 60.0°C. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as control. Sequences for each primer pair were following: VCAM-1, sense 5'-ACAGACAGTCCCTCAATGG-3', antisense 5'-TGAGTAGACCTCCACCTGGG-3'; IL-7R $\alpha$ , sense 5'-AATCAAGGAGGATGGGATCC-3', antisense 5'-TTCAGACTCGTTTTGGCTTC-3'; RANKL, sense 5'-TGTACTIONTCGAGCGCAGATG-3', antisense 5'-CCACAATGTGTTGCAGTTCC-3'; RANK, sense 5'-CCAGGAGAGGCATATGAGC-3', antisense 5'-TTGCCTGCATCACAGACTTT-3' and GAPDH, sense 5'-TGTGTCCGTCGTGGATCTGA-3', antisense 5'-TTGCCTGCATCACAGACTTT-3'.

## Results

#### RANKL expression during LN development in fetal mice

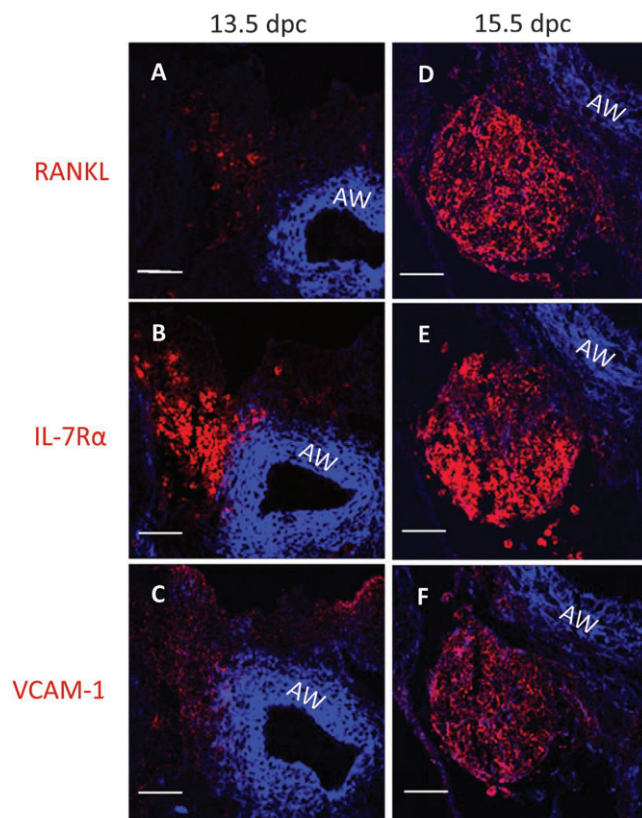
RANKL expression during LN development was precisely followed by analyzing RANKL protein expression through a combination of whole-mount immunostaining, sliced-section immunohistochemistry and flow cytometry. RANKL expression was first monitored in the retroperitoneal and gastrointestinal organs of mouse fetuses by whole-mount immunohistochemistry (Fig. 1). In combination with anti-RANKL antibody staining, the procedure also involved immunostaining for IL-7R $\alpha$  or VCAM-1, as markers of LTi or LTo cells, in those tissues (13). RANKL expression was confirmed as early as 13.5 dpc in the para-aortic mesenteric LNs (MLNs) (Fig. 1A). In contrast, the earliest RANKL expression in the PP anlagen was at 16.0–16.5 dpc (Fig. 1D). VCAM-1 (Fig. 1B and E) or IL-7R $\alpha$  (Fig. 1C and F) immunostaining indicated RANKL expression is found in the same region with LTo cells or LTi cells distributing. The appearance of RANKL immunoreactive cells detected in these whole-mount tissue was similar to IL-7R $\alpha$  immunoreactive cells rather than VCAM-1 immunoreactive cells in this stage (Fig. 1D–F).



**Fig. 1.** Whole-mount immunostaining of RANKL in developing PLO tissues. RANKL (A and D), VCAM-1 (B and E) and IL-7R $\alpha$  (C and F) expression was examined at 13.5 dpc para-aortic LN (PLN) (A–C) or 16.0 dpc PP (D–F) anlagen. Note the appearance of RANKL-expressing cells detected in PP anlagen seemed compatible with IL-7R $\alpha$  expressing cells. Kid: kidney, Ad: Adrenal gland. Scale bar; 100  $\mu$ m.

Sliced sections of the LNs were examined by fluorescent immunohistochemistry to identify the RANKL-expressing cell types. The first cells expressing RANKL in the LN anlagen were leukocyte-like cells, based on their phenotype; these cells appeared to be a part of LTi cells on the basis of their immune-positive staining for IL-7R $\alpha$  expression in serial sections (Fig. 2A and B). At first, these cells were localized around the LN anlagen (Fig. 2A), but they gradually migrated into the central region of the LN anlagen over the subsequent 24-h period (Fig. 2D). After the first wave of RANKL expression and migration of LTi cells from the surface to the interior of the LN anlagen (Fig. 2B and E), mesenchymal cells were also observed to express RANKL in the whole region of the LN anlagen from 15.5 dpc (Fig. 2D). Based on their appearance and their staining with anti-VCAM-1 antibody (Fig. 2C and F), these mesenchymal cells were estimated as LTo cells. The LTo cells, localized across the whole region of the LN anlagen at this stage, expressed VCAM-1; however, RANKL-expressing LTo like cells seemed gradually migrated from the periphery of the LN anlagen to the inside, as was described for the LTi cells (data not shown). By 15.5 dpc and later, it was not possible distinguish which type of cells (LTi, LTo or both) were expressing RANKL. Therefore, flow cytometric analyses of single-cell suspensions of the LN or PP anlagen were conducted on protease-digested tissues.

In accordance with the immunostaining observations, RANKL expression on LTi cells was first detected at 13.0–13.5



**Fig. 2.** Characterization of RANKL expressing cells by fluorescent immunohistochemistry of sliced section. Para-aortic LN anlagen at 13.5 dpc (A–C) and 15.5 dpc (D–F) were fluorescent immunostaining with anti-RANKL (A and D), anti-IL-7R $\alpha$  (B and E) and anti-VCAM-1 (C and F) antibodies, respectively. Images of each embryonic day are serial sections. Blues show F-actin. VW: vascular wall. Scale bar: 50  $\mu$ m.

dpc in the LN anlagen by flow cytometry (Fig. 3) and lasted until 15.5 dpc. However, expression of the ligand gradually decreased from 16.0 dpc and diminished from LTi cells at 16.5 dpc (Fig. 3A). In contrast to the reduction of RANKL on LTi cells, its expression on LTo cell cytoplasmic region was detectable from 14.5 dpc (Fig. 3C) and then surface expression was confirmed from 15.5 to 17.5 dpc (Fig. 3B). The expression of RANKL in this mesenchymal cell population was observed until the end of the fetal stage by both flow cytometry and immunohistochemistry.

Similar to the LN anlagen development, the first RANKL-expressing cells distributed in the PP anlagen were also leukocyte-like cells, and they were found in the anti-mesenteric serosal region of the PP anlagen from 16.0 dpc (Fig. 1D–F, data not shown). Again, these cells were confirmed to be LTi cells by sliced-section immunohistochemistry (Fig. 2) and flow cytometry (Fig. 3). In contrast to the migration of RANKL-expressing LTi cells to the inside of the LN anlagen, these RANKL<sup>+</sup> LTi cells resided on the PP serosal surface and disappeared after 16.5 dpc (Fig. 4A, data not shown). In addition, RANKL-expressing mesenchymal cells were not observed to localize in the PP anlagen until 17.5 dpc, as confirmed by flow cytometry analysis (Fig. 3B and C). These findings indicated that the behavior of LTo cells in the

LN and PP anlagen were completely different during the period reported to be important for the development of those anlagen.

The difference in the expression profiles was also confirmed at the mRNA level by RT-PCR. Expression of RANKL mRNA in the leukocyte population was detectable in both the LN and PP anlagen (Supplementary Figure 1 is available at *International Immunology Online*); however, only the mesenchymal population of the LN anlagen expressed RANKL mRNA.

#### *Perinatal region-specific mesenchymal cell RANKL expression*

RANKL expression was detected in the LN mesenchymal cells from the 15.5 dpc LN anlagen stage until birth. On the day of birth, however, RANKL expression became restricted to the subcapsular region mesenchymal cells, especially those under the sinus and connected to the afferent lymphatic vessels (Fig. 4A–C). In the PP anlagen, LTi like cells distributing in the PP anlagen transiently expressed RANKL for a half day from 16.5 to 17.0 dpc and then mesenchymal-like cells under the subepithelial dome, adjacent to the intestinal lumen, started to express RANKL, at 17.0–18.0 dpc (Fig. 4D and E).

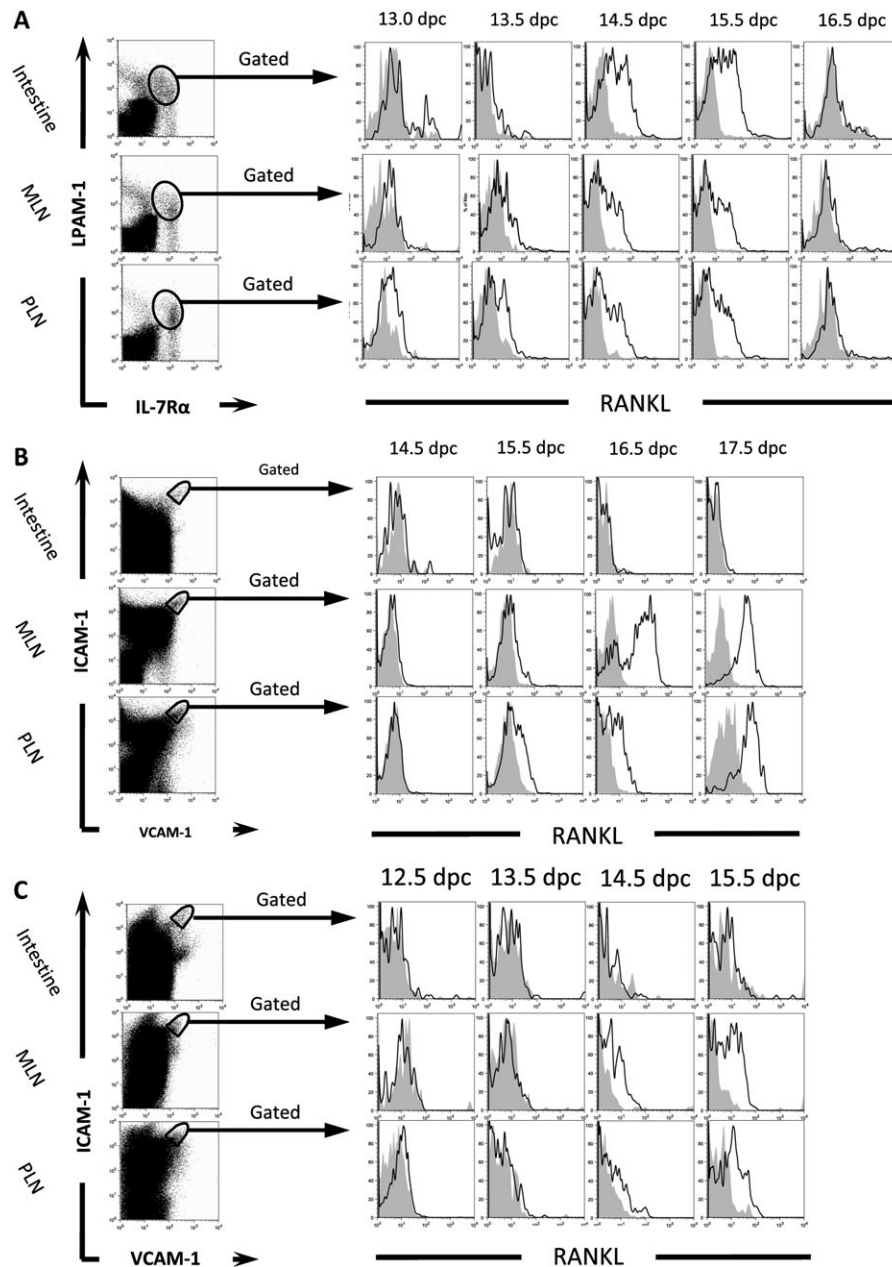
#### *Expression of RANK on endothelial cells in the developing LN anlagen at 13.5–14.0 dpc*

Although RANKL expression in fetal tissue was detected by commercially available anti-RANKL antibody in both immunohistochemical and flow cytometric analyses, commercially available anti-RANK antibody barely detected RANK expression, even on the LTi cells previously reported to express RANK. With augmented fluorescent-labeled antibody immunostaining, however, the endothelial cells surrounding the LN anlagen in 13.5–14.0 dpc fetuses were observed to express RANK but was decreased and almost diminished at 15.5 dpc (Fig. 5).

#### *Functional blockade of RANKL by anti-RANKL antibody*

The temporal changes in detectable RANKL expression mentioned above raised the question as to whether each step of the RANKL expression pattern in the fetal LN anlagen played an important role in the development of the LN anlagen. To detect the function of RANKL at distinct step of LN development, experiments focused on the 13.5 and 16.5 dpc fetuses, the time when only LTi or LTo cells expressed RANKL on their surfaces. Pregnant wild-type mice at 13.5 or 16.5 dpc were injected with 0.02–0.04 mg g<sup>-1</sup> BW of antibody against RANKL at each time point. This resulted in the total absence of LN development in the mesenteric or para-aortic regions (Fig. 6) indicating that RANKL expression at both time points is indispensable. However, an excess dose of antibody administered to the pregnant female may block the target molecule from functioning for a few days (14) and may not be appropriate for RANKL blockade experiments.

RANKL function was also blocked by intra-amniotic injection of the antibody. Since a small amount of antibody injected into amniotic fluid can distribute in the fetus within a few hours and be cleared within the subsequent 24 h

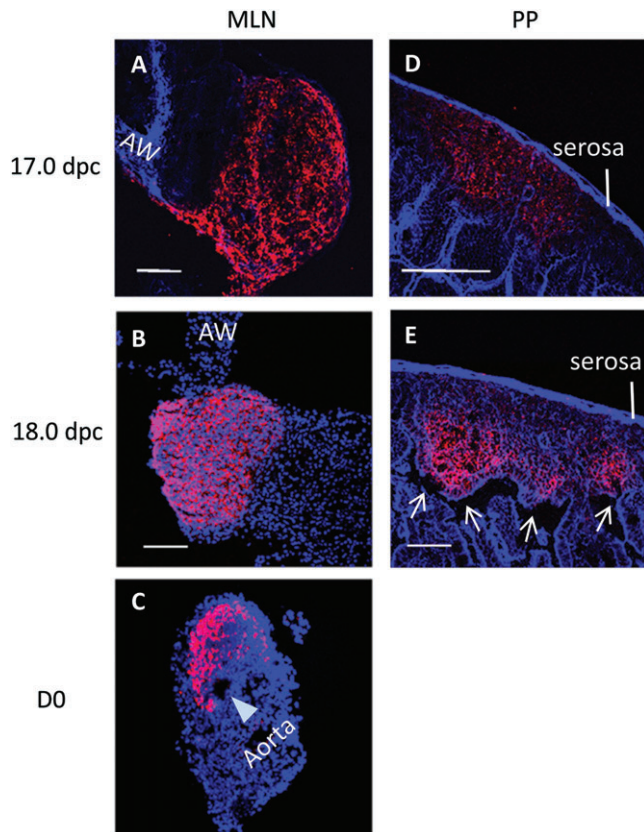


**Fig. 3.** Flowcytometry analysis of RANKL expression in PLO anlagen including tissues. LTi cells in intestine and LNs (MLN and para-aortic LN) were gated with IL-7R $\alpha$ <sup>+</sup>LPAM-1<sup>+</sup> (A). RANKL expression on LTi cells was detected from 13.0 and 13.5 dpc. LTo cells in LNs were gated with VCAM-1<sup>+</sup>ICAM-1<sup>+</sup> (B and C). RANKL expression on the surface LTo cells of LN was found from 15.5 dpc (B). And intracellular RANKL expression in LTo cells was discovered from 14.5 dpc (C). However, RANKL expression in LTo cells of intestine (B and C).

(15), antibody was injected every 24 h between 13.5 and 16.5 dpc. Antibody injection (4  $\mu$ g) at each embryonic stage did not block LN development after birth. RANKL-KO mice exhibited smaller LNs than those of class-matched antibody-injected embryos; however, expression of VCAM-1 (data not shown), a differentiation marker for LTo cells, was absent in the RANKL-KO mice but not disturbed in the antibody-injected embryos.

However, the anti-RANKL-treated pups demonstrated changes in the microarchitecture of their LNs. In wild-type mouse neonatal LN, compartmentalization of B-cell follicles

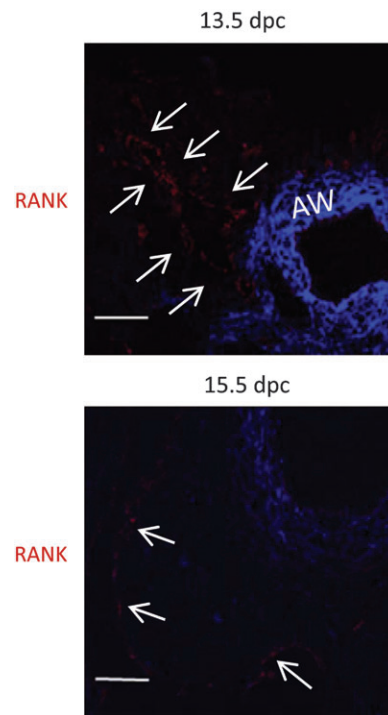
are developed before birth and B-cell follicles are easily detectable at 3 days after birth (16). Wild-type mouse LN (Fig. 7A–C) or control IgG injected mouse LN (Fig. 7D–F) showed B-cell follicle development. On the contrary, neonates that had been injected with antibody at 13.5 dpc displayed an absence of B-cell follicle formation, and all B cells were localized at the periphery of the LN (Fig. 7G–I). This suggested that B-cell follicle microarchitecture development was disturbed, leading to an examination of the expression of the follicular dendritic cell (FDC) markers in these LNs. FDC marker FDC-M1 positive cells were distributed to



**Fig. 4.** Expression pattern shift of RANKL in PLO at perinatal stage. RANKL-expressing mesenchymal cells (red) in mesenteric LN (MLN) were distributed in whole LN region at 17.0 and 18.0 dpc (A and B) but changed to be expressed only in a side of sinus capsule connecting to afferent lymph vessel at 0 days after birth (D0; C). RANKL expression in PP was transiently PP at 17.0 dpc (D) and then dramatically restricted to subepithelial dome region mesenchyme (arrow) at around 18.0 dpc (E). Blue; diamidino-2-phenylindole staining. VW: vascular wall. Scale bar; 100  $\mu$ m.

the same region with B-cell follicles in wild-type mouse LN (Fig. 7P and S); however in 13.5 dpc anti-RANKL antibody-treated mice LN, all FDC-M1 positive FDC like cells were localized at the periphery of the LNs (Fig. 7Q and T). In contrast, antibody treatment at 14.5 or 16.5 dpc had no effect on the distribution of B-cell follicles or FDCs (Fig. 7J–O, R and U), although B-cell numbers in follicles are decreased. These findings indicated that some other component of microarchitecture development or distribution was interrupted.

In order to determine if vasculature development in these LNs was disrupted, high endothelial venule (HEV) markers MAdCAM-1 and PNAd expression (17) in these tissues was examined. Around 4 days after birth, in wild-type mice neonatal LNs, some of the endothelial cells become thicker and express MAdCAM-1; only a small percentage of those MAdCAM-1-expressing cells also express PNAd and differentiate as fully matured HEVs (Fig. 8A–C). However, in RANKL-blocked animals, many of the endothelial cells in the LN showed thick appearance and were immunoreactive with both PNAd and MAdCAM-1, suggested many venules in MLN differentiated into matured HEVs (Fig. 8D–F).

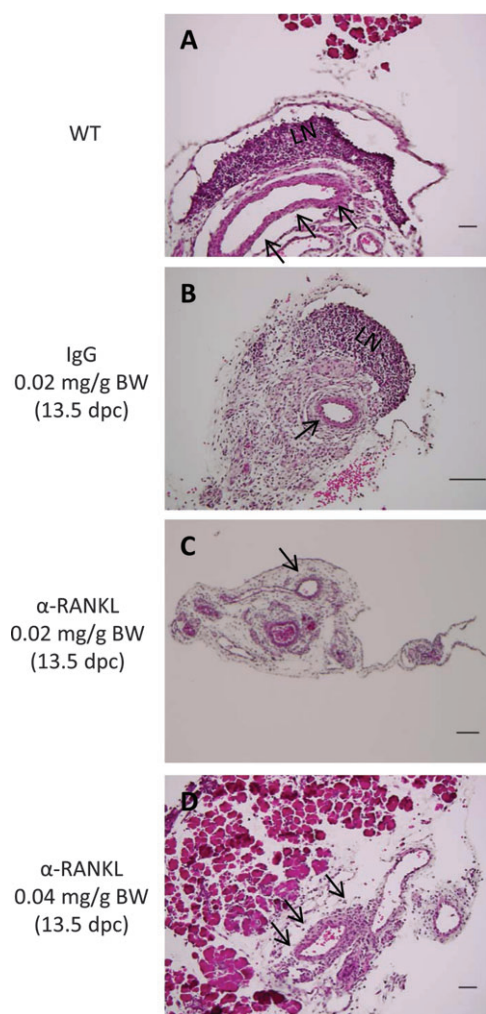


**Fig. 5.** RANK expression in endothelial cells LN anlage at 13.5 dpc. Para-aortic LN anlage at 13.5 dpc was immunostained with anti-RANK antibody. RANK was expressed in vascular endothelium and capsule of LN at 13.5 dpc (arrows). For specifying aortic wall (AW), F-actin was stained with Alexa 647 phalloidin (blue). Scale bar; 50  $\mu$ m.

From 14.5 to 16.5 dpc, anti-RANKL antibody injection also resulted in an increase in MAdCAM-1-expressing, thicker endothelial cells (Fig. 8G–I), but the incidence of PNAd expression was comparable to control specimens and B-cell follicle formation was not disturbed.

## Discussion

In the present study, the stepwise changes in RANKL expression patterns during LN and PP anlagen development were examined in detail, and the functions of those cells expressing RANKL were determined by means of a functional blockade caused by injection of blocking monoclonal antibodies. The first phase of RANKL expression in LNs is its expression on the LT<sub>i</sub> cells near the surface of the LN anlagen at 13.5 dpc. Since LT<sub>i</sub> cells are produced in the fetal liver and migrate to the presumptive LN anlagen region around 13.0 dpc (3, 18), these RANKL-expressing cells were deduced to be the first population to arrive in the developing LN. These first RANKL-expressing cells were not only detected in the LN anlagen but also in the developing PP anlagen. However, only in the LN anlagen did these LT<sub>i</sub> cells start to migrate toward the inside of the organ. It is difficult to distinguish whether an actual migration occurred or if there was only differentiation of the LT<sub>i</sub> cells that began from the outer surface of the anlagen and proceeded toward the interior. Regardless, the LT<sub>i</sub> cells behaved differently between the LN and PP and their arrival preceded the expression of RANKL on LT<sub>o</sub> cells that could not be detected in the LN anlagen until more than a day later. This indicates that the



**Fig. 6.** RANKL blockade effect with excess dose of antibody injection found in neonatal mesenteric tissue. Appearance of mesenteric LNs of a wild-type mouse neonate (A), control IgG antibody-injected mouse neonate:  $0.02 \text{ mg g}^{-1} \text{ BW}$  (B), anti-RANKL antibody-injected neonate;  $0.02 \text{ mg g}^{-1} \text{ BW}$  (C) and anti-RANKL antibody injected:  $0.04 \text{ mg g}^{-1} \text{ BW}$  (D). Antibodies were injected into peritoneal cavity at 13.5 dpc pregnant mice and the materials were harvested at the day 0. Arrows show aorta. Scale bar;  $100 \mu\text{m}$ .

site-specific effect on LTi cells is different between the LN and PP anlagen at this stage of development. Expression pattern shift of RANKL in early LN or PP anlagen are summed as a scheme in Fig. 9. Note the expression of RANKL on LTo cells is detectable only in LN anlagen. Vondenhoff *et al.* (18) also reported similar findings regarding RANKL expression differences between the LN and PP anlagen (19). They also reported the expression of RANKL only on LTo cells in the LN but not in the PP.

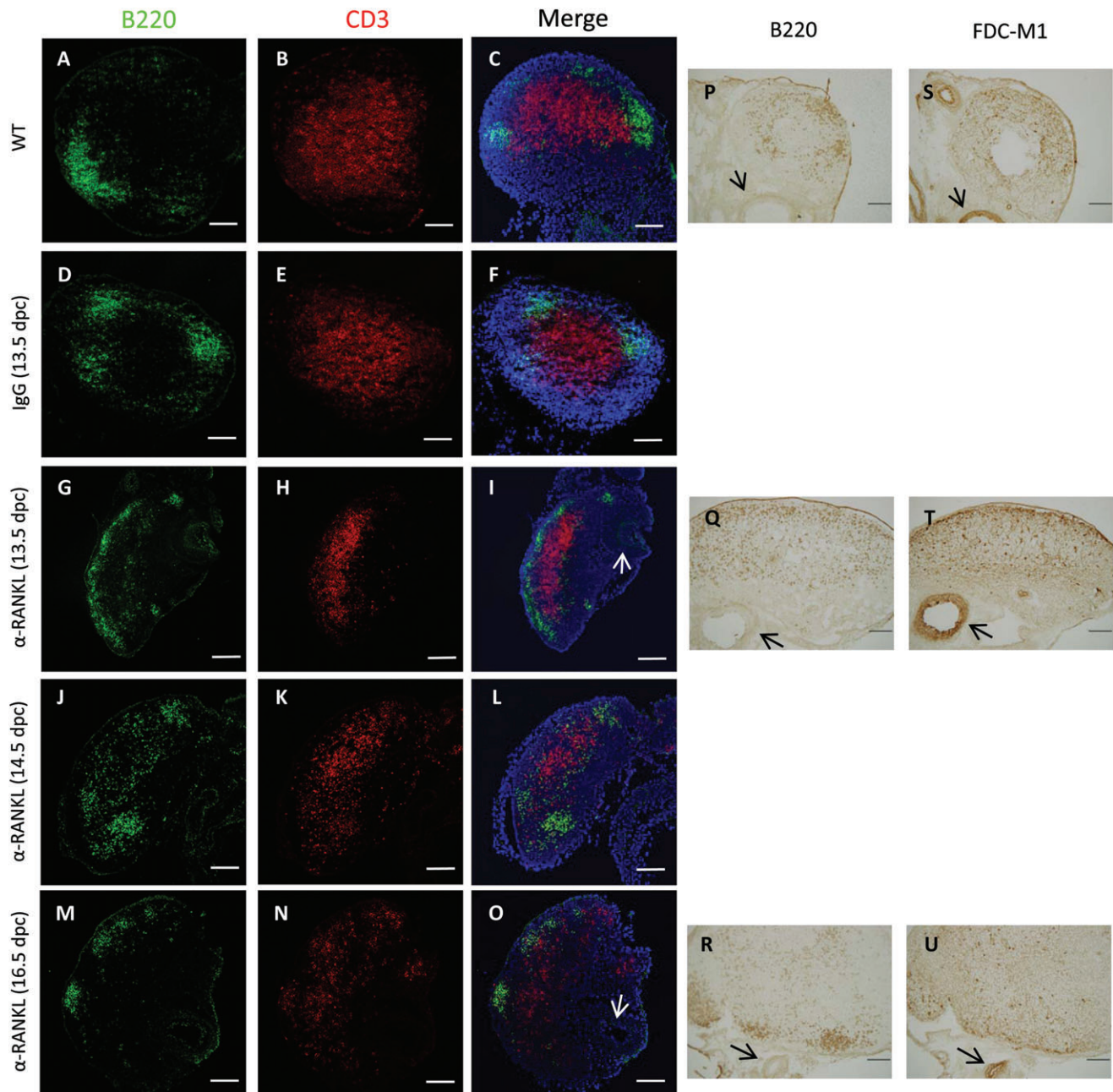
The functional blockade of RANKL expression by injection of excess monoclonal antibody into pregnant females at 13.5 and 16.5 dpc completely blocked formation of LNs. Since the LN defective phenotype obtained by this experiment matched that of RANKL/RANK/TRAF6-KOs (8, 20, 21), this stage must be when RANKL function is indispensable for LN development. These injections also completely blocked intensive VCAM-1 expression in the mesenteric and

para-aortic regions where the LN anlagen should develop (data not shown).

Since excess antibody injection into pregnant females may block the RANKL function for only a few days, it was considered an inappropriate method to examine the function of a molecule like RANKL, where the expression pattern changes within 24 h ((19), this study). Therefore, a small amount of antibody was injected directly into the amniotic fluid; the amount of antibody injected was controlled in an effort to not completely block  $\text{LT}\alpha$  expression in the LN anlagen. Blockade of  $\text{LT}\alpha$  expression would mask the effects of RANKL function because  $\text{LT}\alpha$  expression is critical in PLO development (3). The continued presence of  $\text{LT}\alpha$  was confirmed by quantitative RT-PCR analyses of  $\text{LT}\alpha$  mRNA expression in the LTi population (data not shown).

Methods such as this allow for the fine modification of function by treatment with a blocking antibody and are useful methods to explore the function of a molecule when that cannot be done using a KO mouse or excess dosing of a blocking antibody. For example, the phenotype of B-cell follicle formation disturbance detected with 13.5E antibody treatment, in this study, was similar to that detected in the LNs found in RANKL/RANK KO mice (9) or IL-7-treated LNs of TRAF6-KO mice (3). Previous studies also suggested that RANKL functioned in the formation of LN microarchitecture. Without the present study, however, it would be difficult to know the critical time for this RANKL function in B-cell follicle formation. At present, the precise mechanism of RANKL function in follicle formation is not clear, but this study lays the groundwork for future studies to determine this more precisely.

HEVs are specific PLO microarchitecture components that participate in trapping antigens and facilitating an appropriate immune reaction in these organs. In the MLNs of neonatal mice, the first sign of HEV development is increased endothelial cell height in some vessels and intensive MAdCAM-1 expression in those cells after birth (17). This is followed by PNA<sub>d</sub> expression in a proportion of those MAdCAM-1-expressing cells; the expression of these two adhesion molecules is thought to be a marker for the maturation of HEVs. Blockade of RANKL at 13.5 dpc perturbed this stepwise control of HEV development and induced most of the vessels in the MLN to develop an HEV-like appearance. This suggests that one of the roles of RANKL at this early stage of LN development is the (inhibitory) regulation of HEV development in the LN anlagen. It is interesting to note that the  $\text{LT}\alpha/\text{LT}\beta\text{R}$  signal was reported to be required for normal HEV development (22). Since both  $\text{LT}\alpha$  and RANKL are independently expressed in LTi cells at 13.5 dpc and RANKL expression is continuously maintained in LTo cells at a later stage ((19) and this study), the  $\text{LT}\alpha/\text{LT}\beta\text{R}$  and RANKL/RANK signals may be the positive and negative signals for HEV development in the LN anlagen. Induction of RANKL expression by  $\text{LT}\beta\text{R}$  stimulation (19) also suggested that the RANKL/RANK may be the negative regulator of the  $\text{LT}\alpha/\text{LT}\beta\text{R}$  signal during microarchitecture formation in the fetal LN. These results indicated RANKL/RANK signal transduction system have dual roles in LN anlagen development, first autocrine induction of  $\text{LT}\alpha1\beta2$  expression in LTi cells (3), second paracrine



**Fig. 7.** Microarchitecture development of neonatal LN in intra-amniotic anti-RANKL antibody-injected fetus. Mesenteric LN of wild type (A–C), control antibody injected at 13.5 dpc (D–F), anti-RANKL antibody injected at 13.5 dpc (G–I), 14.5 dpc (J–L) or 16.5 dpc (M–O) *in utero* were harvested at day 9 and immunostained to detect B cells by B220 antibody (left panels, green), T cells by CD3 antibody (second left panels, red) and nucleus was stained by diamidino-2-phenylindole in merged pictures (third left panels blue). Note the B-cell follicle is not formed in the specimen of anti-RANKL antibody injected at 13.5 dpc (G–I). Serial sections of a part of the same specimens were immunostained with anti-B-cell antibody (P–R) or anti-FDC antibody (S–U). Arrows show aortic wall. Scale bars; 200  $\mu\text{m}$  in A–O, 100  $\mu\text{m}$  in P–U.

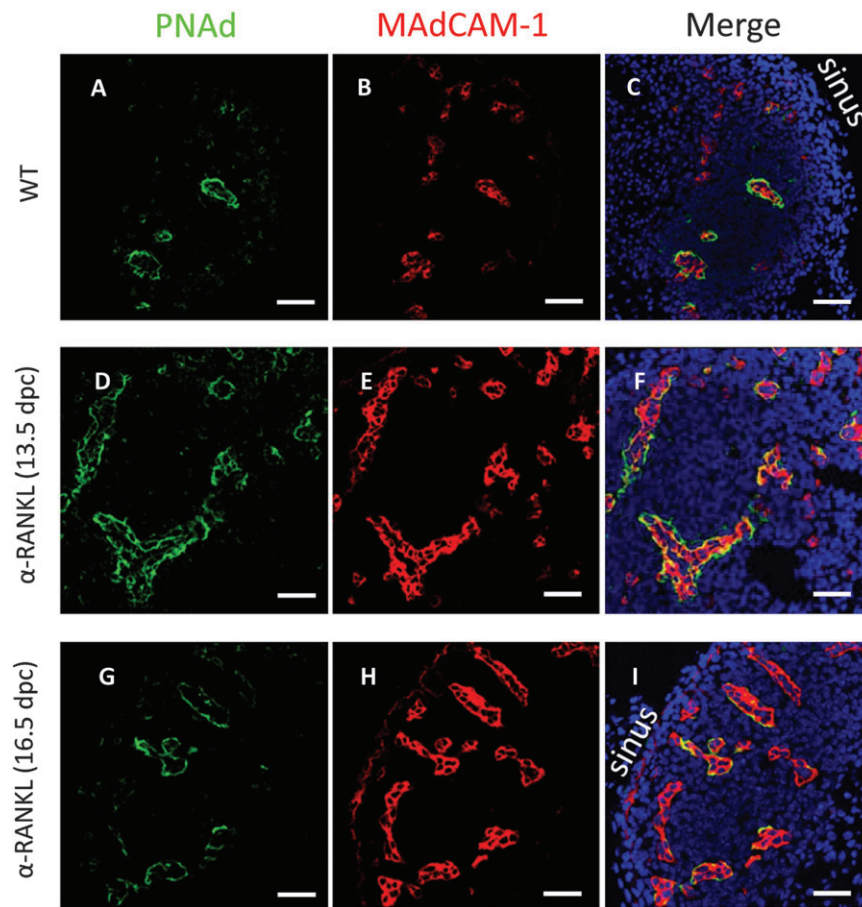
negative regulation of endothelial cell development against  $\text{LT}\alpha 1\beta 2$  function.

It has been reported that RANK, expressed on endothelial cells, is the direct target of RANKL in the adult stage (23). In the present study, endothelial cell-specific expression of RANK at 13.5 dpc in developing LNs was detected in the surrounding vessel endothelial cells, and those are the vessels where RANKL-expressing LTi cells were found during

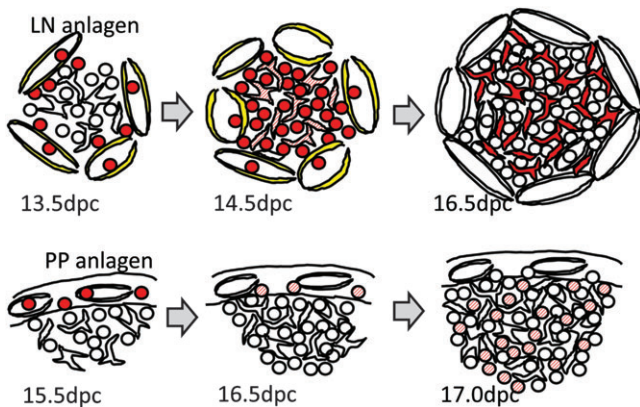
this stage. These findings suggest that RANKL, expressed by LTi cells, directly stimulates RANK-expressing endothelial cells around the LN anlagen to lead this population to become an LN-specific cell population. This possibility will be examined in greater detail once an *in vitro* HEV precursor cell differentiation system is established.

LTi and LTo cells are the sources of the RANKL molecule in developing LNs where this molecule was requisite for LN





**Fig. 8.** Perturbed development of HEV in intra-amniotic anti-RANKL antibody treated neonate LNs. Mesenteric LN of of wild type (A–C) and anti-RANKL antibody treated mice at 13.5 dpc (D–F) or 16.5 dpc (G–I) *in utero* were harvested at day 4 and immunostained by HEV markers PNAd (left panels) and MAdCAM-1 (second left panels), and merged views are shown in right panels. Note the PNAd immunoreactive HEV numbers are increased in 13.5 dpc-treated mice specimen. Blue shows diamidino-2-phenylindole. Scale bar; 50  $\mu$ m.



**Fig. 9.** Scheme of RANKL expression shift in early LN and PP development. Expression pattern change of RANKL in LN or PP is summed as a scheme showing LTI cells (circle), LTo cells (dendritic or bipolar cells) and endothelial cells (elliptical shape). Red color indicates RANKL expression detected by immunohistochemistry but not by flow cytometry and yellow color indicates RANKL expression in endothelial cells. Note the expression of RANK on LTI cells is neglected for comprehensibility.

formation and microarchitecture development. After this stage, the RANKL expression shifted to specific mesenchymal cells in these organs. Specifically, the PP dome region subepithelial space mesenchymal cells and the LN mesenchymal cells just beneath the subcapsular space, with connection to the afferent lymphatics, are the regions that become RANKL expressors during this stage. In LN tissue, this specific expression shift was found just after birth and persisted through to adulthood; in the PP, this specific RANKL expression was detectable at 17.5 dpc when the fetal gut epithelial cells underwent the first wave of apoptosis and meconium became detectable in the intestinal lumen and persisted through the adult stage. These findings indicate that RANKL expression, in a subset of mesenchymal cells in a specific region of the PLO, prepares the organs for mounting an immune reaction against antigens coming into these organs. Close observations of the expression patterns of various markers throughout development allow for the interpretation of expression patterns that have already been described (20).

In summary, we have described changes in the expression pattern of RANKL in the LN anlagen and detected its function in B-cell follicle formation and in the control of HEV

differentiation. The importance of various molecules in the formation of the PLO was previously described using genetically manipulated mice and analysis of the resultant phenotypes. However, analysis of the function of these molecules during organ development by limited-term functional blockades should be utilized to understand the precise function of those molecules.

### Supplementary data

Supplementary data are available at *International Immunology Online*.

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### References

- Nishikawa, S., Honda, K., Vieira, P. and Yoshida, H. 2003. Organogenesis of peripheral lymphoid organs. *Immunol. Rev.* 195:72.
- Mebius, R. E. 2003. Organogenesis of lymphoid tissues. *Nat. Rev. Immunol.* 3:292.
- Yoshida, H., Naito, A., Inoue, J. *et al.* 2002. Different cytokines induce surface lymphotoxin- $\alpha$  on IL-7 receptor- $\alpha$  cells that differentially engender lymph nodes and Peyer's patches. *Immunity* 17:823.
- Yoshida, H., Honda, K., Shinkura, R. *et al.* 1999. IL-7 receptor  $\alpha^+$  CD3 $^-$  cells in the embryonic intestine induces the organizing center of Peyer's patches. *Int. Immunol.* 11:643.
- Mebius, R. E., Schadee-Eestermans, I. L. and Weissman, I. L. 1998. MAdCAM-1 dependent colonization of developing lymph nodes involves a unique subset of CD4 $^+$ CD3 $^-$  hematolymphoid cells. *Cell Adhes. Commun.* 6:97.
- Honda, K., Nakano, H., Yoshida, H. *et al.* 2001. Molecular basis for hematopoietic/mesenchymal interaction during initiation of Peyer's patch organogenesis. *J. Exp. Med.* 193:621.
- Benezech, C., White, A., Mader, E. *et al.* 2010. Ontogeny of stromal organizer cells during lymph node development. *J. Immunol.* 184:4521.
- Naito, A., Yoshida, H., Nishioka, E. *et al.* 2002. TRAF6-deficient mice display hypohidrotic ectodermal dysplasia. *Proc. Natl Acad. Sci. USA* 99:8766.
- Leibbrandt, A. and Penninger, J. M. 2008. RANK/RANKL: regulators of immune responses and bone physiology. *Ann. N. Y. Acad. Sci.* 1143:123.
- Terpos, E., Efstathiou, E., Christoulas, D., Roussou, M., Katodritou, E. and Dimopoulos, M. A. 2009. RANKL inhibition: clinical implications for the management of patients with multiple myeloma and solid tumors with bone metastases. *Expert Opin. Biol. Ther.* 9:465.
- Silva, I. and Branco, J. C. 2011. Rank/Rankl/opg: literature review. *Acta Reumatol. Port.* 36:209.
- Kamijo, S., Nakajima, A., Ikeda, K. *et al.* 2006. Amelioration of bone loss in collagen-induced arthritis by neutralizing anti-RANKL monoclonal antibody. *Biochem. Biophys. Res. Commun.* 347:124.
- Adachi, S., Yoshida, H., Kataoka, H. and Nishikawa, S. 1997. Three distinctive steps in Peyer's patch formation of murine embryo. *Int. Immunol.* 9:507.
- Nishikawa, S., Kusakabe, M., Yoshinaga, K. *et al.* 1991. *In utero* manipulation of coat color formation by a monoclonal anti-c-kit antibody: two distinct waves of c-kit-dependency during melanocyte development. *EMBO J.* 10:2111.
- Yoshida, H., Nishikawa, S., Okamura, H., Sakakura, T. and Kusakabe, M. 1993. The role of c-kit proto-oncogene during melanocyte development in mouse. In vivo approach by the *in utero* microinjection of anti-c-kit antibody. *Dev. Growth Differ.* 35:209.
- Hashi, H., Yoshida, H., Honda, K. *et al.* 2001. Compartmentalization of Peyer's patch anlagen before lymphocyte entry. *J. Immunol.* 166:3702.
- Mebius, R. E., Watson, S. and Kraal, G. 1993. High endothelial venules: regulation of activity and specificity. *Behring Inst. Mitt.* 92:8.
- Yoshida, H., Kawamoto, H., Santee, S. M. *et al.* 2001. Expression of  $\alpha(4)\beta(7)$  integrin defines a distinct pathway of lymphoid progenitors committed to T cells, fetal intestinal lymphotoxin producer, NK, and dendritic cells. *J. Immunol.* 167:2511.
- Vondenhoff, M. F., Greuter, M., Goverse, G. *et al.* 2009. LT $\beta$ R signaling induces cytokine expression and up-regulates lymphangiogenic factors in lymph node anlagen. *J. Immunol.* 182:5439.
- Kong, Y. Y., Yoshida, H., Sarosi, I. *et al.* 1999. OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature* 397:315.
- Li, J., Sarosi, I., Yan, X. Q. *et al.* 2000. RANK is the intrinsic hematopoietic cell surface receptor that controls osteoclastogenesis and regulation of bone mass and calcium metabolism. *Proc. Natl Acad. Sci. USA* 97:1566.
- Browning, J. L., Allaire, N., Ngam-Ek, A. *et al.* 2005. Lymphotoxin- $\beta$  receptor signaling is required for the homeostatic control of HEV differentiation and function. *Immunity* 23:539.
- McGonigle, J. S., Giachelli, C. M. and Scatena, M. 2009. Osteoprotegerin and RANKL differentially regulate angiogenesis and endothelial cell function. *Angiogenesis* 12:35.