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RANKL Is Necessary and Sufficient to Initiate Development of Antigen-Sampling M Cells in the Intestinal Epithelium^1

Kathryn A. Knoop,* Nachiket Kumar,* Betsy R. Butler,* Senthilkumar K. Sakthivel,* Rebekah T. Taylor,* Tomonori Nochi,† Hisaya Akiba,‡ Hideo Yagita,‡ Hiroshi Kiyono,† and Ifor R. Williams2*

Microfold cells (M cells) are specialized epithelial cells situated over Peyer’s patches (PP) and other organized mucosal lymphoid tissues that transport commensal bacteria and other particulate Ags into intraepithelial pockets accessed by APCs. The TNF superfamily member receptor activator of NF-κB ligand (RANKL) is selectively expressed by subepithelial stromal cells in PP domes. We found that RANKL null mice have <2% of wild-type levels of PP M cells and markedly diminished uptake of 200 nm diameter fluorescent beads. Ab-mediated neutralization of RANKL in adult wild-type mice also eliminated most PP M cells. The M cell deficit in RANKL null mice was corrected by systemic administration of exogenous RANKL. Treatment with RANKL also induced the differentiation of villous M cells on all small intestinal villi with the capacity for avid uptake of *Salmonella* and *Yersinia* organisms and fluorescent beads. The RANK receptor for RANKL is expressed by epithelial cells throughout the small intestine. We conclude that availability of RANKL is the critical factor controlling the differentiation of M cells from RANK-expressing intestinal epithelial precursor cells. The Journal of Immunology, 2009, 183: 5738–5747.

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3 Abbreviations used in this paper: FAE, follicle-associated epithelium; PP, Peyer’s patch; ILF, isolated lymphoid follicle; M cell, microfold cell; DC, dendritic cell; RANK, receptor activator of NF-κB; RANKL, RANK ligand; mTEC, medullary thymic epithelial cell; GST, glutathione S-transferase; UEA-I, *Ulex europaeus* agglutinin-I (UEA-I) lectin recognizing α(1,2)-fucose, stubby microvilli, and the capacity to ingest and transcytose particles the size of bacteria (9).

Although the basic functional and ultrastructural features of M cells were initially described over 30 years ago (10), many basic questions about M cell differentiation and function remain unsolved. It has been proposed that specific factors released from the lymphoid microenvironment immediately beneath the FAE have the potential to elicit M cell differentiation in the FAE and promote the function of M cells, but specific signaling mediators with such activity have not been identified to date (11, 12). Debate continues on whether M cells are a distinct lineage arising from crypt stem cells like other differentiated intestinal epithelial cells or whether M cells can instead arise from normal FAE enterocytes with the plasticity to transition into M cells upon encountering the right set of stimuli (13–15).

RANKL (receptor activator of NF-κB ligand) is a member of the TNF superfamily (16) that is also referred to as TNF-related activation-induced cytokine and TNFSF11. Like TNF-α, RANKL is initially synthesized as a transmembrane protein that can be released from the cell surface following cleavage by one of several metalloproteases (17, 18). RANKL signals through its receptor RANK (receptor activator of NF-κB) and a downstream pathway that involves TRAF6 and the activation of NF-κB (19, 20). Osteoprotegerin is a soluble decoy receptor for RANKL that allows for tight regulation of the circulating levels of RANKL (21). A
major breakthrough in establishing a biological role for RANKL-RANK interactions was the discovery that RANKL signaling through RANK is required for normal osteoclast function (22, 23). Mice deficient in either RANKL or RANK have osteopetrosis and severe skeletal abnormalities because they lack the number of osteoclasts needed to remodel bone normally. RANKL-RANK signaling is also involved in several other critical biological processes including development of lymph nodes, development of medullary thymic epithelial cells (mTEC), mammary gland lactation, and provision of survival signals to DCs (22–27). The absence of all lymph nodes in RANKL-deficient mice demonstrates that RANKL is an essential mediator in lymphoid organogenesis (22, 23). RANKL induces lymphoxygenase αβ expression by lymphoid tissue inducer cells in the lymph node anlage (28). RANKL is not required for PP development, but the reduced size of PP reported in two independent lines of RANKL-deficient mice indicates that RANKL contributes to normal PP development (22, 23). Functional studies of PP were not done as part of the initial characterization of these mice.

We previously showed that RANKL is selectively expressed by stromal cells in the subepithelial dome region beneath the FAE of both PP and ILF (29). Stromal cells with phenotypic characteristics similar to neonatal lymph node organizer cells including RANKL expression were recently identified in multiple secondary lymphoid tissues including mucosal-associated lymphoid tissues and lymph nodes (30). The polarized pattern of RANKL expression by stromal cells beneath the FAE of PP and ILF suggested a possible role of RANKL in regulating the induction of mucosal immune responses to particulate luminal Ags taken up through the FAE. In this study, we evaluated the function of PP in RANKL null mice and found that absence of RANKL is associated with loss of the vast majority of UEA-1+ M cells in the FAE. The depletion of M cells correlated with a profound functional defect in FAE following the manufacturer's suggestions.

### Materials and Methods

#### Mouse

Mice carrying a RANKL null mutation on a C57BL/6 background (31) obtained from Dr. Yongwon Choi at the University of Pennsylvania (Philadelphia, PA) were used to establish a breeding colony in a conventional specific pathogen free mouse facility at Emory University. Serological sentinel testing in this facility did not include routine testing for Helicobacter or Pasteurella spp. but no infections attributed to these opportunistic pathogens were demonstrated in the colony of RANKL null mice or their controls. Because RANKL null mice lack teeth, weaning null mice born in this colony are routinely given powdered mouse chow. Mice heterozygous for the RANKL null mutation were also backcrossed to BALB/c mice (Taconic Farms) for a total of four generations. Male C57BL/6 RANKL+/− mice and female BALB/c RANKL+/− mice were intercrossed to produce RANKL null mice and littermate controls on a background roughly equivalent to C57BL/6 × BALB/c F1 mice. RANKL null mice on this F1 equivalent genetic background are closer in weight to their heterozygous and wild-type mice. These findings demonstrate that the RANKL-RANK pathway plays a pivotal non-redundant role in establishing the M cell-mediated pathway of Ag acquisition and handling.

#### Bacterial strains

A wild-type strain of Salmonella enterica serovar Typhimurium (SL3201) transformed with the DiRed-Express plasmid (Clontech) encoding a cytoplasmic red fluorescent protein was provided by Dr. Andrew Neish at Emory University (Atlanta, GA). A Yersinia enterocolitica isolate (ATCC 29913) was purchased from the American Type Culture Collection. For experiments involving injection of bacteria into isolated intestinal loops, these bacteria were grown overnight in LB broth, washed in PBS, and fixed in 2% parformaldehyde for 1 h. The fixed Yersinia were then labeled with Alexa 546-succidymyl ester (Molecular Probes) for 1 h at room temperature following the manufacturer’s suggestions.

#### In vivo assessment of M cell uptake of fluorescent beads and bacteria

The uptake of 200-nm diameter fluorescent polystyrene latex nanoparticles (FluoSpheres YT; Polysciences) and fluorescent bacteria by M cells in the FAE of individual PP or by villous M cells induced by exogenous RANKL treatment was assessed by either oral gavage or by using a modification of previously described isolated small intestinal loop models (33, 34). In oral gavage experiments examining uptake of the by RANKL-induced villous M cells, aliquots of 1 × 10^11 200 nm diameter nanoparticles in a volume of 200 μl were fed to the mice. To prepare isolated small intestinal loops, mice were anesthetized using an isoflurane vaporizer. After opening the peritoneum through a longitudinal midline incision, two or three intestinal sections of small intestine measuring 2–5 cm in length and containing either a single PP (to assess PP M cell uptake) or no PP (to assess villous M cell uptake) were tied off with nylon filament. For bead uptake studies, the loops were injected with 200–400 μl of a suspension of 200 nm nanoparticles diluted in PBS to a concentration of 1 × 10^11 beads/ml and returned to the peri-toneal cavity. The mice were euthanized 90 to 120 min after the loops were injected and the injected intestinal segments were washed in 0.5% Tween 20-PBS, fixed in 4% paraformaldehyde in PBS for 15 min, and embedded in OCT. Frozen sections cut from these intestinal segments were examined by microscopy after counterstaining with 4′,6-diamidino-2-phenylindole (DAPI), leaving out a cold acetone fixation step because acetone dissolved the polystyrene FluoSpheres beads, preventing their visualization. For bacterial uptake studies, loops containing no PP were injected with 300 to 500 μl of bacterial suspension at a concentration of 5 × 10^9 organisms/ml. After 120 min, the mice were euthanized and the intestine tissue within the loop was embedded in OCT as a Swiss roll. Frozen sections from this tissue were fixed with −20°C acetone since this fixation did not interfere with detection of the fluorescent bacteria.

#### Abs and lectins

mAbs for staining were purchased from eBioscience, unless otherwise stated. The mAbs used for immunofluorescent staining of frozen sections were anti-RANKL (IK22-5), anti-RANK (LOB14-8; GeneTex), PE-conjugated anti-B220 (RA3-6B2), biotinylated GL7 (for detection of activated germinal center B cells), and alphalophocytocin-conjugated anti-RANKL IgG (Taconic). The detection of RANKL expression by exogenous RANKL in wild-type mice and the effects of anti-RANKL mAb on PP M cells. All animal studies were reviewed and approved by the Emory University Institutional Animal Care and Use Committee.
anti-Thy-1.2 (53-2.1; BD Biosciences). The rat mAb NK6 16–2–4 specific for mouse M cells was purified from hybridoma supernatant and labeled with FITC (35). A purified rat IgG2a isotype control mAb (BD Biosciences) was used as a control for staining of frozen tissue sections with the rat IgG2a anti-RANKL and anti-RANK mAbs. Biotinylated polyclonal goat anti-rat IgG (BD Biosciences) was used as a secondary reagent for detection of most unconjugated rat primary mAb. Rhodamine-AE was purchased from Vector Laboratories. The anti-RANKL Ab (IK22-S5) used for in vivo RANKL neutralization experiments was prepared as described previously (36). Mice were treated with 250 µg of IK22–5 or a purified functional grade control rat IgG2a mAb (eBioscience) i.p. every 2 days.

Immunofluorescence staining of frozen sections

Frozen sections of PP and adjacent intestinal tissue were cut on a cryostat and prepared for Ab-staining experiments as previously described (29). The sections were washed in PBS and blocked in TNB buffer (PerkinElmer Life Sciences). Abs diluted in TNB buffer were applied for 1 h at room temperature or overnight at 4°C. Biotinylated primary mAbs were detected using streptavidin-conjugated peroxidase followed by FITC-tyramide from the tyramide signal amplification kit (PerkinElmer Life Sciences). Unconjugated primary rat mAbs were detected by a combination of biotinylated streptavidin-conjugated peroxidase followed by FITC-tyramide from the tyramide signal amplification kit (PerkinElmer Life Sciences). Abs diluted in TNB buffer were applied for 1 h at room temperature or overnight at 4°C. Biotinylated primary mAbs were detected using streptavidin-conjugated peroxidase followed by FITC-tyramide from the tyramide signal amplification kit (PerkinElmer Life Sciences). Unconjugated primary rat mAbs were detected by a combination of biotinylated streptavidin-conjugated peroxidase followed by FITC-tyramide from the tyramide signal amplification kit (PerkinElmer Life Sciences).

Electron microscopy

Mice were perfusion fixed using 2.5% glutaraldehyde solution in 0.1 M cacodylate buffer. For transmission electron microscopy, individual PP were isolated, bisected through the center of the domes, and embedded in Epon resin. Thin sections from the PP domes of control and RANKL null mice were examined using a JEOL JEM-1210 microscope. For scanning electron microscopy, small intestinal villi were subjected to critical point drying, sputter coated with gold, and examined on a Topcon DS-130F field emission scanning electron microscope.

Whole mount staining of small intestine tissue for detection of UEA-1+ M cells

For detection of M cells in PP, individual PP were excised and vortexed mixed in 0.5% Tween 20-PBS followed by a shaking incubation with 100 µg/ml DNase for 20 min at 37°C to promote dissociation of mucus from the epithelial layer. The PP were blocked with TNB buffer for 15 min at 4°C, and stained with rhodamine-UEA-1 in TNB for 40 min at 4°C. Each stained PP was mounted under a 20 mm × 20 mm coverslip in 100 µl PBS. A count of UEA-1+ M cells was done for the PP follicle with the most M cells. This method resulted in some degree of underestimation of the full extent of M cell depletion in RANKL null mice because often only one of several PP follicles had any M cells in the mutant mice, while all the follicles in wild-type PP typically had a comparable number of M cells. To examine small intestine tissue for the presence of villous M cells, thin strips of tissue were cut and stained with rhodamine UEA-1 as described above for PP. Villi with M cells on their surface were classified as showing a dense or diffuse pattern of villous M cells using criteria based on the initial description of these patterns by Jang et al. (9). Specifically, villi with one or more clusters of M cells in which 75% or more of the area within the cluster was occupied with M cells were considered to have a dense distribution of villous M cells. Villi with at least one characteristic UEA-1+ M cell on the surface, but not meeting the dense distribution criteria, were considered to have a diffuse distribution.

Quantitative analysis of fluorescent bead and bacteria uptake by M cells

Analysis of the degree of bead uptake from loops containing PP was done by threshold analysis using ImageJ v1.36b software (http://rsb.info.nih.gov/ij/). Images of the fluorescent beads found within sections of individual PP follicles were saved as 8-bit grayscale images and then converted to binary images showing the beads by thresholding at a grayscale cutoff point of 75 of 255. The percentage of the pixels with a signal intensity that exceeded this cutoff was calculated for the area occupied by each PP follicle. Quantitative analysis of bacterial uptake from loops lacking PP was done by counting of the number of organisms present in sections of villi showing the villus from its base to the tip. The data were reported as the percentage of villi that included at least one organism and the average number of organisms per villus. The latter statistic was normalized to a value of 1.0 for loops from mice not treated with RANKL.

ELISA for measurement of fecal IgA

Fecal pellet samples were collected and extracted by making a 1/10 suspension (w/v) with PBS. After the suspension was vortexed and spun for 10 min at 12,000 × g, the supernatant was stored at −70°C. Polyclonal goat anti-mouse IgA Ab (Southern Biotechnology) was used as a capture Ab. The bound mouse IgA was detected with peroxidase-labeled goat anti-mouse IgA Ab (Southern Biotechnology) using TMB (BD Biosciences) as the peroxidase substrate. A mouse IgA isotype control mAb (BD Biosciences) was used to establish a standard curve.

Statistical analysis

Differences between the mean values for groups were analyzed by either two-tailed ANOVA with Tukey correction (for multiple groups), two-tailed Student’s t test, or two-tailed Mann-Whitney U test as calculated using Prism (GraphPad Software). Differences in the frequency of bacterial uptake into villi were analyzed by Fisher’s exact test and also calculated with Prism. A p-value of <0.01 was considered significant.

Results

UEA-1+ M cells are dramatically decreased in the FAE of PP from RANKL null mice

M cells in mouse PP can be detected using the UEA-1 lectin specific for α(1,2)-fucose linkages. In wild-type mice, whole mount microscopy of PP follicles revealed an average of over 100 radially arranged UEA-1+ M cells that extended from the edges of the follicles toward the central subepithelial dome area. In contrast, UEA-1+ M cells were either completely absent or very sparsely represented in individual follicles from the PP RANKL null mice (Fig. 1A). The few remaining UEA-1+ cells in RANKL null mice were mostly located at the periphery of the follicle and did not have the usual polygonal shape of normal M cells, features suggesting these remaining M cells were abnormal. The loss of M cells in PP from RANKL null mice was confirmed by staining PP sections with NKM 16–2–4 (supplementary Fig. S1), a recently described rat mAb that is more selective than UEA-I for the specific α(1,2)-fucose moiety characterizedly displayed by mouse M cells (35). Cells with the defining ultrastructural features of M cells by transmission electron microscopy (i.e., presence of intracellular pockets and blunting of the apical microvilli in comparison to normal enterocytes) were readily apparent in the FAE from control mice, but absent from the FAE of RANKL null mice (Fig. 1B). Although the number of UEA-1+ M cells was significantly decreased in all PP examined from RANKL null mice, a proximal to distal gradient in the number of UEA-1+ cells per dome was observed in RANKL null mice that was not seen in wild-type mice (Fig. 1C). UEA-1+ M cells were almost completely absent in the most proximal PP from RANKL null mice, and progressively increased in more distal PP. In RANKL null mice, the highest number of residual UEA-1+ cells was consistently detected in the most distal ileal PP. Taking into account the decreases in RANKL null mice in the number of PP, the number of follicles in each PP, and the number of M cells per follicle, loss of RANKL is associated with a 73-fold overall depletion of UEA-1+ M cells. This extent of loss of M cells is roughly 10-fold greater than the losses we observed in both μMT B cell deficient mice and CCR6 deficient mice.

The online version of this article contains supplemental material.

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compared with control mice by ANOVA.

RANKL null mice were injected i.p. for 7 consecutive days with RANKL null PP could be restored by replacement of RANKL, to determine whether the deficiency of M cells in the FAE of treatment with exogenous RANKL

BALB/c)F1 RANKL null mice showed a lack of characteristic M cell features by transmission electron microscopy. The long arrowheads indicate intraepithelial pockets within the M cells of the wild-type FAE. The short arrowheads point to the shorter microvilli found on the apical surface of M cells. Scale bars, 50 μm.

To determine whether the villous M cells induced by systemic RANKL treatment were capable of increased transport of particulate Ags across the epithelium, mice were treated with s.c. injections of GST-RANKL or GST as a control (Fig. 2D). Untreated RANKL null mice or those treated with GST had over 10-fold less uptake of beads than control wild-type mice. GST-RANKL treatment for 7 days restored bead uptake to near wild-type levels.

**Systemic administration of RANKL also leads to widespread induction of villous M cells**

In the course of treating RANKL null mice with GST-RANKL and evaluating the reconstitution of M cells in PP, we noticed that the number of UEA-I⁺ M cells present on small intestinal villi was also increased. This effect of RANKL treatment was further evaluated in BALB/c mice, in which <10% of small intestinal villi have any villous M cells at baseline, with most of these rare villous M cells arranged in a diffuse pattern. Treatment with systemic GST-RANKL i.p. for 4 consecutive days induced substantial increase in the number of UEA-I⁺ cells on the surface of the villi (Fig. 3A). Induction of an increased number of villous M cells began by 24 h after the first injection of GST-RANKL; 4 days after the start of RANKL treatment all small intestinal villi had at least some UEA-I⁺ cells present, with 70% of villi showing a diffuse pattern and the remaining 30% exhibiting a dense pattern (Fig. 3B). In villi showing a diffuse pattern of villous M cells, UEA-I⁺ cells represented ~3% of the total number of cells with DAPI⁺ nuclei. Scanning electron microscopy of villi from RANKL-treated mice revealed slightly sunken cells with the characteristic stubby microvilli characteristic of M cells (Fig. 3C).

**RANKL-induced villous M cells are functional M cells capable of taking up 200 nm beads and enteric bacteria**

To determine whether the villous M cells induced by systemic RANKL treatment were capable of increased transport of particulate Ags across the epithelium, mice were treated with s.c. injections of GST-RANKL or GST as a control for 4 consecutive days and gavaged with 200 nm diameter fluorescent nanoparticles at the same time as the last two RANKL injections. Small intestinal segments from these mice were excised 24 h after the second dose of beads and frozen sections cut to identify beads that had been taken
show that the clusters of fluorescent beads (indicated by arrowheads) within two adjacent UEA-I for 5 days. Merged images of bead fluorescence and DAPI fluorescence from frozen sections of PP are shown with the white circles indicating the location of pixels containing green fluorescent beads within the area of the PP follicles. 

Varied lengths of treatment by both UEA-I staining and by uptake of fluorescent 200 nm beads from isolated small intestinal loops.

The number of M cells in the PP follicles was evaluated after a dose previously shown to block the activity of RANKL in vivo (36). The number of M cells in the PP follicles was evaluated after various lengths of treatment by both UEA-I staining and by uptake of fluorescent 200 nm beads from isolated small intestinal loops.

Neutralizing Ab to RANKL reproduces the M cell deficiency observed in RANKL null mice

Some of the developmental defects in RANKL null mice, such as the total absence of lymph nodes, cannot be corrected by simply injecting the mice with the absent cytokine as adults. This raises the issue of whether the M cell defect observed in PP from RANKL null mice might be a byproduct of early developmental alterations in the PP of these mice. To address this issue, wild-type BALB/c mice were treated i.p. with a neutralizing anti-RANKL Ab to determine whether acute blockade of RANKL–RANK signaling would lead to loss of PP M cells. Mice were treated i.p. with 250 μg of the 1K22–5 rat anti-mouse RANKL mAb every 2 days, a dose previously shown to block the activity of RANKL in vivo (36). The number of M cells in the PP follicles was evaluated after various lengths of treatment by both UEA-I staining and by uptake of fluorescent 200 nm beads from isolated small intestinal loops.

After 8 days of Ab treatment, the number of M cells present in the PP and the degree of uptake of fluorescent beads by PP in isolated loops were both dramatically decreased compared with untreated mice or mice treated with an isotype control IgG2a mAb (Fig. 5, A–C). Analysis of the kinetics of the anti-RANKL effects showed that the number of UEA-1+ M cells dropped precipitously between 2 and 4 days, and declined further between 4 and 8 days (Fig. 5D).

Epithelial cells in the small intestine express RANK

RANK is expressed by multiple cell types including osteoclasts, DCs, mammary epithelial cells, and thymic epithelial cells. Because our experiments with RANKL null mice and neutralizing anti-RANKL Ab showed that RANKL is essential for normal M cell development within the FAE, we used immunohistochemical staining with anti-RANK Abs to determine what cells in the vicinity of PP expressed the RANK. Staining for RANK was observed on the apical and basolateral aspects of epithelial cells in the FAE, and was also detected on villous and crypt epithelial cells (Fig. 6). Serial sections of the same PP showed that RANKL expression was restricted to stromal cells concentrated beneath the FAE as previously shown (29). These results suggest that RANKL exerts its effects on M cell differentiation through short-range delivery from the stromal cells to the FAE on the other side of the basement membrane.

RANKL null mice exhibit decreased PP germinal center formation and fecal IgA production

PP were previously reported to be smaller than normal in two independently derived strains of RANKL null mice (22, 23), but other aspects of PP function were not examined in the initial reports. We asked whether the loss of M cell function in RANKL null mice was associated with impaired B cell responses to Ags
pressed surface and attenuated and blunted microvilli characteristic of M cells. Scanning electron microscopy reveals the presence of cells with a de- and dense patterns of distribution following GST-RANKL administration.

FIGURE 3. Administration of rRANKL induces development of villous M cells on all small intestinal villi. A, Whole mount staining of villous M cells in untreated BALB/c mice and mice treated for 4 days with GST-RANKL or GST (an initial injection of 50 μg i.p. followed by 100 μg s.c. every 24 h) with rhodamine-UEA-I and DAPI. In untreated mice, a few villous M cells in a diffuse pattern are present on occasional villi. GST-RANKL treatment leads to an increased fraction of the villi having M cells exhibiting both the diffuse and dense patterns of villous M cell distribution. Scale bars, 200 μm and 500 μm. B, Summary graph showing kinetics of induction of villous M cells in the diffuse and dense patterns of distribution following GST-RANKL administration. C, Scanning electron microscopy reveals the presence of cells with a depressed surface and attenuated and blunted microvilli characteristic of M cells.

Discussion

Ag-sampling M cells have been described in both mammalian and avian species as part of the FAE covering the organized lymphoid structures of the respiratory and digestive tract (3, 39, 40). However, the specific signals and signaling pathways that trigger the differentiation of these M cells from precursor cells located in the stem cell zone of the crypts or from the enterocytes on the surface of the FAE remain to be identified (12). Some clues have emerged from analysis of strains of mutant mice created by gene-targeting that retain PP but exhibit decreased numbers of M cells in these PP. Specifically, B cell-deficient mice such as μMT mice exhibit significantly reduced numbers of M cells in PP (37). Additional support for a role of B cells in promoting M cell development has come from in vitro studies in which coculture of freshly isolated B lymphocyte or B lymphocyte lines with model intestinal epithelial cell lines cultured on semipermeable supports promoted the development of M cell-like features by the epithelial cells, including transcytosis of particulate Ags (11, 41). However, neither in vivo analysis of PP from B cell-deficient mice or experiments based on the in vitro M cell differentiation system have elucidated a specific mechanism by which B cells promote differentiation of M cells in the FAE.

RANKL emerged as a cytokine with a potential role in the differentiation of the FAE and M cells as a result of experiments demonstrating RANKL expression on stromal cells located immediately beneath the FAE in ILF and PP (29). To determine whether PP were functionally compromised in the absence of RANKL, we characterized the PP of RANKL null mice. Staining of PP from RANKL null mice with the UEA-I lectin reactive with murine M cells revealed a profound depletion in UEA-I+ cells compared with wild-type mice. Taking into account all of the factors that contribute to the total number of M cells within small intestinal PP (i.e., number of PP, number of follicles per PP, number of M cells per follicle), we found that RANKL null mice have <2% of the number of UEA-I+ M cells found in wild-type mice. Although the UEA-I lectin was the primary immunohistochemical reagent we used to establish that RANKL null mice are deficient in M cells, we have used several independent means of confirming this deficiency in M cells including functional measurements of M cell activity using uptake of fluorescent nanoparticles and bacteria, transmission electron microscopy, and immunostaining with the NKM 16–2-4 mAb specific for mouse M cells.

RANKL acting through its specific receptor (RANK) plays an important developmental role in multiple tissues. The most striking and best-studied of the deficits in RANKL null mice are the absence of any lymph nodes and the failure of osteoclast development, leading to osteopetrosis and a malformed skeleton. One potential explanation of the loss of M cells we observed in PP from RANKL null mice is an early developmental defect in PP development that permanently compromises the capacity of the FAE to generate conventional M cells. Two types of experiments were
first, we examined whether the M cell defect was reversible if a source of exogenous recombinant RANKL was provided. Daily injections of GST-RANKL given for 5 or more days provided a nearly complete reconstitution of the number of M cells per PP follicle. Second, we used neutralizing mAb to RANKL to test whether acute depletion of RANKL in adult wild-type mice would also cause loss of M cells. After 4 days of anti-RANKL treatment to inhibit normal RANKL-RANK interactions, the number of UEA-1$^+$ M cells in each PP follicle plunged to levels approaching those in the RANKL null mice.

Thus, production of RANKL must be sustained in the adult PP to permit the continued production and/or survival of M cells.

RANK is expressed on multiple cell types including osteoclasts and their precursors, DCs, endothelial cells, mTEC, and mammary epithelial cells. The simplest model to explain the observed effects of RANKL on M cell differentiation is to propose that RANKL derived from the subepithelial dome stromal cells in the PP acts in a paracrine fashion on the adjacent epithelial cells of the FAE. Because RANKL is a type II membrane protein that is synthesized in a transmembrane form, cleavage by metalloproteases is needed.
to generate a soluble form of the cytokine (17, 18). We favor the hypothesis that RANKL is acting directly through RANK on enterocytes because immunohistochemical staining of small intestinal tissue including a PP showed that the bulk of the RANK staining is localized to the epithelium, with roughly equivalent levels of RANK on the FAE and villous epithelium. Gene expression profiling studies comparing flow sorted PP M cells and villous enterocytes revealed that both of these intestinal epithelial cell types express mRNA for RANK (35) (gene expression data for RANK archived in NCBI Gene Expression Omnibus under accession number GSE7838; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=H11005GSE7838). Although the induction of M cells by RANKL appears to be mediated by direct action of RANKL on RANK-expressing epithelial cells, other cell types in the small intestine are known to express RANK and respond to RANKL. For example, RANKL was shown to act on PP DC to enhance IL-10 production (42). In addition, Ab-mediated neutralization of RANKL in a transfer model of colitis resulted in decreased regulatory T cell activity, suggesting that RANKL-RANK signaling contributes to the normal function of regulatory T cells (43).

The capacity of soluble recombinant RANKL injected systemically to induce the appearance of M cells on all small intestinal villi provides further insights into the mechanism of action of RANKL. RANK-expressing epithelial precursor cells located in both dome-associated crypts next to PP follicles and in standard small intestinal crypts have the potential to differentiate into M cells if exposed to sufficient stimulation with RANKL. RANKL-induced villous M cells have most of the same features as PP M cells, including reactivity with UEA-I, stubby surface microvilli observed by scanning electron microscopy, and most importantly the capacity for constitutive uptake of particulate Ags. Under normal conditions, M cell development is primarily restricted (other than a small number of scattered villous M cells) to the organized

**FIGURE 5.** Treatment of wild-type mice with neutralizing anti-RANKL leads to loss of PP M cells. A and B, BALB/c mice were treated i.p. with 250 μg of IKK22–5 mAb or an isotype control rat IgG2a mAb on days 0, 2, 4, and 6. On day 8, isolated bowel loops containing PP were injected with fluorescent beads and the mice euthanized after 90 min. Anti-RANKL treatment led to loss of UEA-I+ M cells detected by whole mount staining (A) and a decrease in the uptake of fluorescent beads detected on frozen sections of PP from the bead-injected loops (B). Scale bar, 200 μm in A and 100 μm in B. C, Summary of data from all PP analyzed in A and B for UEA-I+ cells and fluorescent bead uptake. D, Anti-RANKL-induced loss of UEA-I+ M cells detected by whole mount staining begins by 4 days after start of Ab treatment. ** in C and D indicates p < 0.001 compared with untreated mice by t test (C) or ANOVA (D).

**FIGURE 6.** Intestinal epithelial cells express RANK. Frozen sections of a PP from a single wild-type BALB/c mouse were stained with rat mAbs to mouse RANK (A and B), mouse RANKL (C), or an isotype control rat IgG2a mAb (D), followed by a biotinylated secondary Ab, streptavidin-peroxidase, and FITC-tyramide plus DAPI as a counterstain. A, RANK expression is localized to epithelial cells in the FAE and on the adjacent villi. Scale bar, 100 μm. B, Higher magnification of boxed area from A showing that RANK is present on both the apical and basolateral surfaces of the FAE. Scale bar, 250 μm. C, Reticular stromal cells concentrated immediately beneath the epithelial layer are the only cells on which RANKL is detected. Scale bar, 200 μm. D, No staining is observed with the rat IgG2a isotype control.
lymphoid tissues of the small intestine (i.e., PP and ILF) because constitutive expression of RANKL is restricted to subepithelial stromal cells at these sites. When the spatial restriction of RANKL availability in the small intestine is bypassed by systemic injection, RANKL is able to trigger M cell differentiation in a fraction of epithelial precursors in both dome-associated crypts adjacent to organized lymphoid tissues and normal crypts.

Although our results identify RANKL as a key cytokine signal involved in inducing the differentiation of M cells from precursors in the FAE, we consistently observed a trace number of residual UEA-I$^+$ M cells in the PP. The authors have no financial conflict of interest.

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**Disclosures**

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**References**


Supplementary Figure 1. PP from RANKL null mice have very few cells reactive with the NKM 16-2-4 monoclonal antibody specific for M cells. A,B, Sections of PP from the middle portion of the small intestine from wild type (A) and RANKL null mice (B) were stained with FITC-NKM 16-2-4 and rhodamine-UEA-I. The large arrowheads point to cells that stain for both NKM 16-2-4 and UEA-I. The small arrowhead indicates a cell positive for UEA-I, but negative for NKM 16-2-4. Scale bar, 100 μm.