DCIR Maintains Bone Homeostasis by Regulating IFN-γ Production in T Cells

Takumi Maruhashi, Tomonori Kaifu, Rikio Yabe, Akimasa Seno, Soo-Hyun Chung, Noriyuki Fujikado and Yoichiro Iwakura

J Immunol published online 29 April 2015
http://www.jimmunol.org/content/early/2015/04/28/jimmunol.1500273

Supplementary Material http://www.jimmunol.org/content/suppl/2015/04/28/jimmunol.1500273.DCSupplemental

Subscription Information about subscribing to The Journal of Immunology is online at: http://jimmunol.org/subscription

Permissions Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
DCIR Maintains Bone Homeostasis by Regulating IFN-γ Production in T Cells

Takumi Maruhashi,*†‡¶,1 Tomonori Kaifu,*†‡,§,1 RIKIO YABE,*†‡,§ AKIMASA SENO,*†‡,§,1 SOO-HYUN CHUNG,*†‡,§,1 NORIYUKI FUJIKADO,*†‡,§,1 AND YOICHIRO IWAKURA*†‡,§,1,‡,¶,1

Dendritc cell immunoreceptor (DCIR) is a C-type lectin receptor mainly expressed in DCs. Deir−/− mice spontaneously develop autoimmune enethesitis and ankylosis accompanied by fibrocortilage proliferation and ectopic osification. However, the mechanisms of new bone/cartilage formation in Deir−/− mice remain to be elucidated. In this study, we show that DCIR maintains bone homeostasis by regulating IFN-γ production under pathophysiological conditions. DCIR deficiency increased bone volume in femurs and caused aberrant ossification in joints, whereas these symptoms were abolished in Rag2−/−Deir−/− mice. IFN-γ-producing T cells accumulated in lymph nodes and joints of Deir−/− mice, and purified Deir−/− DCs enhanced IFN-γ+ T cell differentiation. The ankylogic changes and bone volume were suppressed in the absence of IFN-γ. Thus, IFN-γ is a positive chondrogenic and osteoelastic factor, and DCIR is a crucial regulator of bone metabolism; consequently, both factors are potential targets for therapies directed against bone metabolic diseases. The Journal of Immunology, 2015, 194: 000-000.

*Center for Experimental Medicine and Systems Biology, Institute of Medical Science, The University of Tokyo, Tokyo 113-0032, Japan; †Research Institute for Biomedical Sciences, Tokyo University of Science, Chiba 278-0022, Japan; ‡Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Saitama 332-0012, Japan; §Medical Mycology Research Center, Chiba University, Chiba 250-8673, Japan; ¶Tokushima University, Tokushima, Japan; and T.M. and T.K. contributed equally to this work.

1Current address: Division of Immunology, Harvard Medical School, Boston, MA. Received for publication February 4, 2015. Accepted for publication April 3, 2015. This work was supported by Grant-in-Aid for Scientific Research (S) 24220011 (to Y.I.) and Grant-in-Aid for Scientific Research (C) 25500489 (to T.K.) from the Japan Society for the Promotion of Science; a Core Research for Evolutional Science and Technology Grant from the Japan Science and Technology Agency (to Y.I.); the Science and Technology Research Promotion Program for Agriculture, Forestry, Fisheries, and Food Industry (to Y.I.); and by a fellowship from the Japan Society for the Promotion of Science (to T.M.).

T.M. and T.K. designed, performed, and analyzed most of the experiments; R.Y., A.S., S.H.C., and N.F. helped perform experiments and analyzed data; T.M. and T.K. wrote the manuscript; and Y.I. organized and supervised the project and edited the draft manuscript.

Address correspondence and reprint requests to Prof. Yoichiro Iwakura, Research Institute for Biomedical Sciences, Tokyo University of Science, 2669 Yamazaki, Noda, Chiba 278-0022, Japan, E-mail address: iwakura@rs.tus.ac.jp

The online version of this article contains supplemental material.

Abbreviations used in this article: AS, ankylosing spondylitis; CLR, C-type lectin receptor; COL2, type II collagen; DC, dendritic cell; DCIR, dendritic cell immunoreceptor; GMA, glycol methacrylate; LN, lymph node; MMP3, matrix metalloproteinase-3; PCNA, proliferating cell nuclear Ag; RANKL, receptor activator of NF-κB ligand; SHP, Src homology region 2 domain-containing phosphatase; SOX9, sex-determining region Y-box 9; WT, wild-type.

Copyright © 2015 by The American Association of Immunologists, Inc. 0022-1767/15/$25.00
DCIR signaling in osteoclast precursors (19, 20). Th17 cells and IL-17, which induces RANKL expression on osteoblasts and fibroblasts, stimulate osteoclastogenesis, causing bone erosion (17). Therefore, bone metabolism is greatly influenced by the immune system.

Ankylosing spondylitis (AS), a form of seronegative spondyloarthritis, is characterized by axial and peripheral enthesitis, followed by joint immobility due to heterotopic cartilage and bone formation (21, 22). Intensive clinical investigations and genomewide association studies suggest the involvement of HLA-B27, a class I MHC allele, and proinflammatory cytokines such as TNF and IL-23 in the pathogenesis of AS (23–27). Ankylogetic changes of joints are also associated with the bone-remodeling mediators such as bone morphogenetic proteins, wingless proteins, and their endogenous inhibitor dickkopf-1 (28, 29). The expression of these mediators in synovial cells and stromal cells is also regulated by inflammatory cytokines, such as TNF, IL-17, and IL-1 (28, 30–32), suggesting the involvement of these cytokines in the development of ankylogetic changes. However, the precise mechanisms underlying the aberrant cartilage and bone formation seen in AS still remain to be elucidated.

In this study, we analyzed the pathogenic mechanisms of ankylosis in DCIR–/− mice. We showed that articulare changes were completely suppressed in RAG2-deficient (Rag2−/−) mice, indicating an immune-mediated pathology. Furthermore, joint ankylosis was completely abolished in Tnf−/− DCIR−/− and Ifng−/− DCIR−/− mice, but not in Il17a−/− DCIR−/− mice, suggesting that TNF and IFN-γ are crucial for the development of ankylosis in DCIR−/− mice. Furthermore, a mild increase of bone volume was observed in DCIR−/− mice, and this was also normalized in Rag2−/− and Ifng−/− mice. We showed that IFN-γ-producing T cells are effectively induced in DCIR−/− mice, and IFN-γ has potent chondrogenic and osteogenic activity. These observations suggest that DCIR is a possible target for the treatment of bone metabolic diseases.

Materials and Methods

Mice

Deir (Clec4a2−/−) mice (3), Ifng−/− mice (33), and Il17a−/− mice (34) were generated as described and backcrossed to C57BL/6j mice (Japan SLC, Shizuoka, Japan) for 9–12 generations before they were used in this study. Tnf−/− mice (35) were backcrossed to C57BL/6j mice for 12 generations. C57BL/6j Rag2−/− mice were obtained from the Central Institute for Experimental Animals (Kawasaki, Japan). Rag2−/− Il17a−/−, Ifng−/−, and Il17a−/− Rag2−/− mice were generated by intercrossing Rag2−/−, Ifng−/−, Il17a−/−, and Tnf−/− mice with DCIR−/− mice, respectively. C57BL/6j wild-type (WT) mice were purchased from Japan SLC. Male mice of similar ages were used for experiments. These mice were housed under specific pathogen–free conditions in environmentally controlled clean rooms at the Institute of Biomedical Sciences (Tokyo University of Science). All animal experiments were approved by the animal use committees of both universities and were conducted according to the institutional ethics guidelines for animal experiments and safety guidelines for gene manipulation experiments.

Histology and micro–computed tomography analysis

Ankle joints were fixed with neutralized 10% formalin overnight at 4°C, decalcified with 10% EDTA (Nacalai Tesque, Kyoto, Japan) in PBS (pH 7.4) for 2–3 wk, embedded in paraffin, sectioned (5 μm), and stained with H&E or Safranin O/Fast Green (Waldeck, Münster, Germany). For micro–computed tomography (μCT), ankle joints and femurs were fixed and analyzed using an R μXT (Rigaku, Tokyo, Japan) or a Scan Xmate-L090 (Comscantecno, Yokohama, Japan). Three-dimensional image analyses were performed using the TRI/3D-BON software (Ratoc System Engineering, Tokyo, Japan). Bone trabecular bone mineral density was characterized by calculating bone volume density (bone volume/tissue volume), trabecular thickness (2 × bone volume/bone surface), trabecular number ([bone volume/tissue volume] trabecular thickness), and trabecular separation (1/[trabecular number – trabecular thickness]). The trabecular number was defined as the number of trabecular bones.

Quantitative real-time RT-PCR analysis

Total mRNA was extracted from joint-infiltrating cells, chondrocytes, and osteoblasts using the GenElute mammalian mRNA miniprep kit (Sigma-Aldrich, St. Louis, MO). Isolated RNA was then subjected to reverse transcription using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Gene expression was analyzed by quantitative PCR using SYBR premix Ex Taq (Takara Bio, Otsu, Japan) on an iCycler (Bio-Rad, Hercules, CA). Specific primer sets are described in Supplemental Table I. Data were normalized to Gapdh or Actb.

Calcine labeling

Dynamic histomorphometric analysis of bone formation was carried out by Kureha Special Laboratory (Fukushima, Japan). Body weights of WT and Deir−/− mice at 8 wk of age were measured, and the mice were i.p. administered 16 mg/kg (body weight) of calcein (Nacalai Tesque) twice at 2-d intervals. The mice were sacrificed on day 2 after the last injection of calcine. Tibiae were cleaved with a 1.05-mm width at 0.3 mm from the growth plate. The trabecular bones in metaphysis were examined with a Histo-metryRT camera. For studies of dynamic bone formation, the mineralizing surface (mineralization surface/bone surface; %), mineral apposition rate (μm/d), and bone formation rate (bone formation rate/bone surface; μm2/μm2/d) were determined at ×400 magnification using an image analyzer (System Supply, Nagano, Japan).

Bone histomorphometric analysis

A histomorphometric analysis of murine tibiae was performed by Kureha Special Laboratory. The bones of WT and Deir−/− mice were isolated from five to six 8-wk-old mice per group and fixed with 70% ethanol. To measure the histomorphometric parameters of the bone structure, 3-μm sections of GMA-embedded femur tissues were stained with toluidine blue. The trabecular bone parameter was determined in an area of the secondary spongiosa with a 1.05-mm width at 0.3 mm from the growth plate. The trabecular bones in metaphysis were examined with a Histomety RT camera. For assays of osteoclast number (osteoclast number/100 mm) and osteoclast surface (osteoclast surface/bone surface; %), osteoclasts were defined as cells with more than one nucleus that formed resorption lacunae at the surface of the trabeculae. Parameters of bone remodeling, including the osteoclast number (osteoclast number/100 mm) and osteoclast surface (osteoclast surface/bone surface; %), were measured in toluidine blue-stained sections.

Cell preparation and flow cytometry

For joint-infiltrating cell preparations, the ankle joints, after removal of skin and bone marrow, were digested with 5 mg/ml collagenase type II (Worthington Biochemical, Lakewood, NJ) in RPMI 1640 (Life Technologies/Invitrogen, Carlsbad, CA) with 5% FCS (Life Technologies) at 37°C for 90 min. For coculture with T cells and DCs, T cells were purified from spleens of WT mice using an autoMACS Pro separator (Miltenyi Biotec, Bergisch Gladbach, Germany). CD11c−/− DCs were sorted using a FACSaria II (BD Biosciences, Franklin Lakes, NJ) from the spleens of WT and Deir−/− mice after removal of CD90.2+ and B220+ cells by magnetic cell sorting, and 2 × 10^5 cells were cocultured with 2 × 10^5 CD90.2+ T cells for 3 d in the presence of 1 μg/ml soluble anti-CD3 (Ab 145-2C11, ebiosis, San Diego, CA). Fluorescence-activated Abs to CD3 (clone 145-2C11), CD4 (RM4-5), CD8 (53-6.7), IFN-γ (XMG1.2), IL-17 (TC11-1H10), CD45 (30-F11), CD11c (N418), CD11b (M1/70), CD80 (16-10A1), CD86 (GL-1), CD40 (3/23), and I-A/I-E (M5/114.15.2) were obtained from BioLegend (San Diego, CA) or ebiosis. For intracellular cytokine staining, cells were incubated with PMA (50 ng/ml, Sigma-Aldrich), ionomycin (500 ng/ml, Sigma-Aldrich), and monensin (2 μM, Sigma-Aldrich) for 5 h. After surface staining, cells were fixed and permeabilized using an intracellular fixation and permeabilization buffer set (ebiosis) and then stained for intracellular cytokines. Data were acquired on a FACSaria II (BD Biosciences) and analyzed using the FlowJo software (Tree Star, Ashland, OR).
Measurement of IFN-γ-producing T cells in peripheral blood
digested femurs of aged Dcir−/− mice

Whole blood was obtained from mice under anesthesia by cardiac puncture with a 21-gauge needle attached to a syringe containing heparin (Mochida Pharmaceutical, Tokyo, Japan). The blood samples were mixed with 10 volumes of hemolysis buffer and left on ice for 10 min, followed by centrifugation at 1500 rpm at 4°C for 5 min to collect mononuclear cells. The hemolysis buffer treatment was repeated three times. PBMCs underwent stimulation for 5 h with PMA (50 ng/ml), ionomycin (500 ng/ml), and brefeldin A (10 μg/ml) during the entire incubation period. IFN-γ secretion by PBMCs was detected by intracellular molecular staining and flow cytometric analysis.

Joint protein extraction

Ankle joints, after removal of skin and bone marrow, were homogenized in liquid nitrogen, and proteins were extracted with a lysis buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 0.1% Triton X-100.

Serum collection

Whole blood was obtained by cardiac puncture under anesthesia. The blood samples were left to clot at 4°C overnight and then centrifuged at 3000 rpm for 10 min to separate serum from coagulated blood. The serum was collected and stored at −80°C prior to assays.

ELISA

IFN-γ, IL-17A, and TNF were measured using the mouse IFN-γ DuoSet (R&D Systems, Minneapolis, MN), the mouse IL-17A ELISA Ready-SET Go! kit (ebiSciences), and the mouse TNF-α ELISA MAX standard (BioLegend). The serum levels of IFN-γ were determined using the IFN-γ ELISA kit (Mabtech, Nacka Strand, Sweden). Absorbance was measured on an MTP-300 microplate reader (Hitachi, Tokyo, Japan).

Murine chondrocyte primary culture

Primary cultures of murine chondrocytes were performed according to Gosset et al. (36). Murine chondrocytes were prepared from costal cartilage tissue of newborn mice (5–6 d) by enzymatic digestion in DMEM (Life Technologies) with 0.5 mg/ml collagenase D (Roche, Basel, Switzerland) and 0.1% dispase (Roche) for 10 min at 37°C with agitation. The solution was discarded to remove cells to the center of each well of a 24-well plate and then incubated at 37°C for 2 h to allow cells to adhere. To the well was then added 500 μl DMEM containing 10% FCS supplemented with IFN-γ (0, 0.4, 2, 10 ng/ml; PeproTech, Rocky Hill, NJ), TNF (0, 0.4, 2, 10 ng/ml; PeproTech), IL-17 (0, 0.4, 2, 10 ng/ml; R&D Systems), or TGF-β (0, 0.4, 2, 10 ng/ml; PeproTech). After 7 d, cells were fixed in neutralized 10% formalin, rinsed with 0.1 N HCl, followed by staining with 1% Alcian blue 8GX (Sigma-Aldrich) solution. The images were obtained on a BioRevo BZ-9000 microscope (Keyence, Osaka, Japan), and the Alcian blue* area and staining intensity were quantitated using the ImageJ software (National Institutes of Health).

Murine osteoblast primary culture

Calvarial cells were prepared by sequential digestion according to a standard method. Briefly, calvaria from 1- to 2-d-old neonates were surgically isolated, and the adherent mesenchymal tissues were trimmed. The neonatal calvaria was first digested in degradation solution containing 0.1% collagenease (Wako Pure Chemical Industries, Osaka, Japan) and 0.1% dispase (Roche) for 10 min at 37°C with agitation. The solution was discarded to remove debris, and the remaining calvaria were further digested in the same degradation solution for 1 h at 37°C. Cells isolated from serial digestions were suspended in α-MEM containing 10% FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml) and plated at a concentration of 2 × 10^5 cells per well in 12-well plates. After 2 d of culture, the cells were incubated in an osteogenic medium (α-MEM supplemented with 10% FCS, 50 μg/ml ascorbic acid [Sigma-Aldrich], 10 mM β-glycerophosphate [Calbiochem, La Jolla, CA], and antibiotics) for 14 or 21 d, with the medium replaced every 3 d for the entire duration. The primary murine osteoblasts were cultured in the presence of recombinant mouse IFN-γ (PeproTech) from the start of the culture, and IFN-γ was added every 3 d when the osteogenic medium was replaced. The mineralization formation of osteoblastic cells was detected using the von Kossa method and alizarin red S (sodium alizarin sulfonate) staining. For the von Kossa method, osteoblast cultures were fixed in 10% formalin for 10 min at room temperature and then stained with 1% alizarin red S (pH 4.2) for 10 min at room temperature. After washing with distilled water to remove excess dye, the mineralized nodules were visualized as dark brown or black spots. For alizarin red S staining, osteoblasts were fixed with 10% formalin for 10 min at room temperature and then stained with 1% alizarin red S.

Statistical analysis

An unpaired two-tailed Student t test and a Mann–Whitney U test were used for the statistical evaluation of results. Multigroup comparisons were performed by one-way ANOVA followed by a Tukey HSD post hoc test. Incidence of ankylosis was compared using a Fisher exact test. A p value < 0.05 was considered statistically significant.

Results

Ankylosing enthesitis develops spontaneously in aged Dcir−/− mice

Enthesitis of the ankle joints of hindlimbs developed spontaneously in Dcir−/− mice of the 129/Ola × C57BL/6J background, starting at 4 mo of age (3). Enthesitis also developed in aged Dcir−/− mice after backcrossing for 9–12 generations to C57BL/6J mice (Supplemental Fig. 1A, Table I). In those animals, enthesitis was followed by the development of joint ankylosis with limited joint motion (Fig. 1A). Joint ankylosis developed in ~20% of male Dcir−/− mice by 12 mo of age (Fig. 1B), but not in female mice (data not shown). To examine bone microstructure in the affected joints, we performed x-ray and three-dimensional (3D) μCT analyses. Aged Dcir−/− mice exhibited joint deformity associated with accelerated ossification around the joint cavity and tendon, suggesting that heterotopic ossification leads to ankylosis of the joint (Fig. 1C). H&E staining revealed enthesal cell proliferation and ligament thickening (Fig. 1D, left, Table I), and Safranin O/Fast Green staining revealed significant cartilage proteoglycan deposition in the proliferative enthesial tissues of the ankle joints of aged Dcir−/− mice (Fig. 1D, right, arrowheads, Table I). Enthesopathy was also detected in knee joints and thoracic vertebrae, as well as ankle joints, of aged Dcir−/− mice (Supplemental Fig. 1B). These results suggest that enthesis followed by abnormal cartilage formation and heterotopic ossification leads to ankylosis of peripheral and axial joints in aged Dcir−/− mice.

Dcir−/− mice exhibit a mild increase of bone mass

We also investigated the bone architectures of young Dcir−/− mice. A histomorphometric analysis of the tibia distal metaphysis revealed increases in both osteoclastic and osteoblastic parameters (Supplemental Fig. 2A). μCT analyses showed that Dcir−/− mice at 8 wk of age had a mild increase of bone mass in the femur, accompanied by increases in bone volume and trabecular number (Fig. 2A). Remarkably, a dynamic histomorphometric analysis revealed a higher mineral apposition rate and bone formation rate per unit of trabecular bone surface in Dcir−/− mice (Fig. 2B). Thus, DCIR deficiency resulted in higher bone turnover, in which bone formation was dominant over bone destruction. However, DCIR expression was undetectable in osteoblasts (data not shown), and differentiation and mineralization activity were similar between the WT and Dcir−/− osteoblasts in vitro (Supplemental Fig. 2B, 2C). Additionally, mRNA expression of RANKL (Tnfsf11), a differentiation factor of osteoclasts, and OPG (Tnfsf11b), a decoy receptor for RANKL, were comparable upon stimulation with 1,25-(OH)2 vitamin D3, PGE2, and ascorbic acid (Supplemental Fig. 2D), suggesting that DCIR deficiency had no direct effect on osteoblasts.

Rag2 deficiency suppresses the development of ankylosis and increase in bone volume in Dcir−/− mice

Because Dcir−/− mice developed autoimmune-like symptoms due to overexpansion of DCs (3), we examined the possibility that this...
Dcir images of ankle joints from 12-mo-old WT and representative macroscopic images of 12-mo-old mice are shown. (B) Talus, talus.

Because Rag2 WT and Dcir Histopathological analysis confirmed that entheses of aged ankylosing enthesitis and the increased bone mass in Dcir genotype are shown. *

Table 1. Histopathological findings in ankle joints of aged Dcir mice

<table>
<thead>
<tr>
<th>Incidence, % (Affected/Total)</th>
<th>WT</th>
<th>Dcir−/−</th>
<th>Rag2−/− Dcir−/−</th>
<th>Tnf−/− Dcir−/−</th>
<th>Il17a−/− Dcir−/−</th>
<th>Il17g−/− Dcir−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>11.8 (2/17)</td>
<td>57.9 (11/19)**</td>
<td>5.9 (1/17)</td>
<td>36.8 (7/19)**</td>
<td>36.8 (7/19)**</td>
<td>0 (0/17)</td>
</tr>
<tr>
<td>Dcir−/−</td>
<td>52.6 (10/19)***</td>
<td>5.9 (1/17)</td>
<td>36.8 (7/19)**</td>
<td>0 (0/17)†††</td>
<td>10.5 (2/19)†</td>
<td>7.7 (1/13)†</td>
</tr>
<tr>
<td>Rag2−/− / Dcir−/−</td>
<td>0 (0/19)†††</td>
<td>5.9 (1/17)</td>
<td>36.8 (7/19)**</td>
<td>0 (0/19)†††</td>
<td>5.3 (1/19)†</td>
<td>0 (0/13)†</td>
</tr>
<tr>
<td>Tnf−/− / Dcir−/−</td>
<td>0 (0/13)†††</td>
<td>7.7 (1/13)†</td>
<td>0 (0/13)†</td>
<td>0 (0/13)†</td>
<td>0 (0/17)†††</td>
<td>0 (0/17)†††</td>
</tr>
<tr>
<td>Il17a−/− / Dcir−/−</td>
<td>35.3 (6/17)</td>
<td>58.8 (10/17)***</td>
<td>52.9 (9/17)***</td>
<td>41.2 (9/17)***</td>
<td>23.5 (4/17)†</td>
<td>23.5 (4/17)†</td>
</tr>
<tr>
<td>Il17g−/− / Dcir−/−</td>
<td>23.5 (4/17)†</td>
<td>23.5 (4/17)†</td>
<td>23.5 (4/17)†</td>
<td>0 (0/17)††</td>
<td>0 (0/17)†††</td>
<td>0 (0/17)†††</td>
</tr>
</tbody>
</table>

Paraffin sections of ankle joints were prepared from 10- to 12-mo-old WT (n = 17), Dcir−/− (n = 19), Rag2−/− Dcir−/− (n = 19), Tnf−/− Dcir−/− (n = 13), Il17a−/− Dcir−/− (n = 17), and Il17g−/− Dcir−/− (n = 17) mice and stained with H&E. The incidence of enthesitis, tendon or ligament thickening, cartilage formation, and bone fusion in each genotype are shown. *p < 0.05, **p < 0.01, ***p < 0.005 (versus WT), †p < 0.05, ††p < 0.01, †††p < 0.005 (versus Dcir−/−) by Fisher exact test.

abnormality of the immune system is involved in the development of bone abnormalities. Ankylosis of joint ankles was completely suppressed in Rag2−/− Dcir−/− mice, even at 1 y of age (Fig. 3A). Histopathological analysis confirmed that entheses of aged Rag2−/− Dcir−/− mice were normal, without any inflammation or new cartilage formation (Fig. 3B, Table 1). Moreover, μCT analysis revealed that the increase in bone volume was also abolished in Rag2−/− Dcir−/− mice (Fig. 3C). These results suggest that the ankylosing enthesitis and the increased bone mass in Dcir−/− mice are caused by the disturbance of the immune system resulting from DCIR deficiency.

IFN-γ-producing T cells are expanded in Dcir−/− mice

Because Rag2 deficiency abolished the bone abnormalities in Dcir−/− mice, we assumed that T cells and/or B cells were responsible for the ankylosing enthesitis and increased bone mass. However, Clec4a2 mRNA was barely detectable in B cells, but not in T cells (3), and Dcir−/− B cells expressed levels of B cell–specific surface markers, such as IgM, IgD, CD5, and CD43, comparable to those on WT B cells (data not shown).

Therefore, we examined the effect of Dcir deficiency on the activation and differentiation of T cells. The proportion of IFN-γ CD4+ and IFN-γ CD8+ T cells in popliteal lymph nodes (LNs) from 12-wk-old Dcir−/− mice was larger than those in WT mice, whereas the proportion of IL-17 CD4+ T cells was similar (Fig. 4A, 4B). The numbers of IFN-γ CD4+ and IFN-γ CD8+ T cells infiltrating into the ankle joint were also elevated in Dcir−/− mice, whereas the number of IL-17 CD4+ T cells was not (Fig. 4C). The concentration of IFN-γ and TNF was higher in joint extracts from Dcir−/− mice than those from WT mice.

FIGURE 1. Male Dcir−/− mice spontaneously develop joint ankylosis with age. (A) Hindpaws of WT and Dcir−/− mice with ankylosis. Representative macroscopic images of 12-mo-old mice are shown. (B) Incidence of ankylosis in WT and Dcir−/− mice. Groups of age-matched WT (n = 26) and Dcir−/− (n = 30) mice were monitored once a week for 12 mo. Data are representative of three independent groups. (C) Radiographic and 3D µCT images of ankle joints from 12-mo-old WT and Dcir−/− mice. Representative images are shown. (D) Histopathologies of ankle joints from 12-mo-old WT and Dcir−/− mice. Formalin-fixed and paraffin-embedded sections of ankle joints were stained with H&E (left) or Safranin O/Fast Green (right). Representative sections are shown. Arrowheads indicate cartilage matrix deposition. Scale bar, 300 µm. Cal, calcaneum; Nav, navicular; Tib, tibia; Tal, talus.
Additionally, T cells in the peripheral blood and the brachial and axillary, but not mesenteric, LNs of Dcir<sup>−/−</sup> mice secreted higher levels of IFN-γ, and the serum concentration of IFN-γ in Dcir<sup>−/−</sup> mice tended to be higher than that of WT mice (Fig. 4E, 4F, Supplemental Fig. 3). Thus, these observations show that IFN-γ–producing T cells are preferentially expanded in Dcir<sup>−/−</sup> mice, forming an IFN-γ–dominant cytokine milieu.

FIGURE 2. Dcir<sup>−/−</sup> mice exhibit an increase in bone volume. (A) µCT images (left) and bone parameters (right) of femoral trabeculae in 8-wk-old WT and Dcir<sup>−/−</sup> mice (n = 5). Bone volume density (bone volume/tissue volume; BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th.), and trabecular separation (Tb.Sp.) are shown. Representative images are shown. Scale bar, 1 mm. (B) In vivo bone formation in 8-wk-old WT (n = 8) and Dcir<sup>−/−</sup> mice (n = 7) was determined by calcein double-labeling. Calcein staining (left, original magnification ×40) and mineral apposition rate (MAR) and bone formation rate (BFR/bone surface [BS]) (right) are shown. Data from two independent experiments are pooled. Values are mean ± SEM. *p < 0.05, ***p < 0.005 by unpaired two-tailed Student t test.

FIGURE 3. Ankylosis and increase of bone volume are suppressed in Rag2-deficient Dcir<sup>−/−</sup> mice. (A) Incidence of ankylosis in male WT, Dcir<sup>−/−</sup>, and Rag2<sup>−/−</sup>Dcir<sup>−/−</sup> mice. Groups of age-matched WT (n = 26), Dcir<sup>−/−</sup> (n = 26), and Rag2<sup>−/−</sup>Dcir<sup>−/−</sup> (n = 27) mice were monitored once a week for 12 mo. Data are representative of two independent groups. (B) H&E staining of hindpaws from 12-mo-old WT, Dcir<sup>−/−</sup>, and Rag2<sup>−/−</sup>Dcir<sup>−/−</sup> mice. Representative sections from each genotype are shown. Right panels are magnified views (original magnification ×10) of regions of the left panels (original magnification ×4). Arrowhead indicates ligament thickening, and asterisks indicate new cartilage formation. Scale bars, 300 µm (left) and 100 µm (right). (C) µCT images (left) and bone parameters (right) of the distal sites of femurs in 9-wk-old WT, Dcir<sup>−/−</sup>, and Rag2<sup>−/−</sup>Dcir<sup>−/−</sup> mice (n = 4–6). Scale bars, 2 mm. Values are mean ± SEM. *p < 0.05 by one-way ANOVA with Tukey HSD post hoc test. BV/TV, bone volume/tissue volume; Tb.N, trabecular number; Tb.Sp., trabecular separation; Tb.Th., trabecular thickness.
DCIR promotes the differentiation of IFN-γ-producing T cells

To investigate the mechanism by which IFN-γ production is enhanced in \( \text{DCIR}^{−/−} \) mice, we analyzed the expression of activation markers on \( \text{DCIR}^{−/−} \) DCs. The mean fluorescence intensity of CD86, CD40, and MHC class II expression was significantly higher on \( \text{CD11c}^{+} \) DCs from popliteal LNs of \( \text{DCIR}^{−/−} \) mice than on those from WT mice (Fig. 5A), indicating that \( \text{DCIR}^{−/−} \) DCs are activated under physiological conditions. Next, to examine the effect of \( \text{DCIR}^{−/−} \) DCs on T cell differentiation, we cocultured purified splenic DCs from WT and \( \text{DCIR}^{−/−} \) mice with WT T cells, and then measured the proliferation and cytokine production from T cells. The number of T cells was significantly higher after coculture with \( \text{DCIR}^{−/−} \) DCs than after coculture with WT DCs (data not shown). Furthermore, the frequency of IFN-γ-producing T cells and the concentration of IFN-γ in the culture medium were higher in coculture with \( \text{DCIR}^{−/−} \) DCs, whereas there was no difference in the proportion of IL-17+ T cells or the levels of IL-17 secretion (Fig. 5B–D). Thus, DCs isolated from \( \text{DCIR}^{−/−} \) mice promote proliferation of IFN-γ-producing T cells and production of IFN-γ.

**FIGURE 4.** IFN-γ–producing T cells accumulate in LNs and joints of \( \text{DCIR}^{−/−} \) mice. (A and B) Frequency of IFN-γ+ or IL-17+ cells in CD4+ and CD8+ T cells from popliteal LNs of 12-wk-old WT and \( \text{DCIR}^{−/−} \) mice (n = 5 each). Popliteal LN cells were analyzed by intracellular staining (gated on CD3+ CD4+ and CD3+CD8+ cells). (C) Number of joint-infiltrating cells producing IFN-γ and IL-17 in 12-wk-old WT and \( \text{DCIR}^{−/−} \) mice (n = 5 each). Cells were isolated from ankle joints and analyzed by intracellular staining (gated on CD45+CD3+CD4+ and CD45+CD3+CD8+ cells). (D) Cytokine levels in the joints of 16-wk-old WT and \( \text{DCIR}^{−/−} \) mice (n = 5 each). The concentrations of IFN-γ, IL-17, and TNF in the homogenized lysates of ankle joints were determined by ELISA. (E and F) Frequency of IFN-γ+ cells in CD4+ and CD8+ T cells from peripheral blood of 12-wk-old WT and \( \text{DCIR}^{−/−} \) mice (n = 5 each). Peripheral blood cells were analyzed by intracellular staining (gated on CD3+CD4+ and CD3+CD8+ cells). Numbers in (A) and (E) denote the percentage of IFN-γ+ cells within gated population. Each symbol represents an individual mouse, and small horizontal lines indicate the mean. Data are representative of three (A–C) or two (D–F) independent experiments. *p < 0.05, **p < 0.01, ***p < 0.005 by Mann–Whitney U test.

\( \text{DCIR}^{−/−} \) DCs promote the differentiation of IFN-γ–producing T cells
IFN-γ is a pathogenic factor that causes ankylosis and increase of bone volume in Dcir−/− mice

The IL-23/IL-17 axis and TNF have been implicated in the pathogenesis of ankylosis (23–27). To dissect the roles of cytokines in the development of ankylosis in Dcir−/− mice, we intercrossed Dcir−/− mice with Ifng−/−, Il17a−/−, and Tnf−/− mice. Although disease onset age seemed to be different as shown in Figs. 1B, 3A, and 6A, we consistently observed that 20% of Dcir−/− mice developed joint ankylosis by 12 mo of age (Fig. 6A), and there were no significant statistical differences in the incidence of ankylosis in Dcir−/− mice at 4–6 mo of age (Figs. 1B, 6A; p = 0.25, 0.25, and 0.17 at 4, 5, and 6 mo of age, respectively; Figs. 3A, 6A; p = 0.28, 0.28, and 0.21, respectively; Fisher exact test). The development of ankylosis was completely suppressed in Ifng−/− Dcir−/− and Tnf−/− Dcir−/− mice, but not in Il17a−/− Dcir−/− mice (Fig. 6A). Histopathological analysis revealed no inflammatory lesions in the enthesis of Tnf−/− Dcir−/− mice (Fig. 6A). Although the incidence of enthesis in Ifng−/− Dcir−/− mice was greatly reduced, mild enthesis and ligament thickening were still observed in some Ifng−/− Dcir−/− mice (Table I). In contrast, entheseal cell proliferation and bone abnormalities were observed in the joints of Il17a−/− Dcir−/− mice, similar to the case of Dcir−/− mice, indicating that IL-17 is dispensable for the pathogenesis of ankylosing enthesis in Dcir−/− mice (Fig. 6B, arrowhead and asterisk, Table I). Thus, these results indicate that TNF and IFN-γ play important roles in the pathogenesis of ankylosis in Dcir−/− mice. Furthermore, μCT analysis of Ifng−/− Dcir−/− mice revealed that aberrant bone formation was suppressed in these mice, suggesting that the increase of bone volume is also caused by aberrant systemic IFN-γ production in Dcir−/− mice (Fig. 6C).

Chondrocyte proliferation and differentiation and osteoblast differentiation are promoted by IFN-γ

Because IFN-γ is suggested to be involved in joint ankylosis and increased bone volume, we examined the effects of IFN-γ on chondrogenesis and osteoblastogenesis. Because the expression of DCIR was not detected in osteoblasts, and because mineralization, gene expressions responsible for osteoblast differentiation and function, and responses to osteoblast stimulators were comparable between WT and Dcir−/− cells (data not shown, Supplemental Fig. 2B–D), we used primary chondrocytes and osteoblasts from WT mice for these experiments. The effects of IFN-γ on chondrogenesis were examined using micromass culture of murine primary chondrocytes (37). Consistent with previous reports (38, 39), TNF and IL-17 suppressed Alcian blue+ proteoglycan deposition in cultures (Fig. 7A, 7B). In contrast, IFN-γ increased proteoglycan deposition in a dose-dependent manner, similarly to TGF-β, which induces proliferation and differentiation of chondrocytes from mesenchymal stem cells (40) (Fig. 7A, 7B). IFN-γ elicited the expression of proliferating cell nuclear Ag (Pcna), sex-determining region Y–box 9 (Sox9; a transcription factor that regulates chondrocyte differentiation), and type II collagen (Col2a1; a cartilage matrix protein) in chondrocytes, but suppressed the expression of matrix metalloproteinase-3 (Mmp3; an enzyme that degrades collagens and proteoglycans) (Fig. 7C). As in chondrocytes, in calvarial osteoblast primary cultures, exogenous IFN-γ facilitated osteoblast-induced Ca2+ deposition and induced expression of osteogenic genes in a dose-dependent manner (Fig. 7D, 7E). These results indicate that IFN-γ promotes chondrogenesis and osteoblastogenesis by promoting proliferation, differentiation, and extracellular matrix production.

Discussion

Previously, we showed that DCIR contributes to immune homeostasis by regulating DC differentiation and proliferation (3). In this study, we showed that DCIR also plays important roles in bone homeostasis by regulating the production of IFN-γ, which we identified as a potent osteogenic factor that promotes both chondrogenesis and osteoblastogenesis.
DCIR−/− mice develop enthesitis, joint ankylosis, and mild increase of bone volume. We showed that these enthesitis and bone abnormalities in DCIR−/− mice are abolished by Rag2 deficiency, suggesting involvement of an immune-mediated mechanism in pathogenesis. Because enthesitis was completely suppressed in Tnf−/− DCIR−/− mice, TNF may play a major role in the development of enthesitis. Indeed, transgenic mice overexpressing TNF spontaneously develop enthesitis (41, 42) and ankylosis of joints (43). Following the enthesal inflammation, ankylosic changes of joints developed. Joint ankylosis was completely suppressed by Tnf and Ifng deficiency, but not by Il17 deficiency, suggesting the involvement of TNF and IFN-γ, but not IL-17, in the development of ankylosing enthesitis in DCIR−/− mice. Consistent with our observation, systemic IL-17 overexpression does not induce ankylosic changes (44). In contrast, systemic IL-23 overexpression causes enthesal inflammation and other symptoms characteristics of human spondyloarthritides (44). IL-23 acts on newly identified enthesal T cells that produce IL-22, causing new bone formation (44). Systemic IL-23 also increases CD11b+ cells in bone marrow and spleen (45), and IL-23 regulates myeloid cell differentiation via crosstalk with ITAM-coupled receptors (46). Although the role of IL-23 on myeloid cell differentiation is poorly understood, these findings raise the possibility that IL-23 may also be involved in the development of ankylosing enthesitis by acting on myeloid cells in mice lacking DCIR-ITIM pathways, which counterbal-

\[\text{Bone volume/tissue volume (BV/TV)}\]

\[\text{Trabecular number (Tb.N)}\]

\[\text{Trabecular separation (Tb.Sp)}\]

\[\text{Trabecular thickness (Tb.Th)}\]

### FIGURE 6

IFN-γ deficiency prevents the development of ankylosis and increased bone mass in DCIR−/− mice. (A) Incidence of ankylosis in cytokine-deficient DCIR−/− mice. Groups of age-matched WT (n = 10), DCIR−/− (n = 10), Tnf−/− DCIR−/− (n = 18), Il17a−/− DCIR−/− (n = 18), and Ifng−/− DCIR−/− (n = 20) male mice were monitored once a week for 12 mo for the development of ankylosis. (B) Histopathologies in ankle joints of aged Tnf−/− DCIR−/−, Il17a−/− DCIR−/−, and Ifng−/− DCIR−/− mice. Representative sections of H&E staining from each genotype are shown. Lower panels are magnified views (original magnification ×10) of the upper panels (original magnification ×4). The arrowhead indicates enthesal cell proliferation, and the asterisks indicate new cartilage formation and bone fusion. Scale bars, 300 μm (upper) and 100 μm (lower). (C) μCT images (left) and bone parameters (right) of the distal sites of femurs in 9-wk-old WT, DCIR−/−, Ifng−/− DCIR−/−, and Ifng−/− mice (n = 4-5). Scale bars, 2 mm. Values are mean ± SEM. *p < 0.05, **p < 0.01 by one-way ANOVA with Tukey HSD post hoc test.

Bone mineral density is reduced in Ifng1−/− mice (49, 50). We showed that IFN-γ promotes chondrocyte differentiation and proliferation via induction of Pena, Sox9, and Col2a1 expression, thereby increasing extracellular matrix deposition. IFN-γ also induced osteoblast differentiation by inducing Runx2, Osterix, Alp, and Bglap, causing enhanced Ca2+ deposition in osteoblast culture. It was reported, however, that IFN-γ rather inhibits the proliferation and proteoglycan synthesis of chondrocytes in cultures (51). The reason for this discrepancy is not completely clear, but different culture conditions might have affected the results: most previous studies used chondrocyte monolayer cultures, whereas we used a high-density micromass culture system, which provides the 3D environment required for
pallation of DCIR expression was undetectable in osteoblasts and

cytokines that regulate osteoclastogenesis. Thus, these observations suggest that

FIGURE 7. IFN-γ promotes matrix deposition in primary-cultured chondrocytes and osteoblasts. (A) Alcian blue staining of primary chondrocytes in the presence of IFN-γ, IL-17, TNF, or TGF-β (10 ng/ml each). Murine chondrocytes were prepared from costal cartilage of WT newborn mice and then subjected to micromass cultures for 7 d. (B) Quantitation of Alcian blue staining using ImageJ. Murine chondrocytes were cultured in the presence of IFN-γ, IL-17, TNF, or TGF-β (0, 0.4, 2, or 10 ng/ml) for 7 d. (C) Chondrogenic mRNA expressions in IFN-γ-treated chondrocytes. Total mRNA was extracted from primary chondrocytes with or without IFN-γ (10 ng/ml) on day 7. The levels of Pena, Sox9, Col2a1, and Mmp3 mRNAs were determined by real-time PCR. mRNA levels were normalized to Actb. (D) Alizarin red staining of primary osteoblasts supplemented with recombinant IFN-γ (0, 10, 100 ng/ml). Murine osteoblasts were prepared from calvaria of WT newborn mice and cultured for 14 d. The total area of alizarin red staining was analyzed using ImageJ. (E) Osteogenic gene expression in the treatment of IFN-γ at 14 d culture. The levels of Runx2, Osterix, Alp, and Bglap mRNAs were determined by real-time PCR. mRNA levels were normalized to Gapdh. Data are representative of two (B and C) or three (A, D, and E) independent experiments. Values are mean ± SD of triplicate cultures. *p < 0.05, **p < 0.01, ***p < 0.005 by unpaired two-tailed Student t test or one-way ANOVA with Tukey HSD post hoc test.
tially promote differentiation of IFN-γ-producing Th1 cells, but not Th17 cells, resulting in the development of autoimmunity (55, 56). Therefore, the defect in DCIR or its downstream effector SHP-1 causes spontaneous activation of DCs and selectively promotes IFN-γ-producing T cell differentiation by increasing expression of costimulatory molecules, resulting in the induction of excess bone formation and increase of bone volume. In particular, because IFN-γ production is significantly elevated in inflamed joints, enthesis may promote chondrogenesis and osteogenesis around entheseals, causing ankyloitic changes of joints. We also showed that bone volume is increased in young Dcir--/-- mice, suggesting that IFN-γ-producing T cells expand in Dcir--/-- mice even under physiological conditions. However, we cannot exclude the possibility completely that this mild increase in bone volume is a secondary effect of enthesis, in which IFN-γ-producing T cells are expanded. It is possible that invisible inflammation at entheseles in Dcir--/-- mice is already present in young animals, even though their joints apparently look normal.

Joint ankylosis was developed only in 20% of male Dcir--/-- mice by 12 mo of age, although the increase of bone mass and IFN-γ-producing T cells was observed in almost all Dcir--/-- mice. These findings suggest that additional factors, such as environmental cues, may be required for the development of ankylosis. In fact, mechanical stress at the enthesis and/or bacterial infections have been implicated in the pathogenesis of human AS (57, 58). The higher incidence of ankyloitic change in male Dcir--/-- mice than in female mice is similar to the prevalence ratio in human AS patients (59). Elevated levels of androgen dehydroepiandrosterone, which can stimulate Th1 response, have been reported in male patients with AS, suggesting that a sex steroid hormone may be a determinant of male/female difference (60). However, the precise roles of sex steroids in the pathogenesis of AS remain to be elucidated.

Despite intensive studies, it is still unclear how entheseal inflammation is coupled to ankylosis in AS. In this study, we showed that TNF initiates enthesis, and that IFN-γ produced during inflammation plays an important role in the development of ankyloitic changes in Dcir--/-- mice. Consistent with our results, treatment with anti-TNF and nonsteroidal anti-inflammatory drugs ameliorates inflammation in AS, but these drugs cannot suppress progression of ankyloitic bone changes in AS patients and animal models (61, 62). Although the pathology observed in the axial and peripheral joints of Dcir--/-- mice closely resembles that of AS in humans, the etiopathogenesis of AS patients and Dcir--/-- mice seems to be totally different: in contrast to Dcir--/-- mice, AS is closely associated with HLA-B27 and the IL-23/IL-17 axis, and endoplasmic reticulum stress may be involved in AS pathogenesis (63). However, Dcir--/-- mice may provide a disease model for the ankyloitic processes of AS. Expansion of IFN-γ-producing T cells has been observed in patients with AS and in animal models (64, 65), although their pathogenic roles are yet known. Therefore, IFN-γ may also play an important role in the ankyloitic process of AS in humans.

Taken together, our findings show that DCIR plays an important role in maintaining bone homeostasis. Specifically, bone formation is excessively enhanced in Dcir--/-- mice, causing increased bone mass and ankyloitic changes of the joints. We have shown that the development of ankylosis and increased bone mass is completely suppressed in Rag2<sup>−/−</sup> Dcir--/-- mice, suggesting involvement of an immune-mediated mechanism in the pathogenesis. Those pathologies were also suppressed in Tnf<sup>−/−</sup> Dcir--/-- and Ifng<sup>−/−</sup> Dcir--/-- mice, but not in Il17<sup>−/−</sup> Dcir--/-- mice. Furthermore, we showed that IFN-γ is responsible for the excess chondrogenesis and osteogenesis in vitro, and IFN-γ-producing T cells are selectively expanded in Dcir--/-- mice. Thus, DCIR and the downstream mediator IFN-γ are important regulators of bone metabolism, and drugs that control DCIR or IFN-γ activity should be effective for treatment of bone metabolic diseases.

Acknowledgments

We thank H. Kawahara for histopathological analysis, and all the members of the Ikawa laboratory (Tokyo University of Science) for excellent animal care.

Disclosures

The authors have no financial conflicts of interest.

References

**Supplemental Figure 1.** Histopathology of enthesitis in the ankle joint of aged \( Dcir^{-/-} \) mice.  
(A) Paraffin sections of the ankle joints from 12-month-old WT and \( Dcir^{-/-} \) mice were stained with H&E; and representative sections are shown. Images in the lower panels are magnified views (\( \times 20 \)) of the upper panels (\( \times 4 \)). Arrowhead indicates an inflammatory lesion. Tal, talus; Nav, navicular; Cun, cuneiform. Scale bar, 300 \( \mu \text{m} \) (upper) and 60 \( \mu \text{m} \) (lower).  
(B) Histopathology of enthesopathy in knee joint and thoracic vertebra of a 12-month-old \( Dcir^{-/-} \) mouse. Formalin-fixed and paraffin-embedded sections were stained with H&E. Representative sections are shown. Arrowheads indicate enthesopathy. Fem, femur; Tib, tibia; Body, vertebral body; Disk, intervertebral disk. Scale bar, 300 \( \mu \text{m} \) (knee) and 100 \( \mu \text{m} \) (thoracic vertebra).
**Supplemental Figure 2.** Osteoclastic and osteoblastic parameters of tibia in 8-week-old mice and unchanged properties of primary osteoblasts.

(A) Histological analysis of the distal metaphysis of tibia in 8-week-old WT (n = 4) and Dcir<sup>−/−</sup> (n = 6) mice. Paraffin sections of the tibia from 8-week-old mice were stained with TRAP, an osteoclastic marker. Osteoclastic parameters and osteoblastic parameters were measured by bone morphometric analysis. Representative sections are shown. Values are means ± SEM. (B) Alizarin red and von Kossa staining for *in vitro* mineralization of cultured osteoblasts. After 14 days in osteogenic medium, primary osteoblasts were subjected to Alizarin red and von Kossa staining. (C) Osteoblastic gene expression was quantitated in osteoblasts cultured in osteogenic medium for 0 or 7 days. Transcript levels of Runx2, Osterix, Alp, and Bsp were determined by real-time PCR analysis. (D) Effect of VD<sub>3</sub>, PGE<sub>2</sub>, and AA on osteoblast activation and differentiation. Primary osteoblasts were stimulated with VD<sub>3</sub> (10<sup>−8</sup> M) plus either PGE<sub>2</sub> (10<sup>−6</sup> M) or AA (50 μg/ml) for 24 h. Gene expression of Tnfsf11 (RANKL) and Tnfrsf11b (OPG) was determined by real-time PCR analysis. VD<sub>3</sub>, 1,25-(OH)<sub>2</sub> vitamin D<sub>3</sub>; PGE<sub>2</sub>, prostaglandin E2; AA, ascorbic acid. Gene expression is normalized to that of Gapdh (C and D). Values are means ± SD of triplicate cultures. Results are representative of at least three independent experiments (B–D).
**Supplemental Figure 3.** Frequency of IFN-γ–producing T cells in LNs and serum concentration of IFN-γ.

(A) Flow-cytometric analysis of IFN-γ+ T cells in brachial, axillary, and mesenteric LNs. Lymphocytes from 10-week-old WT (n = 5) and Dcir−/− mice (n = 6) underwent stimulation with PMA plus ionomycin for 5 h, and were then analyzed by intracellular staining. Data are presented here as the proportion of IFN-γ+ cells among CD4+ or CD8+ cells (gated on CD3+). *p < 0.05, **p < 0.01 by Mann-Whitney U-test. (B) Serum concentration of IFN-γ in WT and Dcir−/− mice. Serum IFN-γ from 12-week-old WT (n = 10) and Dcir−/− mice (n = 10) was determined by ELISA. Each symbol represents an individual mouse, and small horizontal lines indicate the means. Data are representative of three independent experiments.
<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Runx2</td>
<td>TGGTTCTCTGATCGCCTCAGTG</td>
<td>CCTGGGATCTGTAATCTGACTCT</td>
</tr>
<tr>
<td>Osterix</td>
<td>CGCTTTTGCCCTTTGAAAT</td>
<td>CCGTCAACGACGTTATGC</td>
</tr>
<tr>
<td>Alp</td>
<td>GGACAGGACACACACACACACA</td>
<td>CAAACAGGAGAGCCACTTCA</td>
</tr>
<tr>
<td>Bsp</td>
<td>ACAATCCGTGCCACTCACT</td>
<td>TTTTCATCGAGAAAGCACAGG</td>
</tr>
<tr>
<td>Tnfsf11</td>
<td>CAGCATCGCTCTGTTTCTGTA</td>
<td>CTGCCTTTTCATGGAGCTGCTCA</td>
</tr>
<tr>
<td>Tnfrsf11b</td>
<td>GGGCGTTACCCTGGAGATCG</td>
<td>GAGAAGAACCCATCTGGACATTT</td>
</tr>
<tr>
<td>Pena</td>
<td>TTTGAGGCACGCCTGATCC</td>
<td>GGAGACGTGAGACGAGTCCCAT</td>
</tr>
<tr>
<td>Sox9</td>
<td>GAGCCGGATCTGAAGAGGGA</td>
<td>GCTTGACGTGTTGCTGTTTC</td>
</tr>
<tr>
<td>Col2a1</td>
<td>GGGAGATTGGTCTCTGCGATGAC</td>
<td>GAAGGGGATCTCAGGGGTGG</td>
</tr>
<tr>
<td>Mmp3</td>
<td>ACATGGAGACCTTGTCCCTTTTG</td>
<td>TTGGCTGAGTGGTAGAGTCC</td>
</tr>
<tr>
<td>Bglap</td>
<td>CTGACCTCACAGATGCAAGGC</td>
<td>TGGTCTGATAGCTCGTCACA</td>
</tr>
<tr>
<td>Gapdh</td>
<td>TTCACCACCATGGAGAAGGC</td>
<td>GGCATGGACTGTGGTCATGA</td>
</tr>
<tr>
<td>Actb</td>
<td>CAATAGTGATGACCTGGCCGT</td>
<td>AGAGGGAAATCGTGCGTGAC</td>
</tr>
</tbody>
</table>

**Supplemental Table I.** Primer sets for quantitative real-time PCR.