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*J Immunol* published online 17 July 2013
[http://www.jimmunol.org/content/early/2013/07/17/jimmunol.1202194](http://www.jimmunol.org/content/early/2013/07/17/jimmunol.1202194)
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IL-17 is a pleiotropic cytokine produced by Th17 T cells that induces a myriad of proinflammatory mediators. However, different models of inflammation report opposite functional roles of IL-17 signal in terms of its effects on bone destruction. In this study we determined the role of IL-17RA signal in bone resorption stimulated by dentoalveolar infections. Infrabony resorptive lesions were induced by surgical pulp exposure and microbial infection of mouse molar teeth. IL-17 was strongly induced in periapical lesions in wild-type (WT) mice by 7 d after the infection but was not expressed in uninfected mice. Dentoalveolar infections of IL-17RA knockout (KO) mice demonstrated significantly increased bone destruction and more abscess formation in the apical area compared with WT mice. Infected IL-17RA KO mice exhibited significantly increased neutrophils and macrophages compared with the WT littermates at day 21, suggesting a failure of transition from acute to chronic inflammation in the IL-17RA KO mice. The expression of IL-1 (both α and β isoforms) and MIP2 were significantly upregulated in the IL-17RA KO compared with WT mice at day 21 postinfection. The development of periapical lesions in IL-17RA KO mice was significantly attenuated by neutralization of IL-1β and MIP2. Taken together, these results demonstrate that IL-17RA signal seems to be protective against infection-induced periapical inflammation and bone destruction via suppression of neutrophil and mononuclear inflammation.

The Journal of Immunology, 2013, 191: 000–000.

Various immune cell types, including Th17 cells, γδ T cells, neutrophils, and macrophages, secrete IL-17, a proinflammatory cytokine (1). IL-17 primarily binds to IL-17RA and initiates a proinflammatory signal, which is strongly involved in the progression of many autoimmune or inflammatory diseases in humans such as rheumatoid arthritis, systemic lupus erythematosus, asthma, and allograft rejection (2, 3). However, in terms of inflammatory bone destruction, opposite functional roles for IL-17 have been reported. In different rodent rheumatoid arthritis models, IL-17 was elevated in synovial fluid (4–6), and blocking of IL-17 reduced inflammation and bone damage, whereas IL-17 excess led to disease exacerbation (7, 8). In human periapical lesions, which are caused by an infection in the root canal system of teeth and ultimately result in alveolar bone loss, higher IL-17 levels and greater numbers of neutrophils were observed in symptomatic lesions compared with asymptomatic lesions (9). Additionally, IL-17 expression was higher in the gingival crevicular fluid and tissues of periodontitis patients compared with healthy control subjects (10–12). These findings strongly suggested that IL-17 plays a proinflammatory role in dentoalveolar infection and inflammation. However, the IL-17RA signal is protective in Porphyromonas gingivalis–induced marginal alveolar bone loss in mice (13, 14). In contrast, IL-17 mediates age-related alveolar bone loss in mice (15). Additionally, IL-17 knockout (KO) mice are resistant to develop experimental periapical lesions (16). These basic findings observed in gene-modified mouse models seem to be contradictory and are not always accordant with the clinical findings. Thus, the definitive role of the IL-17RA signal in dentoalveolar inflammation and bone loss is still unclear. In this study, we demonstrate that the IL-17RA signal is protective in the development of periapical lesions using a well-established mouse periapical lesion model (17) in IL-17RA KO and IL-17–neutralized wild-type (WT) mice. Our data also suggest that the IL-17RA signal potently regulating myeloid cell–mediated periapical inflammation.

Materials and Methods

Animals

C57BL/6 (WT) mice were obtained from Charles River Laboratories (Wilmington, MA). IL-17RA KO mice were provided by Dr. Sarah Gaffen (University of Pittsburgh) under authorization from Amgen (Seattle, WA). All animals were maintained in a specific pathogen-free environment at The Forsyth Animal Facility, in accordance with the guidelines of the Institutional Animal Care and Use Committee. All experimental protocols were approved by The Forsyth Institute Institutional Animal Care and Use Committee.

Induction of periapical lesions

Adult male mice, 8 wk of age, on day 0 were anesthetized via i.p. injection with ketamine HCl (80 mg/kg) and xylazine (10 mg/kg) and were placed

Received for publication August 8, 2012. Accepted for publication June 16, 2013.

This work was supported by grants from the Krakow Endodontic Research Fund (E.A., J.C.R.), the Norwegian Research Council (Leiv Eriksson Grant), the Japan Society for the Promotion of Science Strategic Young Researcher Overseas Visits Program for Accelerating Brain Circulation (to H.F.), as well as by National Institute of Dental and Craniofacial Research/National Institute of Health Grant DE099018 (to P.S.) and National Center for Research Resources/National Institutes of Health Grant RR027553 (to H.S.).

Address correspondence and reprint requests to Dr. Hajime Sasaki, Department of Immunology and Infectious Diseases, The Forsyth Institute, 245 First Street, Cambridge, MA 02142. E-mail address: hsasaki@forsyth.org

Abbreviations used in this article: Arg1, arginase 1; μCT, microcomputed tomography; iNOS, inducible NO synthase; KO, knockout; TRAP, tartrate-resistant acidic phosphatase; WT, wild-type.
on a jaw retraction board. The dental pulps of the mandibular first molars were exposed using an electric dental hand piece with a no. 1/4 round bur under a surgical microscope (MC-M92; Seiler Instrument, St. Louis, MO), as described previously (17). The pulp chambers were opened until the entrance of the canals could be visualized and probed with a size 6 endodontic file. Common human pathogens, including Prevotella intermedia (ATCC 25611), Streptococcus intermedius (ATCC 27335), Fusobacterium nucleatum (ATCC 23566), and Peptostreptococcus micros (ATCC 33270), were grown as previously described (18). A total of 10 µl (10^7 cells) of the pathogen mixture in prereduced anaerobically sterilized Ringer’s solution was inoculated into the pulp chamber and introduced into the root canal with the endodontic file. One group of mice from each strain served as noninfected controls. On days 1, 10, and 21 after pulp exposure, all groups of mice were killed and mandibles were isolated and dissected free of soft tissues. The left hemimandibles were fixed in fresh 4% paraformaldehyde in PBS and used for histological analysis. For the right hemimandibles, the periapical tissues surrounding the mesial and distal root apices were carefully extracted, together with surrounding bone in a block specimen under the surgical microscope. Periapical tissues were rinsed in PBS, freed of clots, weighed, and immediately frozen at −70°C for subsequent protein extraction.

**Ab treatment**

In one experiment, infected WT mice were treated with either rat anti-mouse IL-17 mAb (MAB421; R&D Systems, Minneapolis, MN) to neutralize major ligands of IL-17RA, including IL-17A and IL-17A/F heterodimer, or isotype-matched control IgG. Abs were i.m. injected (14 µg/mouse/time) on days −1, 2, 5, 8, 11, 14, and 17 relative to the pulp infection. In the other experiment, infected IL-17RA KO mice received either mouse anti-mouse IL-1α mAb (4-68; Leinco Technologies, St. Louis, MO), rat anti-mouse IL-1β mAb (609; Leinco Technologies, St. Louis, MO), rat anti-mouse MIP2 mAb (MAB452; R&D Systems), or isotype-matched control IgG. Abs were injected into caudal vein (50 µg/mouse/time) on days −1, 2, 7, 12, and 17 relative to pulp infection. Ab-treated mice were killed on day 21 postinfection and subjected to microcomputed tomography (µCT) and histology.

**µCT analysis**

Hemimandibles were scanned as previously described (18) using a compact fan-beam-type tomograph (µCT40; Scanco Medical, Basserdorf, Switzerland) providing a 10-μm nominal resolution. The most centrally located section, which included the distal root canal of the mandibular first molar and that exhibited a patent root canal apex, was selected for quantitation. The cross-sectional area of periapical lesions was selected with Adobe Illustrator (Adobe Systems, San Jose, CA) and measured with ImageJ (Wayne Rasband, National Institutes of Health, Bethesda, MD).

**Protein preparations and ELISA**

For protein extraction, frozen periapical tissue samples were disrupted in a cell lysis buffer (Cell Signaling Technology, Danvers, MA) supplemented with 50 µg/ml gentamicin (Sigma-Aldrich, St. Louis, MO) using FastPrep-24 with matrix A (both MP Biomedicals, Solon, OH). The supernatant was collected after centrifugation and stored at −70°C until assay. Assays for cytokines in extracts employed commercially available ELISA kits obtained from R&D Systems (DuosSets) and were used according to the manufacturer’s instructions to evaluate periapical tissue levels of IL-1α, IL-1β, IFN-γ, TNF-α, RANKL, MIP2, and Gro/KC. The concentration of each cytokine was calculated with reference to a standard curve constructed using recombinant cytokines provided with each kit. Results were expressed as picograms cytokine per milligram periapical tissue.

**Semiquantitative RT-PCR**

To generate IL-17A mRNA for positive controls, spleen cells were obtained from C57BL/6 mice. Mononuclear leukocytes were isolated by Ficoll-Paque centrifugation, washed in RPMI 1640 medium supplemented with 5% FBS, and adjusted to a concentration of 2 × 10^7/ml. Cells were stimulated with Escherichia coli LPS (1 µg/ml) and Con A (1 µg/ml) of 20 h in a humidified atmosphere of 5% CO2/95% air at 37°C. Whole cellular RNA from stimulated cells or from periapical tissue samples was carried out using TRIZol reagent and a PureLink total RNA purification kit (both from Invitrogen). The concentration of RNA was determined spectrophotometrically. cDNA was constructed from mRNA using reverse transcription as described (19). PCR primers specific for IL-17A were chosen using the Primer3 program (Whitehead Institute for Biomedical Research, Cambridge, MA); forward, 5’-CAGCACACGCT-GATCCAGGAC-3’, reverse, 5’-GGGTTCTTCTAGGGTGTCAG-3’ (538 bp). Twenty-eight cycles of PCR amplification was carried out using 1 µl cDNA from the reverse transcription reaction and other reagents as described (19). The β-actin gene served as a reference gene (forward, 5’-ACTGGGACGACATGGAGAAG-3’, reverse, 5’-CTTCACGGTGTTGTTGGAAGG-3’) (384 bp). PCR products were visualized on 2% agarose gels under UV illumination after ethidium bromide staining.

**Macrophage culture**

Resident peritoneal macrophages were harvested from WT and IL-17RA KO mice as we previously described (18, 20). Macrophages (10^6 cells/well in 96-well plate) were stimulated with a mixture of fixed endodontic pathogens described above (10^6 cells/well) for 24 h. The level of IL-1α, IL-1β, and MIP2 in cell culture supernatants was determined by ELISA. The results were expressed as nanograms cytokine per milliliter supernatant.

**Histologic sample preparation**

Fixed hemimandibles samples used for µCT analysis were decalcified using 10% formic acid and sodium citrate, dehydrated in ethanol, and embedded in paraffin. Serial sections of 6-µm thickness were cut; every fifth sample was mounted and stained with H&E. Sections containing the region of interest (patent root canal with localized periapical lesion) were selected, mounted, and stained with tartrate-resistant acid phosphatase (TRAP). Methyl green staining was employed as counterstaining for the TRAP staining. Adjacent sections were used for immunohistochemical analysis. Identification of neutrophils, B cells, and macrophages were performed by help of the Abs Ly-6B.2 (dilution 1:4000; AbD Serotec, Oxford, U.K.), CD20 (1:200; Santa Cruz Biotechnology, Dallas, TX), F4/80 (dilution 1:150; Santa Cruz Biotechnology), respectively. Primary Abs were detected either with biotinylated goat anti-rat Ab or biotinylated rabbit anti-goat using Vector Elite ABC kits (Vector Laboratories, Burlingame, CA).

Of the primary Abs were used as negative controls and the sections showed no positive immune cell staining. Cell enumeration was carried out in grid by light microscopy at ×400 magnification.

**Statistical analysis**

Descriptive statistics, including the mean and SEs or SDs were calculated for all data. Statistical analysis was performed using one-way ANOVA and a Tukey multiple comparison test or Bonferroni post hoc test and an un-
paired t test for comparison between two groups using GraphPad Prism (GraphPad Software, San Diego, CA).

Results

Kinetic expression of IL-17 in periapical granulomas

To determine the kinetics of expression of IL-17 in periapical granuloma, pulpal infection was induced in WT mice. The expression of IL-17 gene and protein was kinetically determined on days 7 and 14 after the infection. As shown in Fig. 1A, the IL-17 gene was strongly induced in all samples in both observation periods, whereas it was not detectable in periapical tissues isolated from noninfected mice. Infection-induced IL-17 expression was also confirmed at a protein level by ELISA. As shown in Fig. 1B, in agreement with the gene expression results, IL-17 protein was significantly upregulated on days 7 and 14 (p < 0.01) compared with the uninfected controls. No significant difference was observed between days 7 and 14.

Effect of genetic deletion of IL-17RA and IL-17 neutralization on infection-induced bone destruction

IL-17 has been reported to exacerbate bone and cartilage destruction in models of experimental autoimmune arthritis (7, 8), but it was protective against alveolar bone loss stimulated by infection (14). Accordingly, we examined the role of IL-17RA signal in dentoalveolar infection using IL-17RA KO and corresponding WT mice. As shown in Fig. 2A, the size of periapical lesion was significantly upregulated in a time-dependent manner in both IL-17RA KO and WT mice. Although the extent of periapical lesion was similar by day 10 postinfection, the KO mice exhibited further extended periapical lesion (∼2-fold) compared with WT mice on day 21. This finding was confirmed in two other separate experiments (data not shown). We further confirmed this finding by functional neutralization of IL-17RA ligands in WT mice. As shown in Fig. 2B, animals treated with anti–IL-17 Ab had a significant increase of periapical inflammatory bone destruction compared with the control group treated with isotype-matched IgG (185% on average, p = 0.0006). Taken together, these results demonstrate that the role of the IL-17RA signal is probably protective in the development of periapical granuloma.

Effect of IL-17RA gene KO on osteoclasts and infiltrating immune cells

The effect of IL-17RA deficiency on osteoclasts and inflammatory cell infiltration in periapical granuloma was histologically examined. At day 10, no obvious histological difference was observed between the genotypes (data not shown). As shown in Fig. 3, all mice with pulp exposures had developed fibrous granulomatous tissue in the apical area, but the KO mice showed more severe inflammatory cell infiltration than did the WT littermates on day 21 after the pulpal infection.

The number of TRAP+ osteoclasts appeared to be increased in IL-17RA KO animals at day 10 (Fig. 3B), whereas at day 21 the bone surrounding the lesions was in part totally resorbed in the KO mice, making controlled comparisons between the two strains impossible. This observation indicates an elevated osteoclastic activity in the KO model. In general, most osteoclasts were associated with bone at the periphery of the periapical granuloma, although some TRAP+ cells were also present within the periapical granuloma, possibly associated with residual bone spicules.

As presented in Fig. 4A, immunohistochemistry revealed such an extensive apical infiltration of Ly-6B.2+ neutrophils in KO mice 21 d after infection that the enumeration of neutrophils was impossible. In WT mice, such severe infiltration of Ly-6B.2+ neutrophils was not observed in the lesioned area. Macrophages play a prominent role in periapical lesion development as an important source of chemotactic factors (21–23) and proinflammatory...
cytokines (24), and they are important in phagocytosis of apoptotic cells (25). Enumeration of F4/80+ cells (Fig. 4B) revealed that macrophages in WT mice were significantly reduced on day 21 versus day 10 postinfection ($p < 0.05$). Such reduction of macrophages was not observed in the KO mice. We also examined the ratio of iNOS+ cells to Arg1+ cells within lesion areas where F4/80+ cells were numerous. The former represents classically activated M1 macrophages and the latter alternatively activated M2 cells. As presented in Fig. 4C, we observed a trend that infected IL-17RA KO mice consistently exhibited an M2-dominant macrophage profile (ratio of $< 1$). The ratio difference between the two genotypes was chronologically increased by day 21 after infection. These findings indicate that the IL-17RA signal seems to play an important role in accumulation and transition of myeloid cells in periapical lesions.

IL-17RA signaling also plays an important role in recruitment of B cells to the site of inflammation (26), and we therefore investigated whether the number of infiltrating CD20+ B cells was different in the KO versus WT mice. As shown in Fig. 4D, we observed that in the absence of IL-17RA activation in the former, a significant increase in the number of CD20+ cells was found 21 d after dental pulp infection.

**Mediator responses in inflammatory granulomas**

We confirmed the level of proinflammatory cytokines in infected mice by cytokine ELISA. As shown in Fig. 5, the production of IL-
1α and IL-1β was upregulated in a time-dependent manner in the infected KO mice. In contrast, the peak of IL-1α production in WT mice was at day 10 after infection, and the level declined thereafter. The progress of periapical inflammation did not affect IL-1β production during 21 d in the WT mice.

MIP2 is a strong chemoattractant of neutrophils (27). The IL-17RA KO mice expressed significantly higher levels of MIP2 (p < 0.001 versus WT), which may contribute to the elevated accumulation of neutrophils. This finding suggests that the IL-17 signal seems to be involved in the recruitment of neutrophils via upregulation of MIP2 in this periapical disease model. The endodontic infection did not modulate the level of another neutrophil chemoattractant, Gro/KC, in any mouse strain.

Alternatively, the effect of IL-17RA deficiency on the level of the proinflammatory cytokine TNF-α and the central Th1 cytokine IFN-γ was redundant in this model (data not shown), suggesting that these cytokines are unlikely to contribute to the active inflammation and upregulated bone resorption in the KO mice. The level of the osteoclast-inducing cytokine RANKL was not different between the strains 21 d after infection (data not shown).

**FIGURE 5.** IL-17RA KO mice exhibited elevated inflammatory cytokine expression profiles compared with WT controls. Protein extracts of periapical lesions were subjected to ELISA to determine IL-1 levels on days 1, 10, and 21 postinfection (n = 6–8/group). Additionally, the expression of neutrophil chemoattractant factors, MIP2 and Groo/KC, was quantified on day 21 (n = 10/group). Bars indicate means ± SEM. *p < 0.05, **p < 0.001 versus corresponding WT control group.

**Discussion**

The IL-17 cytokine family contains six members (IL-17A–F) and five receptors (IL-17RA–RE) (29). The major immunoregulatory ligand is IL-17A that primarily binds IL-17RA. Increasing evidence supports an essential role for the IL-17 family of ligands and receptors in tissue homeostasis in health and disease (1). Differing models of inflammation have reported opposite functional roles for IL-17 in terms of bone resorption. In rodent models of rheumatoid arthritis, IL-17 level was elevated in synovial fluid (4–6), and blocking of IL-17 reduced the inflammation and bone damage, whereas IL-17 excess led to disease exacerbation (7, 8).

In old mice, gingival IL-17 is upregulated by age-related reduction of Del-1, which is an antagonist of a neutrophil chemoattractant LFA-1, and triggers marginal periodontitis with elevated neutrophil accumulation (15). In the same study, IL-17 was also shown to be harmful in induction of ligature-induced bone loss in both young and old mice. In contrast, IL-17 is protective in reducing alveolar bone resorption in periodontal disease induced by F. gingivalis infection, possibly owing to defective neutrophil recruitment (13, 14). According to these findings, we used IL-17RA KO mice and IL-17 neutralization to determine the role of the IL-17RA signal in the development of periapical lesions. Our data suggest that the susceptibility of IL-17RA KO mice to lesion development appeared to be dependent on persistent active inflammation during a 21-d observation period compared with WT mice.

Periapical lesion is a bone-destructive dental disease caused by infection in the dental root canal system. In contrast to periodontal disease, in which the bacteria have to invade the periodontal tissue through the gingival epithelium barrier, endodontic infections involve direct tissue invasion of bacteria and may subsequently lead to different host immune responses. In the present study, infected IL-17RA KO and IL-17–neutralized WT mice exhibited increased periapical lesions. The results show that IL-17RA signaling was protective in the development of periapical lesions in both sit-
In WT mice, the number of macrophages was significantly reduced on day 21 compared with day 10 after infection, whereas the level remained elevated in IL-17RA KO mice in the same period. Furthermore, the level of IL-1α and IL-1β was not significantly changed in any observation period in the WT mice compared with IL-17RA KO mice, which exhibited increased expression of IL-1α and IL-1β from day 10. In the 21-d observation period, the up-regulation of both IL-1α and IL-1β in KO mice was >10-fold compared with its own controls. We previously reported that macrophages are prominent in inflammatory periapical tissues and produce large quantities of IL-1 (24, 31). Macrophages are also an important source of MIP2 (32) as a neutrophil chemoattractant in the present study. The production of these proinflammatory cytokines by IL-17RA KO macrophages in response to fixed endodontic pathogens in vitro was higher compared with that by WT cells. Thus, increased proinflammatory cytokines in periapical lesions in IL-17RA KO mice in this study appeared to be dependent on the elevated cytokine productivity and the number of macrophages.

The kinetics of macrophages and neutrophils in IL-17RA KO mice indicate a failure of resolution of inflammation. A serial transition of inflammation involving predominantly proinflammatory M1 and anti-inflammatory/profibrotic M2 macrophages is important for resolution of inflammation and tissue repair (33). IL-17RA KO mice exhibited prolonged infiltration of macrophages with an M2-dominant profile in response to endodontic infection. It is also likely that M2 macrophages can derive IL-1 as a pro-fibrotic cytokine (34, 35). However, IL-1β may play a harmful role in the development of periapical lesions in IL-17RA KO mice. A potential pathway for the susceptibility of IL-17RA KO to endodontic infection involving elevated proinflammatory cytokines and persistent neutrophil infiltration was further supported by our data showing a dramatic reduction of neutrophilic inflammation and periapical bone loss by neutralization of MIP2 and IL-1β in this mouse strain. Our data demonstrate that the IL-17RA signal contributes to the resolution of periapical inflammation.

We previously demonstrated that B cells are important in prevention of dissemination of endodontic infection (36). In this study, we observed an augmented mobilization of B cells in periapical lesions of IL-17RA KO mice. This finding is in agreement with...
inclusion of other immune cells, and effect of pathogen-dependent proinflammatory mechanisms is still unclear. In particular, the mechanism of upregulated proinflammatory cytokine production by IL-17RA KO mice. IL-17F utilizes IL-17RA and has similar biological effects compared with IL-17A (38). These factors might affect the discordant outcome.

We conclude that the IL-17 signal is protective in the development of periapical lesions depending on its regulation of myeloid cell-mediated inflammation. The detailed mechanism behind the IL-17 signal-mediated protection in periapical lesions is, however, still unclear. In particular, the mechanism of upregulated proinflammatory cytokine production by IL-17RA-null macrophages, inclusion of other immune cells, and effect of pathogen-dependent immunomodulation need to be determined in future studies.

Acknowledgments
We acknowledge Amgen, Inc. and Dr. Sarah Gaffen (Division of Rheumatology and Clinical Immunology, University of Pittsburgh School of Medicine) for providing IL-17RA KO mice. We also acknowledge Drs. Thomas Kohler (cubic-AG, Bruttisellen, Switzerland) and Ralph Müller (Institute for Biomechanics, ETH Zurich, Zurich, Switzerland) for μCT scanning. We thank Drs. Antonio Campos-Neto and Thomas Van Dyke (both from The Forsyth Institute) for helpful discussions. We are grateful to Susan Orlando for assistance in preparation of this manuscript.

Disclosures
The authors have no financial conflicts of interest.

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