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*J Immunol* 2011; 186:2602-2612; Prepublished online 7 January 2011; doi: 10.4049/jimmunol.1003370
http://www.jimmunol.org/content/186/4/2602

Supplementary Material
http://www.jimmunol.org/content/suppl/2011/01/07/jimmunol.1003370.DC1

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Th17 Cells, Not IL-17+ γδ T Cells, Drive Arthritic Bone Destruction in Mice and Humans

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The mechanism whereby IL-17 drives rheumatoid arthritis remains incompletely understood. We demonstrate that anti–IL-17 therapy in collagen-induced arthritis ameliorates bone damage by reducing the number of osteoclasts in joints. We found equal numbers of CD4+ Th17 and IL-17 producing γδ T cells in the joints of arthritic mice, and in vitro, both populations similarly induced osteoclastogenesis. However, individual depletion and adoptive transfer studies revealed that in vivo, Th17 cells dominated with regard to bone destruction. Unlike γδ T cells, Th17 cells were found in apposition to tartrate-resistant acid phosphatase positive osteoclasts in subchondral areas of inflamed joints, a pattern reproduced in patient biopsies. This localization was caused by Ag-specific retention, because OVA-primed Th17 cells showed a γδ T cell-like diffuse distribution. Because IL-23, as produced by osteoclasts, enhanced T cell-mediated osteoclastogenesis, we propose that Ag-specific juxtaposition is key to foster the molecular cross talk of Th17 cells and osteoclasts, thus driving arthritic bone destruction. *The Journal of Immunology, 2011, 186: 2602–2612.

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by immune cell invasion into the joint space, cytokine imbalance, inflammation of the synovial membranes, and progressive synovial fibroblast- and osteoclast-mediated joint destruction (1). There is mounting evidence that osteoclasts, synoviocytes, and immune cells communicate via cytokines and other signaling molecules. Factors derived from activated T cells are known to affect the differentiation and activation of pathogenic osteoclasts (2, 3). The role of IL-17, a T cell-derived inflammatory cytokine has been of particular interest recently because it was found that it exacerbates autoimmune diseases such as RA (4). The relevance of this cytokine in RA is highlighted by the current success of clinical trials with neutralizing Abs to IL-17 (5, 6). This cytokine has been described to orchestrate diverse immune functions such as elicitation of proinflammatory mediators from joint-resident cells, chemotraction of neutrophils, stimulation of receptor activator for osteoclastogenesis (RANKL) expression, a factor that is crucial for osteoclastogenesis, as well as mediate direct pro-osteoclastogenic effects (7–12).

In collagen-induced arthritis (CIA), disease was markedly suppressed in IL-17–deficient mice (13). Major sources of IL-17 in this model are CD4+ T cells (Th17) and IL-17–secreting γδ T cells. Although both cell types have the potential to exacerbate CIA (14, 15), the relative significance of IL-17+ γδ T cells and Th17 cells in arthritis remains to be shown (16–18).

A strong link between the proinflammatory activity of IL-23 and cells of the Th17 lineage has been established. IL-23 stabilizes the Th17 lineage, thereby generating large numbers of IL-17–producing effector cells (19). Consequently, IL-23–deficient mice are protected from autoimmune diseases such as CIA (20) and resistance correlated with an absence of IL-17–producing CD4+ T cells. In addition, there is evidence that IL-23 affects cells of the innate immune system. For example, IL-23 is required to induce IL-17A production from NKT cells, γδ T cells, and Thy1+ SCA1+ innate immune cells (21–25). The IL-23/IL-17 axis has been described to play a pivotal role in the autoimmune inflammation in joints. Indeed, in RA, IL-23p19 levels correlate with those of IL-17 in the synovial fluid and with IL-17, TNF-α, and IL-1β levels in the serum. Furthermore, synovial IL-23p19 levels were found to be greater in patients with bone erosions (26). Cross talk between IL-23 and IL-17 is particularly associated with the destruction phase of RA characterized by the T cell-mediated activation of osteoclastogenesis (27).

In this study, we identified the relative pro-osteoclastogenic contributions of IL-17–secreting γδ and CD4+ T cells in the CIA model and relate these findings to RA.

Materials and Methods

Mice

Eight- to 10-wk-old DBA/1 male mice (Janvier) were used in accordance with Swiss Federal and Cantonal Authorities.

Human samples

Synovial fluid from three patients (60–68 y old) with RA based on American College of Rheumatology criteria were obtained from the Felix-Platter and University Hospital Basel. Duration of RA ranged from 16 to
20 y. Patients were undergoing cortisone treatment and one received abateccept. Informed consent from patients was obtained, and studies were conducted under guidance/agreement from the Basel Ethical Commission. Paraffin-embedded RA synovial tissue from a patient with chronic RA (53 y) treated with prednisolone, diclofenac, and leflunomide was purchased from Asterand. Activities of Asterand and their collaborators were conducted in accordance with applicable laws, regulations, and ordinances. Human metacarpal joints were obtained by informed consent from five RA patients (four women and one man) undergoing joint replacement surgery under the terms of the ethical approval granted by the combined office of research ethics committee (CORERC No. 07/Q0411/30). Patients were aged 44–65 y of age at the time of surgery.

### CIA and histopathology

The CIA model and histopathology were performed as described previously by others (28). In brief, 100 μg bovine collagen type II (Chondrex, Redmond, WA) emulsified in complete Freund’s adjuvant was administered intradermally in the base of the tail of anesthetized DBA/1 mice. Twenty-one days later, a boost of collagen (100 μg) in PBS was given i.p. Mice generally developed arthritis shortly after boost. Histopathology was scored for bone erosion by two independent researchers with blinded samples: 0 = no bone erosion; 1 = mild (only surface erosion, not overall); 2 = moderate (moderate surface erosion, overall, and/or 1–2 breakthroughs of inflammatory cells into the bone marrow); 3 = strong (strong surface erosion, overall, and/or 1–2 breakthroughs of inflammatory cells in the bone marrow). Tartrate-resistant acid phosphatase (TRAP)-positive stained osteoclasts were assessed in tissue sections covering tarsus, tibia, calcaneus, and metatarsal bones. Infiltration of cells was scored on a scale of 0–3, depending on the amount of inflammatory cells in the joint cavity. Cartilage proteoglycan loss as assessed by Safranin O staining was: 0 = fully stained cartilage; 1 = 10–20%; 2 = 20–60%; 3 = 60–100% proteoglycan loss visible.

### Depletion of IL-17A in CIA

CIA mice were treated i.p. three times per week from days 19–40 with 200 μg/mouse of anti-mouse IL-17A (mAb 3-784-9; Novartis), anti-mouse IL-17A (mAb 421; R&D Systems), and mouse IgG1 and rat IgG2a control Abs.

### Depletion of γδ and CD4+ T cells in CIA

CIA mice were injected on day 28 with 500 μg/mouse anti-mouse CD4 (clone GK1.5), anti-γδ TCR Abs (clone UC7-1D3; BioLegend), or isotype control Ab (anti-chicken lysozyme IgG1; Novartis), followed by 200 μg/mouse every third day until day 40. Depletion efficacy was tested by FACS analysis of PBLs.

### Immunohistochemistry

For mouse, serial frozen unfixed sections were stained with H&E and TRAP according to standard techniques. Another serial section was mounted for immunohistochemistry (IHC) according to standard techniques. Rabbit anti-human IL-17 Ab (Santa Cruz Biotechnology) with biotinylated anti-rabbit IgG Ab (Vector Laboratories) was used for both human and mouse. Mouse anti-human CD4 (RPA-T4) purified anti-mouse CD4 (RM4-5), mouse anti-human γδ TCR (B1), and hamster anti-mouse γδ TCR (GL3) were all from BD Pharmingen.

### Microcomputed tomography analysis

Paws were scanned on a VIVA MicroCT40 machine (Scando Medical) with calibration settings of 70 kV, 200 mg HA, 4096 scaling, and diameter of 21 mm. Optical slice thickness was 10 μm. High-quality three-dimensional images of the calcaneus bone were produced.

### Isolation of cells from joints, spleen, and lymph nodes

Cells were digested with 0.125% Dispase II (Roche), 0.2% collagenase II, and 0.2% collagenase IV (Sigma-Aldrich) in HBSS for 75 min at 37°C and passed through a cell strainer. Spleen and draining lymph nodes were directly minced through a cell strainer. RBCs were lysed by incubating the preparations in 0.83% NH4Cl for 3 min on ice.

### Intracellular cytokine staining

For intracellular cytokine staining, 1 × 10^6 cells/ml were cultured at 37°C for 4 h in medium containing 5 μg/ml brefeldin A, 50 ng/ml PMA, and 0.5 μg/ml ionomycin (Sigma-Aldrich). After activation, cells were stained for intracellular cytokines using an intracellular cytokine staining kit (BD Pharmingen). Abs were purchased from BD Pharmingen or BioLegend. Before staining, Fc-blockade (anti-CD16/32) was performed. For FACS, T cell populations were first gated on CD45 and subsequently on CD3^+CD4^+ and CD3^+γδTCR^+CD4^−, respectively. For mouse, we employed the following Abs: FITC–IFN-γ, PE–IL-17, FITC–or PerCP-CED4, allopolyacycin-CD3, biotinylated γδ TCR, Alexa 488– or PerCP-CD4, PE-CD3, CD11b–PerCP, FITC–Ki-67, allopolyacycin-Gr1, streptavidin–PerCP, and streptavidin–allopolyacycin. For human, we used the following Abs: FITC–IFN-γ, PE–γδ TCR, PE–CD4, PerCP-CD3, and AlexaFluor674–IL-17A. Flow cytometry data were acquired on a FACSCalibur (BD Bioscience) and analyzed with FlowJo 6.42 software (Tree Star). Cell sorting was performed on a FACSaria II (BD Bioscience).

### Quantitative real-time PCR analysis of FACS-sorted T cells

FACS-sorted spleen, lymph nodes, or joint cells were placed in RNeasy Micro RNA Isolation Kit (Qiagen). cDNA was generated using the High Capacity cDNA RT Kit (Applied Biosystems). Quantitative real-time PCR (qRT-PCR) analyses were performed using a TaqMan Low Density Array (Applied Biosystems) with the following assays (assay ID): IL-17A (Mm00439618_m1), IL-17B (Mm00444686_m1), IL-17C (Mm00521397_m1), IL-17D (Mm01313472_m1), IL-17F (Mm00521423_m1), IL-23A (p19) (Mm00518984_m1), IL-23R (Mm00519943_m1), RANKL (Mm004191-08_m1), IL-1b (Mm01336189_m1), TNFα (Mm00435258_m1), IL-6 (Mm01999906_m1), IL-21 (Mm00517640_m1), IL-22 (Mm00444241_m1), retinoic acid-related orphan receptor α (RORa; Mm00443103_m1), RORγ (Mm01264022_m1), CCR6 (Mm09999114_s1), and 18S rRNA (Hs99999901_s1) for normalization.

### Adoptive transfer

A total of 1 × 10^6 FACS-purified γδ and CD4+ T cells from spleen and lymph nodes of CIA mice or CD4+ T cells from spleen and lymph nodes of collagen type II or OVA-primed mice (day 10) were labeled with CellTracker CM-DIL (Invitrogen) and injected i.p. into CIA recipient mice. Forty-eight hours later, animals were sacrificed, paws snap-frozen, and 20-μm sections generated on a cryostat (Microm).

### In vitro induction of osteoclasts

FACS-sorted γδ or CD4+ T cells (5 × 10^3) isolated from organs of arthritic mice were cocultured with bone marrow cells (2 × 10^6) for 6–10 d. In one experiment, FACS-purified γδ or CD4+ T cells were preincubated with 10 ng/ml recombinant mouse IL-23 for 24 h before coculture. Positive control was bone marrow cells incubated with M-CSF (10 μg/ml), RANKL (10 μg/ml), and IL-1α (5 μg/ml; R&D Systems). Tartrate-resistant acid phosphatase-positive cells were counted.

### Statistical analysis

Unless otherwise noted, data are presented as means with the error bars representing SEM. Data were analyzed either by two sample tests (t test for normally distributed data, and rank sum test for data that was either not normally distributed or the groups had unequal variance), or by ANOVA (one way for normally distributed data of equal variance or ANOVA on ranks when data were not normally distributed or of unequal variance). In addition, two-way ANOVA was used as appropriate (see figure legends). Post hoc tests for one-way ANOVA used Tukey’s test and for ANOVA on ranks used Dunn’s test, the latter was used because of unequal group sizes. Normality was detected by Shapiro–Wilk tests, and equal variance among groups was determined by an F-test. χ^2 test was used to determine the ratio of human T cells in proximity to bone. Global p value was set at 0.05.

### Results

Anti–IL-17A therapy protects bone destruction in CIA

IL-17 is known to be involved in the pathogenesis of arthritis. To understand the role of this cytokine with regard to bone destruction, we treated arthritic mice with two different neutralizing anti–IL-17A Abs (mAb 3-784-9 and mAb 421). Anti–IL-17A treatment before onset of clinical signs of disease potently inhibited disease severity (Fig. 1A). This was accompanied by a lower histopathological disease score with respect to inflammatory cell infiltrate, cartilage damage, and bone loss (Fig. 1B, 1C, Supplemental Fig.)
1). Because osteoclast-mediated bone destruction is an integral component of CIA, TRAP+ cells were quantified in paw sections. As expected, osteoclast numbers were significantly reduced in joints from anti–IL-17A–treated animals (Fig. 1D). This confirmed the relevance of IL-17A for the induction of osteoclast-mediated bone destruction in arthritis.

Arthritic joint-derived γδ and CD4+ T cells take on Th17 characteristics, express pro-osteoclastogenic cytokines, and induce TRAP+ osteoclast formation

Next, we defined the source of IL-17 in arthritic joints. We observed that in CIA, IL-17 is produced solely by γδ and CD4+ T cells (data not shown). To define the net contribution of both T cell populations, we characterized these cells on isolation from different locations during CIA. This enabled us to compare peripherally primed cells (in spleen and draining lymph nodes) with pathogenic effector T cells after invasion into arthritic joints. To evaluate any potential cross-contamination caused by preparative procedures when analyzing joint-derived lymphocytes, we determined the frequency of γδ and CD4+ T cells in purified bone marrow preparations. We found that the frequencies were low (~0.4% γδ and ~7.4% CD4+ T cells), and of these low percentages, ~7.6 and ~1.0%, respectively, were IL-17+ (data not shown).

Within the γδ T cell population, IL-17–producing cells were 3-fold enriched in arthritic joints as compared with the periphery. This enrichment was more pronounced for the CD4+ T cell fraction where a 14-fold greater accumulation of the percentage of IL-17+ cells was observed in the joint (Fig. 2A).

Although there were about 4-fold more CD4+ T cells than γδ T cells in the arthritic joints (Fig. 2B, left column), the number of IL-17–producing CD4+ and γδ T cells was almost equal (Fig. 2B, right column, 2C) because the latter population contained a greater proportion of IL-17+ cells compared with the CD4+ counterpart.

To gain insight into the effector mechanisms used by these two T cell types in CIA, we performed qRT-PCR analysis for expression patterns of Th17 lineage markers on FACS-sorted γδ and CD4+ T cells from the spleen, lymph nodes, and joints of CIA mice (Supplemental Fig. 2). γδ T cells in arthritic mice always displayed features of Th17 cells, most notably in inflamed joints. Both T cell populations when isolated from this tissue showed an upregulation of IL-17A, IL-17F, and IL-22, and transcription factors RORγt and RORα when compared with peripherally derived counterparts (Fig. 3A). Of note, both γδ and CD4+ T cells from arthritic joints expressed high and similar levels of IL-23R and the Th17-associated CCR6 (Fig. 3A).

Th17 cells display a pro-osteoclastogenic potential and enhance osteoclast activity via the induction of RANKL in osteoclastogenesis-supporting cells, thus linking T cell activation and bone destruction (29). However, the participation of IL-17–producing γδ T cells in this paradigm is not described. To compare γδ and CD4+ T cells for their potential to contribute to bone damage, they were isolated from the spleen, draining lymph nodes, or arthritic joints of CIA mice, and analyzed for the expression of cytokines known to be involved in osteoclast activation. In addition to IL-17, γδ and CD4+ T cells from the arthritic joints displayed a comparable expression pattern of other pro-osteoclastogenic factors such as RANKL, TNF-α, IL-1β, and IL-6 (Fig. 3B). To address the functional significance of these results, we assessed the bone pathogenic potential of γδ and CD4+ T cells. These cells were cocultured with bone marrow cells using TRAP staining to indicate de novo osteoclast formation. Because there is as yet no means to enrich for the IL-17+ subpopulations, we performed our studies with whole preparations of γδ and CD4+ T cells. Identical numbers of FACS-sorted γδ and CD4+ T cells isolated from the peripheral organs could induce some TRAP+ cells. However, in line with the previous molecular findings, both
γδ and CD4+ T cells exerted a higher potential to induce osteoclasts when derived from arthritic joints (Fig. 3C). We saw an equipotent ability of γδ and CD4+ T cells from arthritis joints to induce osteoclast formation despite the knowledge that a greater percentage of γδ T cells produce IL-17 in this organ, highlighting the fact that IL-17 may not be the only driver of osteoclastogenesis.

Because of experimental procedures, we cannot exclude that a low percentage of contaminating non-T cells such as macrophages and fibroblasts were contributing to the pro-osteoclastogenic signature of joint-derived T cell populations (Supplemental Fig. 2). Nevertheless, these experiments demonstrate that γδ T cells analogous to CD4+ T cells exhibit an IL-17–biased and pro-osteoclastogenic signature especially on isolation from arthritic joints and have the potential to compromise bone integrity.

Depletion of CD4+ T cells protects bone damage in CIA
The in vitro data could not entirely exclude pro-osteoclastogenic effects from contaminating cells. Thus, to unequivocally define the individual relevance of the pathogenic capability of γδ and CD4+ T cells in vivo, cell type-specific depletion studies were performed on early onset of disease in arthritic mice. These experiments revealed a strikingly distinct role for these T cell populations. Whereas ablation of γδ T cells delayed disease, albeit nonsignificantly, depletion of CD4+ T cells suppressed arthritis progression (Fig. 4A, Supplemental Fig. 3A, 3B). This was reflected in the histopathological and microcomputerized tomography analysis where anti-CD4+ T cell treatment led to a strong protection of bone integrity (Fig. 4B–D). These dichotomous in vivo results were unexpected because our previous experiments revealed comparable numbers of IL-17–producing CD4+ and γδ
T cells in the arthritic joints (Fig. 2B) with in vitro capacities to affect bone integrity (Fig. 3C). In histopathological joint sections, TRAP+ osteoclasts were prominent within areas of high bone turnover in control and anti-γδ T cell-treated animals. However, ablation of CD4+ T cells led to a drastic reduction in osteoclast number (Fig. 4E). This indicates that presence of CD4+ but not γδ T cells is an essential component for osteoclast activity in murine arthritis.

Differential localization of γδ and CD4+ T cells within the arthritic joint

To define the mechanism behind these in vivo findings, we investigated whether there may be a differential localization of γδ and CD4+ T cells within the inflamed tissue. Labeled γδ or CD4+ T cells from collagen-primed animals were adoptively transferred into arthritic recipients to assess their migration patterns. Although both cell types possessed comparable capacities to migrate to the spleen (Supplemental Fig. 4A, 4B), there were markedly less total γδ T cells infiltrating the joint (Fig. 5A), in line with our findings in Fig. 2B. These adoptive transfer studies revealed major differences in the localization of γδ or CD4+ T cells within the inflamed joint. Although about half of the CD4+ T cell population was found in close proximity (within 100 μm) to bone, only 10% of the γδ T cells could be identified in this area (Fig. 5B, 5C, Supplemental Fig. 4C). Histopathological analysis of serial sections revealed that the labeled γδ T cells were predominantly positioned near soft tissue structures such as tendons whereas osteoclasts and other bone-associated cells were absent. Thus, although γδ T cells were found in areas of IL-17 staining, they were not associated with TRAP+ osteoclasts (Fig. 5C, left column, Supplemental Fig. 4C). In contrast, many fluorescently labeled CD4+ T cells (confirmed by IHC analysis in Supplemental Fig. 4D) could be found adjacent to TRAP+ osteoclasts in areas of IL-17 positivity (Fig. 5C, middle and right columns). However, we assume that not all of the CD4+ T cells were positive for this cytokine.

One major difference between γδ and CD4+ T cell subsets is their responsiveness to the priming Ag collagen type II, with only the latter mounting an Ag-specific response (30, 31). Proliferation (thymidine incorporation) assays with CD4+ T cells were performed to monitor the collagen-specific recall response using splenocytes and lymphocytes from collagen-immunized DBA/1 animals. In both organs, an Ag-specific response with a stimulation index between 2 and 3 was routinely measured on incubation with 50 μg/ml collagen II over baseline (data not shown). To dissect whether Ag-specific retention of CD4+ T cells is an

FIGURE 3. Arthritic joint-derived γδ and CD4+ T cells take on Th17 characteristics, express pro-osteoclastogenic cytokines, and induce TRAP+ osteoclast formation. qRT-PCR analysis for the expression of (A) IL-17 signature genes and (B) genes involved in osteoclastogenesis on FACS-sorted γδ T cells and CD4+ T cells from spleen, lymph nodes, and joints of acute CIA mice. A and B, Mean ± SEM of two independent experiments. C, In vitro coculture of mouse bone marrow cells either left unstimulated (negative control [−]); stimulated with RANKL, IL-1α, and M-CSF (positive control [+]); or cultured in the presence of FACS-sorted γδ or CD4+ T cells from organs of acute CIA mice. TRAP+ multinucleated cells (MNC) were counted after 6–10 d. Data are average ± SEM of three independent experiments. Statistical analysis used ANOVA on ranks (overall p = 0.027) with Dunn’s test for comparisons versus control (cultures minus growth factor).
underlying cause for the observed phenotype, we performed adoptive transfer studies using CD4+ T cells from donors primed with collagen II or OVA in adjuvant. Although both Ag-primed CD4+ T cells had an equal capacity to migrate into the arthritic joints (data not shown), only the collagen type II-specific T cells associated with bone. In contrast, OVA-primed CD4+ T cells exhibited a similar localization pattern as the innate γδ T cells (Fig. 5D).

Because we have previously shown that IL-17–producing cells were enriched in joint versus peripheral CD4+ T cell fractions (Fig. 2), we also wanted to determine how many of the adoptively transferred collagen II- and OVA-primed peripheral CD4+ T cells became IL-17+ on invasion into arthritic joints. Although we managed to recover the transferred CFSE-labeled cells, their numbers were so low that a solid determination of IL-17 positivity was not possible.

These data suggest that γδ and CD4+ T cells have different functions in the pathogenesis of arthritis. Collagen-specific CD4+ T cells have, by nature of their localization, a greater capacity to interact with osteoclasts in vivo and thus accentuate joint pathology.

**CD4+ T cells in close apposition to osteoclasts in human RA**

To assess whether our findings translate to human RA, we analyzed γδ and CD4+ T cells in synovium and synovial fluid of RA patients. Analogous to studies by Ito et al. (18), we found that in the synovial fluid, the percentage of IFN-γ-positive γδ or CD4+ T cells far outnumbered those producing IL-17 (Supplemental Fig. 5). To study the localization of γδ and CD4+ T cell populations, we performed IHC on biopsy sections of RA synovial tissue showing bone destruction, vessel-rich regions, and inflammatory cell infiltrates. Similar to the CIA mouse model, there were less total numbers of infiltrating γδ versus CD4+ T cells in the inflamed tissue (Figs. 2B, 6A). Many CD4+ T cells were localized in perivascular cuffs in blood vessel-rich areas, whereas the γδ T cells were more disperse within similar regions (Fig. 6A). In addition, and in striking analogy to the mouse system, we found ~55% of CD4+ T cells in close proximity to bone (Fig. 6B), often in clusters surrounding multinucleated osteoclasts (Fig. 6C). γδ T cells were more diffusely distributed with only ~35% of the cells close to bone surfaces. Similar to the CIA model, IL-17 immunoreactivity was detected in proximity of the bone (Fig. 6D), highlighting the potential for bone-resorbing osteoclasts to respond to this secreted proinflammatory cytokine. These findings again point toward a differential contribution of γδ and CD4+ T cells to bone pathology in RA patients in analogy to the CIA model.

**IL-23 produced by osteoclasts enhances the osteoclastogenic profile of T cells**

Increasing evidence points toward a bidirectional cytokine communication between cells of the immune system and osteoclasts. In human RA, the relevance of the IL-23/IL-17 axis has been...
FIGURE 5. Differential localization of γδ and Ag-specific CD4+ T cells within the arthritic joint. A, Quantification of total infiltrating CM-DIL+ γδ and CD4+ T cells in CIA joints after adoptive transfer. Statistical analysis was a rank sum test. B, Percentage of bone-associated (within 100 μm) γδ and CD4+ T cells in CIA joints after adoptive transfer. A and B, Data are average + SEM. Blinded scoring of three midsections per inflamed paw with n = 3–6 animals per adoptive transfer group. Pooled data from two separate experiments. C, Histopathological analysis of joints from CIA recipient mice after adoptive transfer of γδ T cells (left column) or CD4+ T cells (middle and right columns). Photomicrograph showing H&E-stained frozen sections. Boxes in first row indicate areas displayed in rows 3–5. Influx of fluorescently labeled CM-DIL γδ T cells or CD4+ T cells. Dashed lines show outline of bone/tendon. Asterisk shows autofluorescence in the lower power CM-DIL images. IHC for IL-17 (faint staining in γδ T cell section [arrows]). Asterisk shows nonspecific staining of tendon with anti–IL-17 Ab. TRAP staining for presence of active osteoclasts. Scale bars, 50 μm. D, Percentage of bone-associated (within 100 μm) collagen II- or OVA-primed CD4+ T cells in CIA joints after adoptive transfer. Data are average + SEM. Blinded scoring of a midsection per inflamed paw with n = 6–7 paws per group. Statistical analysis for B and D was a two-way ANOVA using Tukey’s test for pairwise comparisons. B, bone; T, tendon.
described to be associated with the T cell-mediated bone destruction phase of the disease (26, 27). Studies with IL-23–deficient mice further support the requirement for this cytokine toward development of arthritis (20). We found that IL-23p19 mRNA was highly expressed in the joints of arthritic mice, whereas it was only marginally present in the periphery of arthritic animals and joints of naive mice (Fig. 7A). By RT-PCR, we found expression of IL-23p19 mRNA on FACS-sorted cells from arthritic joints. Monocytic cells (Gr1med CD11bhigh) and neutrophils (Gr1high CD11bhigh) were positive for this cytokine (Fig. 7B).

Because osteoclasts are derived from the monocytic lineage, we were interested to investigate whether IL-23 is also produced by the mature bone-resorbing cells. A mixture of IL-1β, RANKL, and M-CSF induced de novo generation of TRAP+ osteoclasts, which was accompanied by a significant upregulation of IL-23p19 mRNA as the cells matured in culture (Fig. 7C).

It is known that IL-23 is essential to generate pathogenic IL-17+ effector T cells (19). Indeed, conditioning peripheral CD4+ T cells with recombinant mouse IL-23 overnight led to a significant increase in IL-17 production (data not shown) and an enhancement of TRAP+ osteoclasts on coculture with bone marrow cells (Fig. 7D). We conclude that there is a cross talk between IL-17+ Ag-specific CD4+ T cells and IL-23–producing osteoclasts that leads to aggravated bone erosion (Fig. 8).

Discussion

We demonstrate that in arthritic joints, collagen II-specific Th17 cells rather than IL-17+ γδ T cells are the main effectors that trigger osteoclast-mediated joint destruction in an IL-23–dependent manner.

Pathogenic bone erosion in RA is mediated by the mobilization and subsequent differentiation of osteoclast precursors within the
inflamed synovial tissue. Active osteoclasts localize at the boundary between synovium and articular bone, leading to erosion (32). These cells require complex cytokine signals from surrounding stroma and immune cells, to generate and maintain osteoclast activity (2). In RA patients, pro-osteoclastogenic cytokines such as RANKL, IL-23, and IL-17 are increased in synovial fluid and synovium compared with osteoarthritic and normal counterparts (11, 26, 27, 33, 34), and are predictive for bone destruction (35).

We confirmed that blockade of endogenous IL-17A significantly reduced bone erosion in arthritic mice (28, 36, 37), and that \( \gamma^d \) and CD4+ T cells are responsible for production of this cytokine. Furthermore, both T cell populations, when derived from the arthritic joint, displayed a remarkably similar in vitro profile with respect to Th17 gene signatures; however, because our study inhibited only IL-17A, we cannot exclude an additive effect by blocking other Th17-associated cytokines.

Both joint-derived CD4+ and \( \gamma^d \) T cells similarly upregulated pro-osteoclastogenic genes including RANKL and induced osteoclastogenesis in vitro cocultures, revealing a comparable potential to destroy bone. IL-17 can exert its osteoclastogenic effects via RANKL (11), but the cellular source thereof is under debate (3). Sato et al. (29) used in vitro polarized Th17 cells and showed that these induced osteoclastogenesis only in the presence of osteoblasts. In our studies, we used cell populations isolated from arthritic animals to show ex vivo osteoclastogenic capabilities of CD4+ and \( \gamma^d \) T cells, which may highlight a direct function for T cells in the disease situation, in line with a role of IL-17 in osteoclastogenesis (12). Interestingly, the mRNA of IL-1b, although rarely recognized as a product of T cells, was present in low and high levels in the spleen and joint-derived T cell populations, respectively. However, studies in other disease settings have indicated that IL-1 may be produced by T cells (38–40). Therefore, we cannot eliminate the possibility that Ag-specific T cells are producing this cytokine in the CIA model.

Despite the many aforementioned similarities of the IL-17–producing CD4+ and \( \gamma^d \) T cells, our studies highlighted the particular importance of CD4+ T cells over \( \gamma^d \) T cells for bone pathology. Ag-specific retention appeared to account for the

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**FIGURE 7.** Osteoclast-derived IL-23 enhances the osteoclastogenic profile of T cells. A, qRT-PCR analysis for IL-23p19 mRNA from total cell preparations of arthritic spleen, lymph nodes, joints, and naive joints. Data show mean ± SEM from \( n = 2–4 \) mice. B, qRT-PCR for IL-23p19 mRNA on FACS-sorted inflammatory cells from arthritic joints and periphery of CIA mice. Typical FACS plot showing gates for neutrophils and monocytes. One representative experiment with mean ± SEM of three independent experiments is shown. C, qRT-PCR for IL-23mRNA on de novo formed osteoclasts from mouse bone marrow cultures ± RANKL, IL-1\( \alpha \), and M-CSF. Statistical analysis is by Student t test. Representative TRAP stained sections showing cultures ± growth factors. Scale bars, 10 \( \mu \)m. Insert in bottom right panel shows the multinuclei from TRAP+ multinucleated cells. Scale bar, 5 \( \mu \)m. D, Bone marrow cells either left unstimulated (negative control [−]); stimulated with RANKL, IL-1\( \alpha \), and M-CSF (positive control [+]); or cocultured with isolated peripheral CD4+ T cells from arthritic mice that had been incubated with or without recombinant IL-23 (10 ng/ml) for 24 h before the initiation of coculture. TRAP+ multinucleated cells were counted. Data from one experiment with three to four replicates. Statistical analysis is by ANOVA with Tukey.

**FIGURE 8.** Self-perpetuating feedback loop in CIA. 1, Peripheral CD4+ and IL-23R+ \( \gamma^d \) T cells release low levels of cytokines. 2, On formation of the immunological synapse with Ag-presenting osteoclasts, IL-17+ collagen-specific CD4+ T cells stay at bone surfaces. 3, IL-17+ CD4+ T cells differentiate/activate osteoclasts via osteoclastogenic cytokine release. 4, IL-23+ osteoclasts, in turn, maintain/enhance the pathogenic capacity of IL-17+ CD4+ T cells generating a positive feedback loop of joint destruction. 5, IL-17+ \( \gamma^d \) T cells recruit IL-23–producing neutrophils to the joint cavity.
accumulation of CD4+ T cells to the collagenous surfaces of bone and cartilage in the CIA mouse because CD4+ T cells primed for an irrelevant epitope were less frequently found near bone. Indeed, osteoclasts may serve as APCs: they express MHC class I and II, CD80, CD86, and CD40, and have the capacity to present soluble Ags and activate T cells (41). We speculate that in CIA, osteoclasts, among other APCs, present peptides to CD4+ T cells leading to their subsequent activation and local retention on formation of a stable immunological synapse. This preferential retention of Ag-specific Th17 cells in the arthritic joint may explain the enrichment of IL-17+ CD4+ T cells in arthritic joints and in proximity to the bone.

In human RA, the close proximity of CD4+ T cells and osteoclasts in areas of IL-17 production within the rheumatoid synovium closely resembled our observations in the CIA model. This would imply that targeting CD4+ T cells might be a successful therapeutic strategy for RA. However, clinical RA trials with depleting anti-CD4 Abs were not successful (42, 43). This has been attributed to inadequate dosing, duration of treatment, or because the anti-CD4 Abs did not reach the synovium (44). Nevertheless, the therapeutic principle of interference with the biological synapse has been validated clinically by the successful use of CTLA4-Ig in RA patients (45). Whether formation of the biological synapse is blocked in the joint or in the lymphoid organs is not known. In two clinical trials, however, structural joint changes were significantly slowed down in patients treated with abatacept versus placebo controls (46, 47), pointing toward a potential effect of the inhibitor at the T cell–osteoclast interface. The relevant pathogenic Ag(s) in RA remain under debate (48).

However, our data and the results from the abatacept trials suggest that bone-derived Ags are involved in pathology, at least within the joint, and that bone-associated CD4+ T cells may be key contributors toward bone erosion in RA.

Although under nonpathological conditions IL-23 has been described to have a protective effect on bone mass via its inhibitory effect on osteoclastogenesis (49), a pathogenic role for the IL-17/IL-23 axis is well established in autoimmune diseases, including arthritis. In a pathogenic setting, IL-23 is known to enhance the effector functions of Th17 cells (19, 20, 50, 51), as well as IL-23 production by γδ T cells (18, 22, 52). Our data are in line with studies by Yago et al., who demonstrated that IL-23 stimulates human osteoclasts, among other APCs, present peptides to CD4+ T cells (19, 20, 50, 51), as well as IL-17 (53). We therefore speculate that CIA, as well as RA, IL-17 produced by CD4+ T cells plays the dominant role with respect to osteoclast function and bone destruction. It is still too early to evaluate the prevention of structural damage with anti–IL-17 therapies in clinical RA trials; however, our findings would suggest that a bone-protective mechanism is involved.

Acknowledgments

We thank Rita Nagele, Grazyna Wieczorek, Nadja Mamber, Melanie Ceci, Catherine Huck, and Kathrin Wagner for technical assistance and Dr. Daniele Benz for provision of human samples. We also thank Dr. José Carballo for fruitful scientific discussions.

Disclosures

B.P., T.J., B.M., C.A., S.B., R.K., F.R., F.D.P., T.O.R., D.D.P., and A.L.-E. are or were employees of Novartis Institutes for BioMedical Research and/or own Novartis shares.

References


Supplementary Figure Legends

**Suppl. Fig. 1 Histopathological analysis of anti-IL-17 therapy in CIA.** CIA mice were treated with isotype control (mouse IgG1, rat IgG2a) anti-IL-17 (mAB 3-784-9) and anti-IL-17 (mAB 421) antibodies. Individual scoring of histopathological analysis of inflammatory cell influx, bone erosion and cartilage destruction (proteoglycan loss) from histopathological sections of hind paws (6 animals per group).

**Suppl. Fig. 2 Purity control of FACS-sorted γδ and CD4 T cells from spleen lymph node and joint of arthritic CIA mice.** γδ (top) and CD4+ (bottom) T cells were FACS-sorted from the spleen, lymph node and joint of CIA animals by first gating on CD45 and subsequently on CD3+CD4+ and CD3+γδTCR+CD4- respectively. Numbers indicate percentage of cells within gate.

**Suppl. Fig. 3 Analysis of T cell depletion efficacy upon treatment with anti-γδ TCR and anti-CD4 antibodies.** (a) FACS-analysis of peripheral blood lymphocytes for anti-γδ TCR (left) and anti-CD4 (right) treated animals at the end of the experiment. n=3 animals were analyzed per group. The percentages within the lymphocyte population ± SEM are indicated. Student T test was performed for statistical analysis. (b) Representative FACS profile of peripheral blood lymphocytes from control IgG, anti-γδ T cell and anti-CD4 T cell treated animals.

**Suppl. Fig. 4 Both γδ and CD4+ T cells possess an equal capacity to migrate to the spleen.** a) Purity control of FACS-sorted γδ (top) and CD4+ (bottom) T cells from a mix of spleen and lymph node of collagen II-immunized mice. γδ and CD4+ T cells were FACS-sorted by first
gating on CD45 and subsequently on CD3^+CD4^+ and CD3^+γδTCR^CD4^- . Numbers indicate percentage of cells within gate. (b) Photomicrograph showing fluorescently labeled adoptively transferred T cells infiltrating the spleen. γδ T cells (top) and CD4^+ T cells (bottom). Size bar is 200 μm. (c) Histopathological analysis of joints from CIA recipient mice after adoptive transfer. Photomicrograph showing Hematoxylin and Eosin stained frozen sections. B is bone, T indicates tendon. Dashed lines show outline of bone/tendon. Influx of fluorescently labeled CM-DIL positive γδ or CD4^+ T cells (arrows). Only sporadic localization of γδ T cells near bone (top row), few CD4^+ T cells in proximity to tendon (bottom row). Scale bar is 50 μm. (d) All secondary antibodies showed no binding; a secondary antibody for IL-17 staining is shown as an example (top panel). Immunohistochemistry for CD4 is shown (bottom panel). Size bar is 50 μm.

**Suppl. Fig. 5 Synovial fluid from RA patients contains predominantly IFNγ producing T cells.** Intracellular co-staining for IL-17 and IFNγ in γδ and CD4^+ T cells from synovial fluid from patients with rheumatoid arthritis. The percentages ± SEM of IL-17 and IFNγ^+ positive cells are indicated. Student T test was performed for statistical analysis. For IFNγ^+ γδ versus CD4^+ T cells p=0.13, for double positive γδ versus CD4^+ T cells p=0.659.
### Suppl. Fig. 1

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<tr>
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Suppl. Fig. 2

γδ T cells

Spleen

Lymp node

Joint

CD3

γδ TCR

90.0%

79.2%

82.3%

CD4+ T cells

CD4

CD3

98.2%

99.2%

90.9%
Suppl. Fig. 4a

γδ T cells

Suppl. Fig. 4b

γδ T cells

Suppl. Fig. 4c

H&E

CM-DIL* cells

γδ T cells near bone

Suppl. Fig. 4d

Negative control

CD4+ T cells near tendon

CD4+ T cells