

# Activated $\gamma\delta$ T cells inhibit osteoclast differentiation and resorptive activity *in vitro*

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## Summary

Extensive evidence suggests that the immune system exerts powerful effects on bone cells, particularly in chronic disease pathologies such as rheumatoid arthritis (RA). The chronic inflammatory state in RA, particularly the excessive production of T cell-derived proinflammatory cytokines such as tumour necrosis factor (TNF)- $\alpha$  and interleukin (IL)-17, triggers bone erosions through the increased stimulation of osteoclast formation and activity. While evidence supports a role for IL-17 and TNF- $\alpha$  secreted by conventional CD4<sup>+</sup> T cells in RA, recent evidence in animal models of RA have implicated  $\gamma\delta$  T cells as a major producer of pathogenic IL-17. However, the capacity of  $\gamma\delta$  T cells to influence osteoclast formation and activity in humans has not yet been investigated widely. To address this issue we investigated the effects of  $\gamma\delta$  T cells on osteoclast differentiation and resorptive activity. We have demonstrated that anti-CD3/CD28-stimulated  $\gamma\delta$  T cells or CD4<sup>+</sup> T cells inhibit human osteoclast formation and resorptive activity *in vitro*. Furthermore, we assessed cytokine production by CD3/CD28-stimulated  $\gamma\delta$  T cells and observed a lack of IL-17 production, with activated  $\gamma\delta$  T cells producing abundant interferon (IFN)- $\gamma$ . The neutralization of IFN- $\gamma$  markedly restored the formation of osteoclasts from precursor cells and the resorptive activity of mature osteoclasts, suggesting that IFN- $\gamma$  is the major factor responsible for the inhibitory role of activated  $\gamma\delta$  T cells on osteoclastogenesis and resorptive activity of mature osteoclasts. Our work therefore provides new insights on the interactions between  $\gamma\delta$  T cells and osteoclasts in humans.

**Keywords:**  $\gamma\delta$  T cells, CD4<sup>+</sup> T cells, IFN- $\gamma$ , osteoclast, osteoimmunology

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## Introduction

Bone tissue is constantly remodelled during life through the activity of bone matrix-producing osteoblasts and bone-resorbing osteoclasts [1]. Osteoclasts are large multinucleated cells that are generated from the fusion of monocytic-lineage precursor cells, the process of which relies on the availability on two essential factors: macrophage colony-stimulating factor (M-CSF), which binds the c-Fms receptor expressed on osteoclast precursors [2], and receptor activator of nuclear factor  $\kappa$ B ligand (RANKL), which binds the RANK receptor [3].

In recent years a variety of studies have demonstrated that the immune system is involved intimately in the regulation of bone cells, with a particular focus on how T cells

interact with osteoclasts and influence their formation and/or activity. In chronic inflammatory disease states, such as rheumatoid arthritis (RA), the prevalent effect of activated CD4<sup>+</sup> T cells is stimulatory towards the formation and activity of osteoclasts, resulting in bone loss and degradation of joint architecture [4]. However, evidence surrounding the exact role of activated T cells for influencing osteoclast formation is currently conflicting, with numerous *in-vitro* studies reporting either pro- or anti-osteoclastogenic effects of activated CD4<sup>+</sup> T cells [5,6]. Activated CD4<sup>+</sup> T cells produce RANKL [4] and various cytokines, including interleukin (IL)-17 [7], tumour necrosis factor (TNF)- $\alpha$  [8] and IL-1 $\beta$  [9] that support osteoclast formation and activity through direct or indirect mechanisms. However, CD4<sup>+</sup> T cells also produce a variety

of cytokines with anti-osteoclastogenic effects, such as interferon (IFN)- $\gamma$  [6], granulocyte-macrophage colony-stimulating factor (GM-CSF) [10], IL-4 [11] and IL-10 [12].

While research has focused predominantly on the role of CD4<sup>+</sup> T cells in inflammatory arthritis, a recent study implicated a further subset of T cells, so-called  $\gamma\delta$  T cells, as important mediators of the disease pathology in the collagen-induced arthritis (CIA) model of inflammatory arthritis [13]. Through production of IL-17,  $\gamma\delta$  T cells drive bone loss by increasing the production of RANKL by osteoblasts and/or stromal cells and inducing sustained osteoclast formation. In humans,  $\gamma\delta$  T cells represent a small subset of T cells (up to 5% of the total peripheral blood T cells), which express a heterodimeric T cell receptor (TCR) composed of a particular  $\gamma$  and  $\delta$  chain, in contrast to conventional CD4<sup>+</sup> T cells that express a heterodimeric TCR composed of an  $\alpha$  and a  $\beta$  chain [14]. In support of a role of  $\gamma\delta$  T cells in the disease pathology of human RA,  $\gamma\delta$  T cells are present in the inflamed joints of rheumatoid arthritis patients [15,16], and have been shown to be capable of producing IL-17 upon activation under extreme polarizing conditions [17,18]. Furthermore, the activation of  $\gamma\delta$  T cells and its potential relevance to human health is of great clinical interest, particularly as the major subset of  $\gamma\delta$  T cells in human peripheral blood (V $\gamma$ 9V $\delta$ 2<sup>+</sup>) are activated by anti-resorptive nitrogen-bisphosphonate drugs (N-BPs) [19,20], which are used widely to treat a variety of bone diseases characterized by excessive osteoclast activity. However, currently the role of human  $\gamma\delta$  T cells for influencing osteoclast formation and activity has not been elucidated.

In this study we show that activated  $\gamma\delta$  T cells exert inhibitory effects on osteoclast formation and resorptive activity comparable to activated CD4<sup>+</sup> T cells, which is mediated primarily through production of IFN- $\gamma$  by activated  $\gamma\delta$  T cells. Despite producing a variety of pro-osteoclastogenic cytokines upon activation, freshly isolated  $\gamma\delta$  T cells consistently failed to produce IL-17 *ex vivo*, suggesting important functional differences between murine and human  $\gamma\delta$  T cells for influencing osteoclastogenesis in inflammatory conditions.

## Materials and methods

### T cell isolation and activation

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats obtained from healthy donors by density-gradient separation using Lymphoprep™ reagent (Axis-Shield, Dundee, UK). Buffy coats were provided by the Scottish National Blood Transfusion Service and all studies were approved by the North of Scotland Research Ethics Committee.  $\gamma\delta$  T cells and CD4<sup>+</sup> T cells were positively isolated using magnetic-activated cell sorting (MACS®) (Miltenyi Biotec, Bergisch Gladbach, Germany) using anti-

$\gamma\delta$ -TCR and anti-CD4-TCR beads, respectively. To increase the purity of  $\gamma\delta$  and CD4<sup>+</sup> T cell isolations, CD14<sup>+</sup> cells were depleted prior to T cell isolation, using anti-CD14<sup>+</sup> Dynabeads® (Invitrogen, Carlsbad, CA, USA). The purity of the isolated T cell subsets was assessed by flow cytometry, with routine purities of  $\geq$ 95% obtained for  $\gamma\delta$  T cells and >98% for CD4<sup>+</sup> T cells. Assessment of monocyte and B cell contamination in the  $\gamma\delta$  T cell cultures was investigated using anti-CD14-Pacific Orange (Invitrogen) and anti-CD19-phycoerythrin (PE)-CF594 (BD Biosciences, San Jose, CA, USA) antibodies, respectively, and was found routinely to be approximately 1% for both monocytes and B cells.  $\gamma\delta$  and CD4<sup>+</sup> T cells were activated with anti-CD3/anti-CD28-coated T-Activator Dynabeads® (Invitrogen) at a bead-to-cell ratio of 1:1 for 24 h, prior to incubation with autologous osteoclast precursors or mature osteoclasts. Activation of T cells was assessed using an anti-human CD69-PE antibody (BD Biosciences) and subsequent flow cytometric analysis using a LSR II flow cytometer (BD Biosciences).

### Isolation of osteoclast precursors and generation of mature osteoclasts

Osteoclast precursors were isolated from buffy coats from healthy donors using Lymphoprep™ density-gradient separation and cultured in  $\alpha$ -minimum essential medium (Sigma, St Louis, MO, USA) supplemented with 100  $\mu$ g/ml L-glutamine (Sigma), 100 U/ml penicillin/streptomycin (Sigma) and 10% (v/v) heat-inactivated fetal bovine serum (FBS). Osteoclast precursors were expanded prior to generation of osteoclasts as reported previously [21]. Briefly, osteoclast precursors were treated with 20 ng/ml recombinant human (rh)M-CSF (R&D Systems, Abingdon, UK) for 5–7 days. To stimulate osteoclast formation, osteoclast precursors were cultured in a 96-well plate at  $2 \times 10^4$  cells/well and treated with 2 ng/ml recombinant mouse (rm)RANKL (R&D Systems) for 5–6 days in the presence of  $5 \times 10^4$  autologous unstimulated or activated  $\gamma\delta$  T cells or CD4<sup>+</sup> T cells. Cells were subject to half-medium changes and supplemented with fresh cytokines every 48 h. In some experiments osteoclast precursor cells were incubated with 10% (v/v) conditioned media from  $\gamma\delta$  or CD4<sup>+</sup> T cells stimulated for 24 h with anti-CD3/anti-CD28-coated T-Activator Dynabeads® (Invitrogen) and 10 U/ml of IL-2 (Sigma-Aldrich, Gillingham, UK).

For cytokine neutralization studies, the following antibodies were used: affinity-purified polyclonal goat anti-human IFN- $\gamma$  antibody (10  $\mu$ g/ml; R&D Systems), anti-human GM-CSF (1  $\mu$ g/ml) and rat IgG2a isotype control (1  $\mu$ g/ml) (both eBioscience, Vienna, Austria).

### Osteoclast resorptive activity

Mature osteoclasts were generated on plastic for 5 days in the presence of 20 ng/ml rhM-CSF and 2 ng/ml rmRANKL,

as reported previously [21]. Following this period, differentiated osteoclasts were detached with trypsin and plated onto dentine discs (obtained from elephant ivory) in a 96-well plate, at a density of  $2 \times 10^4$  cells/well. Following a period of attachment (4 h), osteoclasts were co-cultured for 5 days with  $5 \times 10^4$  autologous activated  $\gamma\delta$  T cells or  $CD4^+$  T cells in the presence of 20 ng/ml rhM-CSF and 2 ng/ml rmRANKL. In some experiments mature osteoclasts were cultured in the presence of 10% (v/v) conditioned media from  $\gamma\delta$  or  $CD4^+$  T cells generated as described above. Cultures were subject to half-medium changes with fresh cytokines and conditioned medium added every 48 h. To assess osteoclast resorptive activity, images of the dentine discs were captured using reflected light microscopy and the total resorbed surface area of dentine was quantified using in-house software to establish a grey threshold to determine the area resorbed by osteoclasts (in black) and non-resorbed surfaces (in white).

### Immunohistochemistry

Osteoclast formation was assessed using immunohistochemical staining for expression of the vitronectin receptor (VNR) by mature osteoclasts using a mouse anti-CD51/CD61 antibody (AbD Serotec, Oxford, UK) and goat anti-mouse Alexa Fluor<sup>®</sup>-488 antibody (Molecular Probes, Carlsbad, CA, USA). Osteoclast formation was quantified by assessing multi-nucleated ( $\geq 3$  nuclei) osteoclast number using fluorescence microscopy after nuclear counterstaining with 4',6-diamidino-2-phenylindole (DAPI). To visualize polarized osteoclasts on dentine, the osteoclasts were fixed with 4% formaldehyde, prior to immunohistochemical staining for F-actin rings using tetramethylrhodamineisothiocyanate (TRITC)-phalloidin (Sigma-Aldrich) and quantification using fluorescence microscopy.

### Cytokine production

Conditioned medium generated from activated  $\gamma\delta$  T cells and  $CD4^+$  T cells was assessed for levels of IL-4, IL-10, IFN- $\gamma$ , TNF- $\alpha$ , IL-6 and IL-1 $\beta$  using a cytometric bead array (BD Biosciences) and analysed using a fluorescence activated cell sorter (FACS) Array flow cytometer. Production of IL-17 was determined using a R&D Systems Quantikine enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's instructions. Production of GM-CSF was assessed using a Milliplex Bead Assay (Millipore, Billerica, MA, USA) and a Bio-Plex 200 analyser (Bio-Rad, Hercules, CA, USA).

### Statistical analysis

Data were analysed using the Kruskal–Wallis one-way analysis of variance on ranks (SigmaPlot<sup>®</sup>11.0), with inter-

group comparisons analysed using the Wilcoxon matched-pairs rank test. *P*-values  $\leq 0.05$  were considered statistically significant.

## Results

### Activated $\gamma\delta$ T cells inhibit osteoclast formation

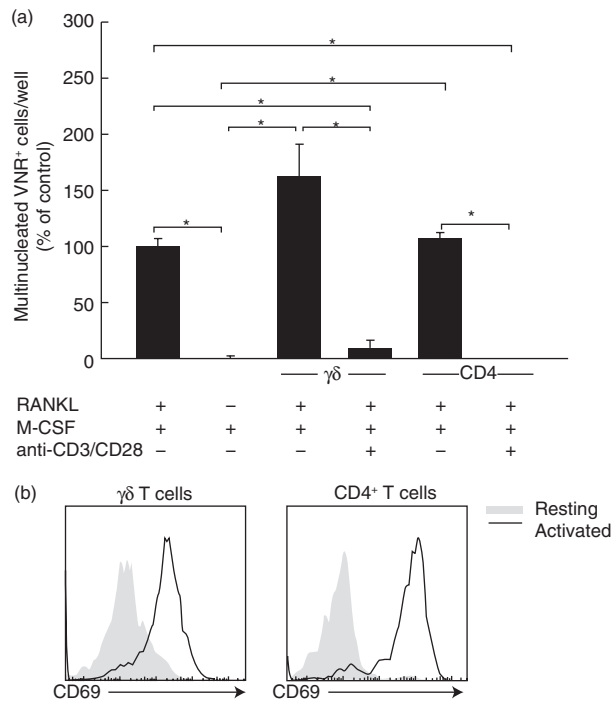
To determine the effects of  $\gamma\delta$  T cells on osteoclast formation, purified  $\gamma\delta$  T cells (unstimulated or activated with anti-CD3/anti-CD28-coated Dynabeads<sup>®</sup>) were cultured with autologous M-CSF-dependent osteoclast precursors for 6 days in the presence of M-CSF and RANKL. Because VNR is also expressed on activated macrophages [22], we used multi-nuclearity ( $\geq 3$  nuclei) in combination with VNR positivity as a determinant of osteoclast formation in our assays, as reported previously [23]. Co-cultures of osteoclast precursors and T cells were supplemented with exogenous RANKL as activated  $\gamma\delta$  or  $CD4^+$  T cells consistently failed to induce osteoclast formation in the absence of RANKL in our *in-vitro* assay system (data not shown). Osteoclast precursors were cultured with M-CSF alone to assess basal levels of spontaneous osteoclast formation.

The presence of unstimulated  $\gamma\delta$  T cells resulted in a non-statistically significant trend towards an increased number of VNR<sup>+</sup> osteoclasts compared to RANKL + M-CSF alone, suggesting a potential stimulatory effect of unstimulated  $\gamma\delta$  T cells on osteoclast formation (Fig. 1a), whereas unstimulated  $CD4^+$  T cells had no stimulatory effect on osteoclast formation. Conversely, the addition of anti-CD3/CD28 stimulated  $\gamma\delta$  T cells or  $CD4^+$  T cells (Fig. 1b) resulted consistently in a significant inhibition of multi-nucleated VNR<sup>+</sup> osteoclast formation (Fig. 1a). The marked inhibitory effect of activated T cells (both  $\gamma\delta$  and  $CD4^+$ ) on osteoclast formation was found to be independent of cell–cell contact, as the addition of 10% (v/v) conditioned medium from activated  $\gamma\delta$  T cells (Fig. 2a,c) or  $CD4^+$  T cells (Fig. 2b,c) was sufficient to markedly inhibit osteoclast formation. Furthermore, using Transwell inserts to isolate activated T cells from osteoclast precursors, we observed no decrease in the capacity of activated  $\gamma\delta$  T cells or  $CD4^+$  T cells for inhibiting osteoclast formation (Fig. S1).

### Cytokine production by activated $\gamma\delta$ T cells and activated $CD4^+$ T cells

To identify potential mediators of the inhibitory effects of T cells on osteoclast formation we assessed the levels of bone-active cytokines produced by unstimulated  $\gamma\delta$  and  $CD4^+$  T cells and following 24 h stimulation with anti-CD3/anti-CD28-coated Dynabeads (Table 1).

Following activation,  $\gamma\delta$  T cells and  $CD4^+$  T cells were found to produce significant levels of IFN- $\gamma$ , IL-10, TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and GM-CSF compared to unstimulated cells. GM-CSF production was not detected in unstimulated



**Fig. 1.** Activated  $\gamma\delta$  T cells and CD4<sup>+</sup> T cells inhibit osteoclast formation. (a) Quantification of osteoclast formation following incubation of osteoclast precursor cells with resting or activated  $\gamma\delta$  or CD4<sup>+</sup> T cells. Macrophage colony-stimulating factor (M-CSF)-dependent osteoclast precursors were cultured in the presence of M-CSF and receptor activator of nuclear factor  $\kappa$ B-ligand (RANKL) and co-cultured with resting or anti-CD3/CD28-stimulated  $\gamma\delta$  T cells or CD4<sup>+</sup> T cells, for 5 days. Osteoclast formation was assessed using immunohistochemical staining for vitronectin receptor (VNR) and counterstaining with 4',6-diamidino-2-phenylindole (DAPI). Multi-nucleated ( $\geq 3$  nuclei) VNR<sup>+</sup> cells per well were quantified using fluorescence microscopy. Data shown are the mean  $\pm$  standard error of the mean from three experiments from independent donors. \* $P < 0.05$ . (b) Activation of  $\gamma\delta$  T cells and CD4<sup>+</sup> T cells following activation with anti-CD3/CD28 T activator beads. Purified T cells were stimulated with anti-CD3/CD28 beads (1:1 ratio) for 24 h, prior to staining with anti-human CD69-phycoerythrin (PE) antibody and flow cytometric analysis. Data shown are from one experiment and are representative of two further experiments from independent donors.

CD4<sup>+</sup> T cells, but was increased markedly following activation, with levels increased markedly (~20-fold) compared to activated  $\gamma\delta$  T cells. While IL-4 production was not increased significantly in  $\gamma\delta$  T cells, CD4<sup>+</sup> T cells increased IL-4 production significantly following activation. The marked production of IFN- $\gamma$ , IL-4, IL-17 and IL-10 by activated CD4<sup>+</sup> T cells reflects the heterogeneous nature of cells in this population, i.e. T helper type 1 (Th1), Th2, Th17 and regulatory T cells (T<sub>reg</sub>). Interestingly, we observed marked production of IL-17 in activated CD4<sup>+</sup> T cells, but not activated  $\gamma\delta$  T cells, suggesting that  $\gamma\delta$  T cells isolated from

healthy volunteers do not produce IL-17 *ex vivo* following anti-CD3/CD28 stimulation. Of further note, unstimulated  $\gamma\delta$  T cells produced increased levels of IL-6 (~10-fold higher) compared to unstimulated CD4<sup>+</sup> T cells, which may explain the tendency of unstimulated  $\gamma\delta$  T cells to enhance RANKL-induced osteoclastogenesis (Fig. 1a). This is consistent with a previous study documenting the heterogeneity of  $\gamma\delta$  T cells for producing IL-6 in healthy donors [24].

### IFN- $\gamma$ is the primary mediator of the anti-osteoclastogenic effect of activated $\gamma\delta$ T cells

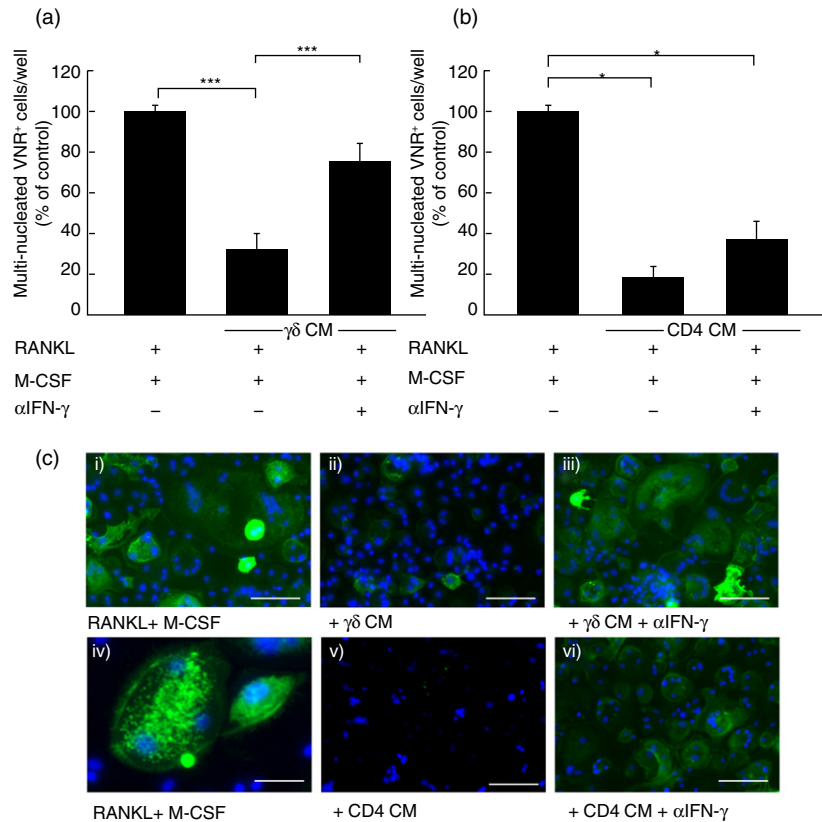
Following the observation that high levels of IFN- $\gamma$  are produced by activated  $\gamma\delta$  and CD4<sup>+</sup> T cells and this is reported to inhibit osteoclastogenesis [6], we sought to determine if neutralization of IFN- $\gamma$  could prevent the inhibitory effects of activated T cell conditioned medium. The addition of an anti-IFN- $\gamma$  antibody restored osteoclast formation significantly in the presence of activated  $\gamma\delta$  T cell conditioned medium (Fig. 2a,c), suggesting that the soluble factor responsible for the inhibitory effects of activated  $\gamma\delta$  T cell conditioned medium on osteoclastogenesis is IFN- $\gamma$ . Anti-IFN- $\gamma$  treatment only partially overcame the inhibitory effects of activated CD4<sup>+</sup> T cell conditioned medium on osteoclastogenesis (Fig. 2b,c) suggesting that, while IFN- $\gamma$  mediated some inhibitory effects of activated CD4<sup>+</sup> T cells, there are further inhibitory factors produced by CD4<sup>+</sup> T cells that potentially inhibit osteoclastogenesis.

A further potential mechanism through which activated T cells may inhibit osteoclast formation is through production of GM-CSF which, in the presence of RANKL, induces osteoclast precursors to differentiate down the dendritic cell lineage [10]. However, the neutralization of GM-CSF did not overcome the inhibitory effects of conditioned medium from activated  $\gamma\delta$  T cells (Fig. S2a) or CD4<sup>+</sup> T cells (Fig. S2b) on osteoclast formation. This suggests that IFN- $\gamma$  is the primary mediator of the inhibitory effects of activated  $\gamma\delta$  T cells on osteoclastogenesis, while diversion of osteoclast precursor differentiation towards the dendritic cell lineage by GM-CSF had no effect in our *in-vitro* assay system.

Given the powerful inhibitory effects of  $\gamma\delta$  T cells stimulated with anti-CD3/CD28 beads for affecting osteoclast formation, we then sought to determine whether  $\gamma\delta$  T cells activated with N-BPs [such as zoledronic acid (ZOL)] had similar inhibitory effects on osteoclast formation. ZOL-expanded V $\gamma$ 9V $\delta$ 2 T cells consistently inhibited RANKL-induced osteoclast formation, and even more pronounced effects on osteoclast formation were observed following the addition of 10% (v/v) conditioned medium obtained from these expanded cultures (Fig. S3). The inhibitory effects of ZOL-activated V $\gamma$ 9V $\delta$ 2 T cells (or conditioned medium from these cultures) on osteoclast formation could be markedly prevented following neutralization of IFN- $\gamma$ , indicating that IFN- $\gamma$  is also the major soluble factor responsible



**Fig. 2.** The blockade of interferon (IFN)-γ partially overcomes the inhibitory effect of activated γδ T cells, but not CD4<sup>+</sup> T cells. Macrophage colony-stimulating factor (M-CSF)-dependent osteoclast precursors were cultured in the presence of receptor activator of nuclear factor κB-ligand (RANKL) and co-cultured with 10% (v/v) conditioned medium (CM) from activated γδ T cells (a) or 10% (v/v) CM from activated CD4<sup>+</sup> T cells (b), in the presence or absence of α-IFN-γ. Following immunohistochemical staining for vitronectin receptor (VNR), cells were counterstained with DAPI and multinucleated (≥3 nuclei) VNR<sup>+</sup> cells per well were quantified using fluorescence microscopy. Data shown are the mean ± standard error of the mean from three experiments from independent donors. \**P* < 0.05; \*\*\**P* < 0.001. (c) Representative images showing anti-VNR staining (green) and nuclei (blue) of osteoclast precursor cells treated with M-CSF and RANKL (i), and higher magnification view in (iv); 10% (v/v) CM from activated γδ T cells alone (ii) or in the presence of α-IFN-γ (iii); 10% (v/v) CM from activated CD4<sup>+</sup> T cells alone (v) or in the presence of α-IFN-γ (vi). Scale bars are 50 μM (i–iii; v–vi) and 20 μM (iv).



for the inhibitory effects of ZOL-activated Vγ9Vδ2 T cells on osteoclast formation.

**Activated γδ T cells inhibit osteoclast resorptive activity**

To assess whether activated γδ T cells can influence osteoclast resorption, we seeded mature osteoclasts onto dentine discs, co-cultured them with γδ T cells or CD4<sup>+</sup> T cells for 6 days and assessed resorptive capacity. Both activated γδ (Fig. S4a) and CD4<sup>+</sup> T cells (Fig. S4b) reduced the osteoclast

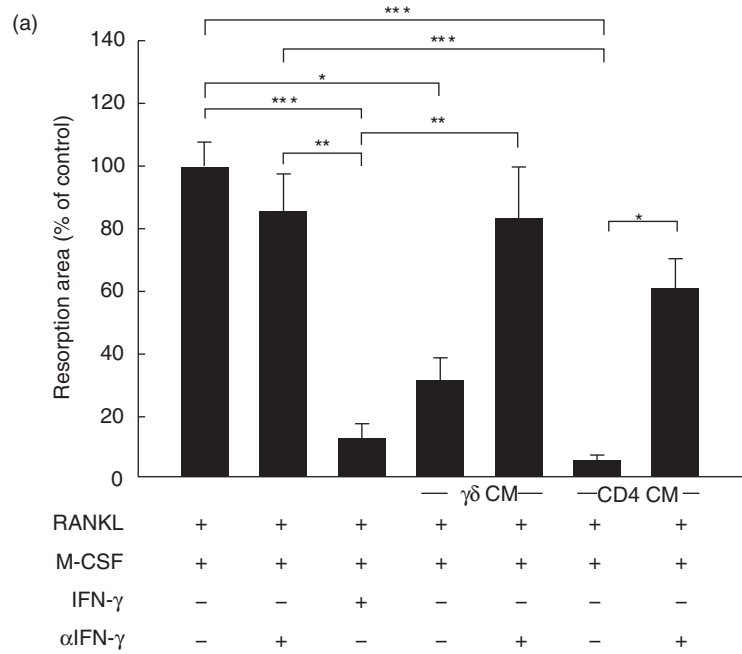
resorption area significantly by ~60 and 70%, respectively. To assess whether the observed inhibitory effects of activated T cells were again due to the release of soluble inhibitors we conducted similar experiments using activated T cell-derived conditioned medium. In the presence of either activated γδ T cell or CD4<sup>+</sup> T cell conditioned medium we observed markedly reduced resorptive activity compared to control osteoclasts (Fig. 3a,b).

Due to the powerful inhibitory role of γδ T cell-derived IFN-γ on osteoclastogenesis we assessed whether IFN-γ was

**Table 1.** Cytokine production from activated γδ and CD4<sup>+</sup> T cells.

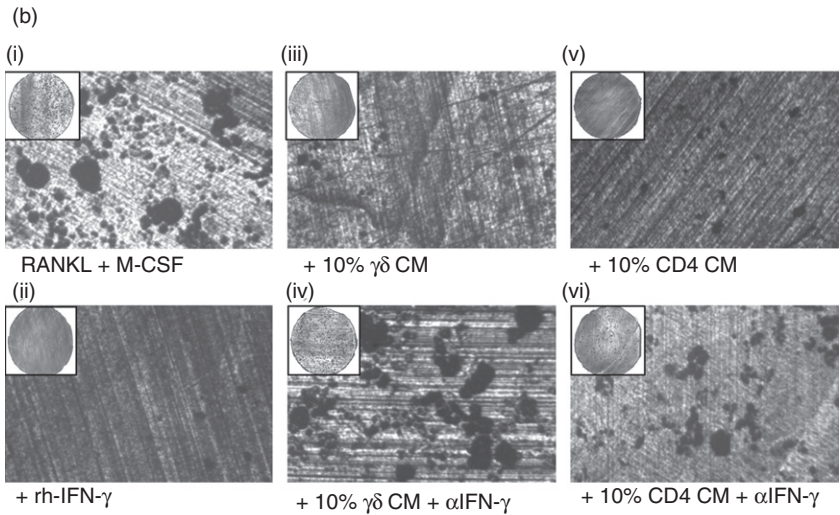
	Cytokine concentration (pg/ml)			<i>n</i>	Cytokine concentration (pg/ml)			<i>n</i>
	Resting γδ	Activated γδ	<i>P</i>		Resting CD4	Activated CD4	<i>P</i>	
IFN-γ	21.8 ± 8.0	1124.6 ± 513.3	<0.001	10	3.0 ± 2.2	1605.1 ± 423.9	<0.001	8
IL-4	3.8 ± 1.6	10.0 ± 2.6	n.s.	8	3.0 ± 1.8	107.6 ± 37.9	<0.001	8
IL-10	5.5 ± 0.9	30.7 ± 8.1	<0.05	9	2.5 ± 1.3	630.5 ± 173.9	<0.001	8
TNF-α	33.4 ± 8.5	1050.4 ± 256.9	<0.001	10	4.4 ± 2.1	2630.1 ± 440.4	<0.001	8
IL-1β	12.6 ± 2.8	54.9 ± 11.9	<0.05	6	2.4 ± 0.8	68.4 ± 21.8	<0.05	6
IL-6	151.7 ± 33.3	430.1 ± 131.1	<0.05	10	14.2 ± 4.4	327.6 ± 94.3	<0.05	8
IL-17	4.3 ± 4.2	13.5 ± 10.9	n.s.	16	9.7 ± 9.7	354.6 ± 118.5	<0.001	14
GM-CSF	0.7 ± 0.4	209.1 ± 60.9	<0.05	3	n.d.	4412.5 ± 1575.9	-	3

The production of pro- and anti-osteoclastogenic cytokines was determined for resting γδ and CD4<sup>+</sup> T cells, and following anti-CD3/anti-CD28 stimulation for 24 h. Production of interferon (IFN)-γ, interleukin (IL)-10, tumour necrosis factor (TNF)-α, IL-1β, IL-6 and granulocyte-macrophage colony-stimulating factor (GM-CSF) was assessed using a multiplexed cytometric bead array. IL-17 production was assessed by enzyme-linked immunosorbent assay (ELISA). Data shown are mean ± standard error of the mean from independent experiments from *n* number of independent donors (n.s. = not significant; n.d. = not determined).



**Fig. 3.** Activated  $\gamma\delta$  T cells and CD4<sup>+</sup> T cells inhibit the resorptive activity of mature osteoclasts. (a) Mature osteoclasts were seeded onto dentine discs and cultured with 10% (v/v) activated  $\gamma\delta$  T cell conditioned medium (CM) or activated CD4<sup>+</sup> T cell CM, in the presence or absence of  $\alpha$ -interferon (IFN)- $\gamma$ , for 6 days. 100 U/ml recombinant human (rh)-IFN- $\gamma$  (R&D Systems) was used as a positive control. Resorptive area was quantified using reflected light microscopy and in-house software. Data shown are the mean  $\pm$  standard error of the mean from three experiments from independent donors. \* $P$  < 0.05. (b)

Representative images showing osteoclast resorption pits on dentine discs: receptor activator of nuclear factor  $\kappa$ B-ligand (RANKL) and M-CSF alone (i); rhIFN- $\gamma$  (ii); 10% (v/v) CM from activated  $\gamma\delta$  T cells alone (iii), or in the presence of  $\alpha$ -IFN- $\gamma$  (iv); 10% (v/v) CM from activated CD4<sup>+</sup> T cells alone (v), or in the presence of  $\alpha$ -IFN- $\gamma$  (vi).

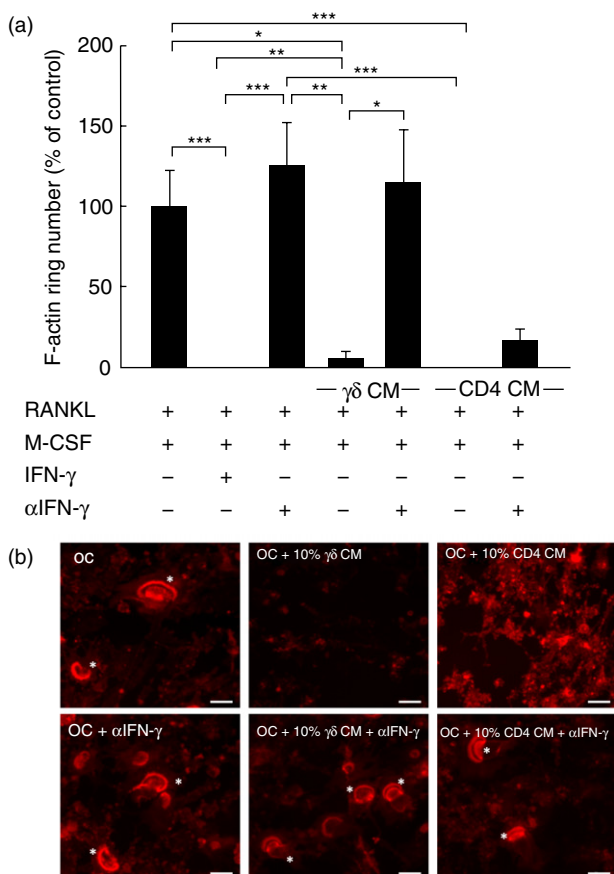


responsible for the inhibitory effects on osteoclast resorption. Neutralization of IFN- $\gamma$  resulted in the almost complete restoration of the resorption area in osteoclasts treated with activated  $\gamma\delta$  T cell conditioned medium and markedly overcame the inhibitory effects of CD4<sup>+</sup> T cell conditioned medium (Fig. 3a,b), suggesting that IFN- $\gamma$  is the predominant soluble factor responsible for the inhibitory effect of both activated  $\gamma\delta$  and CD4<sup>+</sup> T cell conditioned medium on resorption of mature osteoclasts. The marked inhibitory effect on osteoclast resorption was paralleled by a profound reduction in F-actin ring number in cultures treated with either activated  $\gamma\delta$  T cell conditioned medium or activated CD4<sup>+</sup> T cell conditioned medium (Fig. 4a,b), which was comparable in efficacy to rhIFN- $\gamma$  treatment, and thereby demonstrates the importance of F-actin ring formation for osteoclastic resorptive activity. In agreement with the quan-

tified resorption data, this inhibitory effect of  $\gamma\delta$  T cell conditioned medium on F-actin ring number was completely abolished when IFN- $\gamma$  was neutralized (Fig. 4a,b).

### Discussion

Numerous studies have reported that activated T cells are capable of influencing osteoclastogenesis either positively or negatively, with a recent focus on the stimulatory nature of the IL-17-producing Th17 subset of CD4<sup>+</sup> T cells on the pathological bone loss associated with chronic inflammatory disease states [7,25]. IL-17 stimulates RANKL expression by osteoblasts and stromal cells indirectly, thereby increasing osteoclastogenesis, and thus Th17 cells may play a pathological role in bone loss associated with chronic inflammatory disease states, such as RA. However, in the



**Fig. 4.** F-actin ring formation in osteoclasts is disrupted by interferon (IFN)- $\gamma$  derived from activated  $\gamma\delta$  T cells. Mature osteoclasts were seeded onto dentine discs, supplemented with receptor activator of nuclear factor  $\kappa$ B-ligand (RANKL) and macrophage colony-stimulating factor (M-CSF), and treated with 10% (v/v) conditioned medium (CM) from activated  $\gamma\delta$  or CD4<sup>+</sup> T cells, in the presence or absence of  $\alpha$ -IFN- $\gamma$ , for 6 days. (a) F-actin ring number was quantified by fluorescence microscopy following immunohistochemical staining using tetramethylrhodamineisothiocyanate (TRITC)-phalloidin. Data shown are the mean  $\pm$  standard error of the mean from three experiments from independent donors. \**P* < 0.05; \*\**P* < 0.05; \*\*\**P* < 0.001. (b) Representative images of osteoclasts (left panels) treated with CM from activated  $\gamma\delta$  (middle panels) or CD4<sup>+</sup> T cells (right panels), in the presence or absence of  $\alpha$ -interferon (IFN)- $\gamma$ . Asterisks indicate the F-actin rings. Images are from one experiment and are representative of two further experiments from independent donors. Scale bar = 50  $\mu$ M.

murine model of human RA, collagen-induced arthritis (CIA),  $\gamma\delta$  T cells were identified as a major cell type responsible for IL-17 production [13]. As yet, the contribution of  $\gamma\delta$  T cells to chronic autoimmune conditions in humans, particularly their capacity to influence osteoclastogenesis, is unknown.

In this study, we identified that activated  $\gamma\delta$  T cells inhibit the formation of mature osteoclasts from monocytic lineage precursors via a cell-contact independent mechanism.  $\gamma\delta$  T

cells were found to increase production of a variety of bone-active cytokines with both pro- and anti-osteoclastogenic effects following anti-CD3/CD28 stimulation, but failed consistently to produce IL-17. This was in marked contrast to CD4<sup>+</sup> T cells, which produced abundant IL-17 upon activation. Using neutralizing antibodies we determined that the inhibitory effect of activated  $\gamma\delta$  T cells on osteoclastogenesis was mediated predominantly via IFN- $\gamma$ , while IFN- $\gamma$  played a relatively minor role in the inhibitory effect of activated CD4<sup>+</sup> T cells. Finally, we identified that activated  $\gamma\delta$  and CD4<sup>+</sup> T cells inhibit the resorptive activity of mature osteoclasts, which is mediated predominantly by production of IFN- $\gamma$ .

To date, the effect of T cells on osteoclast formation has focused predominantly on conventional T cell subsets, such as CD4<sup>+</sup> T cells, while the effects of non-conventional T cell subsets, such as  $\gamma\delta$  T cells, for influencing osteoclastogenesis are currently ill-defined, particularly in humans. We have found that activation of  $\gamma\delta$  T cells with anti-CD3/CD28 stimulation results in potent inhibitory effects on osteoclastogenesis, with similar inhibitory effects observed with activated CD4<sup>+</sup> T cells. The inhibitory effects of activated CD4<sup>+</sup> T cells on osteoclastogenesis are consistent with previous studies [6,26], and highlight further the fundamental importance of route of T cell activation for influencing osteoclastogenesis. Using murine cells, Wyzga *et al.* demonstrated that the influence of activated CD4<sup>+</sup> T cells on osteoclastogenesis was crucially dependent upon the mechanism of activation, with anti-CD3 stimulation resulting in an inhibitory effect on osteoclastogenesis, whereas concanavalin A or phytohaemagglutinin treatment resulted in stimulatory effects. Thus, it is likely that the numerous conflicting studies documenting both stimulatory [5] and inhibitory effects [6] of activated T cells on osteoclastogenesis reflect the use of a variety of stimulation protocols, as well as the utilization of both murine and human assay systems.

By analysing cytokine production in both unstimulated and anti-CD3/CD28 activated  $\gamma\delta$  T cells and CD4<sup>+</sup> T cells we observed that, unlike their murine counterparts, purified human  $\gamma\delta$  T cells failed consistently to produce detectable IL-17 *ex vivo* following stimulation but produced abundant levels of IFN- $\gamma$ , thereby displaying a marked Th1 bias, as reported previously [27]. IL-17 production has been reported recently in the predominant subset of  $\gamma\delta$  T cells in human peripheral blood, V $\gamma$ 9V $\delta$ 2 T cells (also referred to as V $\gamma$ 2V $\delta$ 2), although typically only <1% of cells from healthy donors demonstrated IL-17 production following non-specific activation with phorbol myristate acetate (PMA) plus ionomycin [17]. Therefore, although it is apparent that V $\gamma$ 9V $\delta$ 2 T cells can acquire the ability to produce IL-17 following culture with a variety of Th17 differentiation-promoting cytokines [such as IL-1 $\beta$ , IL-6, IL-23 and transforming growth factor (TGF- $\beta$ )] [18], it is evident that freshly isolated human  $\gamma\delta$  T cells from healthy donors

produce IFN- $\gamma$  preferentially, with potential IL-17 release probably influenced by the method of stimulation and culture conditions. Thus, it is possible that in the local microenvironment of inflamed joints the abundance of proinflammatory cytokines, coupled with the liberation of TGF- $\beta$  from bone matrix due to excessive osteoclast activity, may provide a suitable environment for the generation of IL-17-producing  $\gamma\delta$  T cells.

In support of a pathological role for  $\gamma\delta$  T cells in human autoimmune disease,  $\gamma\delta$  T cells have been reported in the synovial fluid of RA patients [15,16,28], and an increased prevalence of IL-17-producing  $\gamma\delta$  T cells have been detected very recently in the peripheral blood of patients with ankylosing spondylitis [28]. Consistent with our findings, Kenna *et al.* demonstrated that anti-CD3/CD28 stimulation of  $\gamma\delta$  T cells resulted in abundant IFN- $\gamma$  production in the absence of increased IL-17 production in healthy controls, yet in ankylosing spondylitis patients the pattern was reversed, with a profound reduction in IFN- $\gamma$  release and a marked increase in IL-17 production, which was potentiated by IL-23 treatment [29]. However, recent studies in RA patients have suggested that IL-17 production is due exclusively to CD4<sup>+</sup> Th17 cells [30], with no IL-17-producing  $\gamma\delta$  T cells resident in synovial fluid or infiltrating the rheumatoid synovium [31]. Therefore, despite compelling evidence for a pathological IL-17-producing role of  $\gamma\delta$  T cells in murine experimental arthritis, to date the evidence for a pathological role of  $\gamma\delta$  T cells in stimulating bone loss associated with chronic inflammatory diseases is currently lacking. Further studies are therefore warranted to determine the contribution of  $\gamma\delta$  T cell-derived IL-17 to chronic human autoimmune diseases.

During this study IFN- $\gamma$  was revealed as the major mediator of the inhibitory effects of activated  $\gamma\delta$  T cells on osteoclast formation and resorption. By stimulating the degradation of the TNF receptor-associated factor 6 (TRAF-6) protein through activation of the ubiquitin-proteasome system, IFN- $\gamma$  potently inhibits RANK signaling required for osteoclast formation [6]. Further inhibitory effects of IFN- $\gamma$  on osteoclast formation have also been postulated, including stimulation of apoptosis through Fas/FasL interactions [32] and decreased expression of RANKL and c-Fms [33]. Interestingly, despite producing similar levels of IFN- $\gamma$  as activated  $\gamma\delta$  T cells, neutralization of IFN- $\gamma$  in activated CD4<sup>+</sup> T cell cultures had only a partial effect on recovering osteoclastogenesis, suggesting that activated CD4<sup>+</sup> T cells are able to suppress osteoclastogenesis via multiple mechanisms, such as through production of the well-characterized inhibitors IL-4 [34], and/or IL-10 [35].

The net contribution of IFN- $\gamma$  to bone metabolism *in vivo* is currently an area of intensive debate, with conflicting reports documenting both pro- and anti-osteoclastogenic effects of IFN- $\gamma$ . The anti-osteoclastogenic effects of IFN- $\gamma$  *in vivo* are supported by the finding that IFN- $\gamma$ <sup>-/-</sup> mice expe-

rience enhanced bone resorption during collagen-induced arthritis [36]. Conversely, a number of studies have reported pro-osteoclastogenic effects of IFN- $\gamma$ , as IFN- $\gamma$  is an effective treatment for osteopetrosis in humans [37], and IFN- $\gamma$ <sup>-/-</sup> mice are protected from ovariectomy-induced bone loss [38]. These data suggest that IFN- $\gamma$  has multiple roles in pathological bone diseases and further supports the notion that there exists a complex interplay between the immune system and regulation of bone homeostasis, particularly in pathological inflammatory disease states.

The profound inhibitory effect of IFN- $\gamma$  derived from activated  $\gamma\delta$  and CD4<sup>+</sup> T cells on resorption by mature osteoclasts is supported by a previous study showing that IFN- $\gamma$  decreases expression of the secreted protease cathepsin K in RANKL-treated RAW 264.7 cells (a murine pre-osteoclastic cell line) [39]. As cathepsin K plays an important role in the degradation of bone matrix proteins during bone resorption, this provides a direct link by which T cell-derived IFN- $\gamma$  may directly impair resorption by mature osteoclasts. The inhibitory effects on resorption may be due partially to an inhibition of further osteoclast formation during the culture period, rather than direct inhibition of osteoclast resorptive function *per se*. However, the profound inhibitory effect of rhIFN- $\gamma$  on resorption observed in our assays is strongly indicative of a direct effect of IFN- $\gamma$  on resorptive activity by mature osteoclasts, rather than an inhibitory effect on further osteoclast formation in our assay system. Furthermore, the importance of IFN- $\gamma$  to the efficacy of CD4<sup>+</sup> T cells for inhibiting resorption was in marked contrast to its relative lack of effect for inhibiting osteoclastogenesis. Taken together, this suggests that IFN- $\gamma$  is specifically inhibiting osteoclast resorptive function via a direct effect on the resorptive capacity of mature osteoclasts.

The importance of  $\gamma\delta$  T cells for influencing bone biology is currently poorly understood, but may be of specific interest in the context of therapeutic administration of anti-resorptive aminobisphosphonates drugs (N-BPs). We and others have shown previously that N-BPs, such as ZOL, potently induce an IFN- $\gamma$ -producing phenotype in  $\gamma\delta$  T cells *in vitro* [20] and *in vivo* [40,41]. By inducing the accumulation of the  $\gamma\delta$ -TCR agonists isopentenyl pyrophosphate (IPP) and dimethylallyl diphosphate (DMAPP) [42] in monocytic-lineage cells [43], N-BPs selectively activate the major subset of  $\gamma\delta$  T cells in peripheral blood, so-called V $\gamma$ 9V $\delta$ 2 T cells [19]. Thus N-BPs, by virtue of their high bone affinity and relatively selective targeting of osteoclasts, potentially induce an IFN- $\gamma$ -effector phenotype in V $\gamma$ 9V $\delta$ 2 T cells specifically in the local bone microenvironment, which may be of relevance *in vivo* given the inhibitory effects of ZOL-induced IFN- $\gamma$  production by V $\gamma$ 9V $\delta$ 2 T cells on osteoclast formation that we observed in this study. Such a mechanism may further potentiate the anti-resorptive effects of N-BPs by inhibiting osteoclast formation and resorption through the direct effects of  $\gamma\delta$  T



cell-derived IFN- $\gamma$ . This further highlights the important links between the immune system and bone resorbing cells such as osteoclasts, and suggests that pharmacological manipulation of innate immune cells, for example with N-BPs, may have important consequences for host immunity and bone health.

In conclusion, this study demonstrates that activated human  $\gamma\delta$  T cells are capable of potent inhibitory effects on osteoclast formation, and the resorptive activity of mature osteoclasts, mediated via production of IFN- $\gamma$ .

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## Disclosure

The authors declare no financial or commercial conflicts of interest.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

**Fig. S1.** The inhibitory effect of activated  $\gamma\delta$  T cells and CD4<sup>+</sup> T cells on osteoclast formation is independent of cell–cell contact. Quantification of osteoclast formation following incubation of osteoclast precursor cells with anti-CD3/CD28-activated  $\gamma\delta$  or CD4<sup>+</sup> T cells for 5 days, in the presence of macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor  $\kappa$ B-ligand (RANKL). Activated  $\gamma\delta$  T cells and activated CD4<sup>+</sup> T cells were co-cultured with osteoclast precursor cells to permit cell contact, or cells were separated using a Transwell insert into which the activated T cells were placed. Osteoclast formation was assessed using immunohistochemical staining for vitronectin receptor (VNR) and counterstaining with 4',6-diamidino-2-phenylindole (DAPI). Multi-nucleated ( $\geq 3$  nuclei) VNR<sup>+</sup> cells per well were quantified using fluorescence microscopy. Data shown are the mean  $\pm$  standard deviation from two experiments from independent donors.

**Fig. S2.** The blockade of granulocyte–macrophage colony-stimulating factor (GM-CSF) does not overcome the inhibitory effect of activated  $\gamma\delta$  T cells or CD4<sup>+</sup> T cells on osteoclastogenesis. Osteoclast precursors were cultured in the presence of receptor activator of nuclear factor  $\kappa$ B-ligand (RANKL) + macrophage colony-stimulating factor (M-CSF) and with 10% (v/v) conditioned medium (CM) from activated  $\gamma\delta$  T cells (a), or 10% (v/v) CM from activated CD4<sup>+</sup> T cells (b), in the presence or absence of  $\alpha$ GM-CSF or isotype control [immunoglobulin (Ig)G<sub>1</sub>], for 5–6 days. Following immunohistochemical staining for vitronectin receptor (VNR) and nuclear counterstaining with 4',6-diamidino-2-phenylindole (DAPI), multi-nucleated ( $\geq 3$  nuclei) VNR<sup>+</sup> cells per well were quantified using fluorescence microscopy. Data shown are the mean  $\pm$  standard error of the mean from three experiments from independent donors. \* $P < 0.05$ ; \*\*\* $P < 0.001$ .

**Fig. S3.** Zoledronate-activated  $\gamma\delta$  T cells inhibit osteoclast differentiation via interferon (IFN)- $\gamma$ . Peripheral blood mononuclear cells (PBMCs) were seeded in a 24-well plate at a density of  $1 \times 10^6$  cells/ml (1 ml/well) in  $\alpha$ -minimum essential medium (MEM) supplemented with 1  $\mu$ M zoledronic acid (ZOL) plus 10 U/ml interleukin (IL)-2 and were cultured for 7 days. Cells were subjected to half-medium changes every 3 days. After 7 days  $\gamma\delta$  T cells were positively isolated using  $\gamma\delta$  T cell magnetic affinity cell sorter (MACS) beads. Macrophage colony-stimulating factor (M-CSF)-expanded osteoclast precursors were treated with M-CSF and receptor activator of nuclear factor  $\kappa$ B-ligand (RANKL) for 5 days, in the presence or absence of autologous ZOL-activated  $\gamma\delta$  T cells. In some wells osteoclast precursors were incubated with 10% (v/v) conditioned

medium from 72 h cultures of PBMCs treated with ZOL, as described above. To assess the contribution of interferon (IFN)- $\gamma$  to the inhibitory effect of  $\gamma\delta$  T cells (or conditioned medium) on osteoclast formation, affinity-purified polyclonal goat anti-human IFN- $\gamma$  antibody (10  $\mu$ g/ml) was added to the cultures. Data shown are the mean  $\pm$  standard deviation from two experiments from independent donors. **Fig. S4.** Activated  $\gamma\delta$  T cells and CD4<sup>+</sup> T cells inhibit the resorptive activity of mature osteoclasts. Mature osteoclasts

were seeded onto dentine discs and cultured with resting or activated  $\gamma\delta$  T cells (a), or resting or activated CD4<sup>+</sup> T cells (b), for 6 days. The resorptive area was quantified using reflected light microscopy and in-house software to determine resorption pits. Data shown are the mean  $\pm$  standard error of the mean from three experiments from independent donors. \*\*\* $P < 0.001$ .