



# Activation status of $\gamma\delta$ T cells dictates their effect on osteoclast generation and bone resorption



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## ABSTRACT

$\gamma\delta$  T cells, a small subset of T cell population (5–10%), forms a bridge between innate and adaptive immunity. Although the role of  $\gamma\delta$  T cells in infectious diseases and antitumor immunity is well investigated, their role in bone biology needs to be explored. Aminobisphosphonates are used as a standard treatment modality for bone related disorders and are potent activators of  $\gamma\delta$  T cells. In the present study, we have compared the effect of “activated” and “freshly isolated”  $\gamma\delta$  T cells on osteoclast generation and function. We have shown that “activated” ( $\alpha$ CD3/CD28 + rhIL2 or BrHPP + rhIL2 stimulated)  $\gamma\delta$  T cells inhibit osteoclastogenesis, while “freshly isolated”  $\gamma\delta$  T cells enhance osteoclast generation and function. Upon stimulation with phosphoantigen (BrHPP), “freshly isolated”  $\gamma\delta$  T cells were also able to suppress osteoclast generation and function. Cytokine profiles of these cells revealed that, “freshly isolated”  $\gamma\delta$  T cells secrete higher amounts of IL6 (pro-osteoclastogenic), while “activated”  $\gamma\delta$  T cells secrete high IFN $\gamma$  levels (anti-osteoclastogenic). Neutralization of IFN $\gamma$  and IL6 reversed the “inhibitory” or “stimulatory” effect of  $\gamma\delta$  T cells on osteoclastogenesis. In conclusion, we have shown that, activation status and dynamics of IL6 and IFN $\gamma$  secretion dictate pro and anti-osteoclastogenic role of  $\gamma\delta$  T cells.

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## 1. Introduction

$\gamma\delta$  T cells represent a unique subset of immune cells accounting for 5–10% of total T cell population in peripheral blood of humans.  $\gamma\delta$  T cells are unique as compared to  $\alpha\beta$  T cells due to their T-cell receptor (TCR) gene usage, tissue tropism and MHC independent antigen recognition (Chiplunkar et al., 2009; Hayday, 2000). Major subtype of  $\gamma\delta$  T cells in human peripheral blood express V $\gamma$ 9V $\delta$ 2 TCR (also called as V $\gamma$ 2V $\delta$ 2) and are typically of Th1 type secreting copious amount of IFN $\gamma$  upon stimulation (Caccamo et al., 2013; Beetz et al., 2007). Similar to  $\alpha\beta$  T cells,  $\gamma\delta$  T cells exhibit plasticity and can differentiate into Th2 (Wesch et al., 2001; Sireci et al., 1997), Th17 (Ness-Schwickerath et al., 2010; Caccamo et al., 2011), Tfh (Caccamo et al., 2013) and T

regulatory (Casetti et al., 2009) type.  $\gamma\delta$  T cells play an important role in antitumor cytotoxicity (Konigshofer and Chien, 2006; Kabelitz et al., 2013), wound healing and tissue repair (Sharp et al., 2005; Jameson et al., 2005; Havran et al., 2005). These cells express natural killer receptors (Born et al., 2006; Hayday, 2009) and recognize stressed/tumour cells expressing MICA/B and ULBPs (Wu et al., 2014).  $\gamma\delta$  T cells are increased in bacterial, viral and parasitic infections (Born et al., 2006). Antitumor ability of  $\gamma\delta$  T cells against solid tumours and leukaemia's has been widely reported (Wrobel et al., 2007; Todaro et al., 2009; Gomes et al., 2010; Gertner-Dardenne et al., 2012; Dhar and Chiplunkar, 2010; Thomas et al., 2000; Laad et al., 1999). A unique set of antigens recognized by  $\gamma\delta$  T cells include intermediate products of eukaryotic mevalonate pathway (isopentenyl pyrophosphate or its synthetic analogue bromohydrin pyrophosphate) and bacterial Rohmer pathway (HMBPP [(E)-4-hydroxy-3-methyl-but-2-enylpyrophosphate]). Plant derived alkylamines and aminobisphosphonates are also known to activate  $\gamma\delta$  T cells (Born et al., 2006). Aminobisphosphonates and anti-RANKL therapies are used to treat patients with bone metastasis to reduce the skeletal complications and tumour burden (Lipton and Goessl, 2011; Roodman and Dougall, 2008; Lee et al., 2011a; Neville-Webbe and Coleman, 2010). The meta-analysis done to analyse the safety and efficacy of V $\gamma$ 9V $\delta$ 2 based immunotherapy has shown improved overall survival in patients compared to conventional therapies (Buccheri et al., 2014).

Though bone appears to be static in nature, it is a dynamic structure which undergoes continuous remodelling. The process of bone

*Abbreviations:* PBMCs, peripheral blood mononuclear cells; FH, Ficoll-Hypaque; RPMI, Roswell Park Memorial Institute medium; BrHPP, bromohydrin pyrophosphate; OPCs, osteoclast precursor cells; TRAP, tartarate resistant acid phosphatase; rhIL2, recombinant human interleukin 2; IL6, interleukin 6; IFN $\gamma$ , interferon gamma;  $\alpha$ IL6, anti-interleukin 6;  $\alpha$ IFN $\gamma$ , anti-interferon gamma; rhM-CSF, recombinant human macrophage-colony stimulating factor; rhRANKL, recombinant human receptor activator of nuclear factor kappa-B ligand;  $\alpha$ MEM, complete minimum essential medium with alpha modification; FCS, Fetal calf serum; PBS, phosphate buffered saline; CBA, cytometric bead array; OAS, osteoclast activity assay substrate; MACS, magnetic-activated cell sorting; MFI, mean fluorescent intensity; SEM, standard error of mean.

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remodelling is under the tight control of osteoblasts and osteoclasts. Osteoblasts are derived from mesenchymal cells, secrete bone matrix proteins and promote mineralization. Osteoclasts are large multinucleated cells generated by fusion of monocyte-macrophage precursor cells (Soysa et al., 2012) and are known to decalcify and degrade the bone by secreting the lysosomal proteases (Teitelbaum, 2007). In normal conditions, osteoclasts and osteoblasts work in coordinated manner to maintain normal bone physiology, while their imbalance results in pathological conditions, such as osteoporosis, rheumatoid arthritis (RA), Paget's disease and osteopetrosis. Immune system and skeletal system are influenced by a number of common cytokines, chemokines, signalling molecules and transcription factors (Takayanagi, 2007). Activated T lymphocytes support osteoclastogenesis through production of IL6 (Kurihara et al., 1990), IL17 (Sato et al., 2006), RANKL (Horwood et al., 1999; Weitzmann et al., 2001; Takayanagi et al., 2000) and TNF $\alpha$  (Lam et al., 2000). Inflamed synovial joints of rheumatoid arthritic patients showed presence of  $\gamma\delta$  T cells (Keystone et al., 1991), though  $\gamma\delta$  T cells were not recognized as major contributors of arthritic bone destruction (Pollinger et al., 2011). It was recently demonstrated that activated  $\gamma\delta$  T cells inhibit osteoclast differentiation and their resorptive function hence have important implications in understanding pathogenesis of rheumatoid arthritis (Pappalardo and Thompson, 2013). In disease free breast cancer patients, a single dose of aminobisphosphonate (zoledronic acid) induces a long lasting activation of effector subsets of  $\gamma\delta$  T cells (Santini et al., 2009). These studies indicate that activation status of  $\gamma\delta$  T cells is responsible for their antiosteoclastogenic activity. The objective of the present study was to have a comparative analysis of "activated" and "freshly isolated"  $\gamma\delta$  T cells with respect to their effect on generation and function of osteoclasts. Our results demonstrate that "activated"  $\gamma\delta$  T cells inhibit generation and resorptive ability of osteoclasts, while "freshly isolated"  $\gamma\delta$  T cells promote osteoclast formation and function.

## 2. Materials and methods

### 2.1. Study group

Peripheral blood from healthy volunteers (age, 25–45 years) was collected in heparin containing vacutainers. The present study was approved by the Institutional Ethics Committee and informed consents were taken from the healthy volunteers before collecting the blood sample.

### 2.2. Expansion and isolation of $\gamma\delta$ T cells

$\gamma\delta$  T cells were either directly isolated from peripheral blood mononuclear cells (PBMCs) or from ex-vivo expanded PBMC cultures. Briefly, PBMCs were separated from heparinised peripheral blood of healthy individuals by Ficoll-Hypaque (Sigma-Aldrich, USA) density gradient centrifugation. PBMCs ( $5 \times 10^6$ ), resuspended in 5 ml cRPMI (10% heat-inactivated human AB serum, 2 mM glutamine, and antibiotics) were stimulated for 12 days with rhIL-2 (30 IU/ml) in 25 mm<sup>2</sup> tissue culture flasks precoated with  $\alpha$ CD3 (1  $\mu$ g) +  $\alpha$ CD28 (1  $\mu$ g), with intermediate feedings with rhIL2. These cells were subcultured after 6 days. On the 12th day, cultures were terminated and  $\gamma\delta$  T cells were immunomagnetically separated (positive selection) by using  $\gamma\delta$ -TCR MACS kit (magnetic activated cell sorting, Milteney Biotech, Germany) as per the kit instructions. On the 12th day,  $13 \pm 2.5 \times 10^6$   $\gamma\delta$  T cells were obtained from  $\alpha$ CD3/CD28 expanded PBMCs ( $150 \pm 40 \times 10^6$ ). "Freshly isolated"  $\gamma\delta$  T cells were obtained from PBMCs without expansion. About  $3.8 \pm 1.6 \times 10^6$   $\gamma\delta$  T cells were isolated directly from PBMCs ( $130 \pm 52 \times 10^6$ ) by MACS purification. The purity of the MACS separated  $\gamma\delta$  T cells (FITC labelled) was analysed by flow cytometry and ranged from 95 to 98%. The  $\gamma\delta$  T cells were rested overnight in medium before using them for further experiments.  $\gamma\delta$  T cells purified from  $\alpha$ CD3/CD28 + rhIL2 expanded PBMCs are termed as "activated"  $\gamma\delta$  T cells,

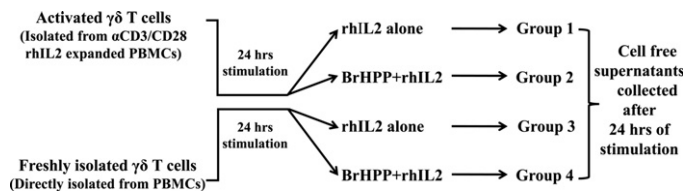
while  $\gamma\delta$  T cells isolated directly from PBMCs are referred as "freshly isolated"  $\gamma\delta$  T cells.

### 2.3. Flow cytometry

Expression of CD69, CD25 and RANKL was analysed on "activated" and "freshly isolated"  $\gamma\delta$  T cells. To analyse expression of CD69, CD25 and RANKL on "activated"  $\gamma\delta$  T cells, PBMCs ( $1 \times 10^6$ /ml) were cultured in cRPMI supplemented with either rhIL2 (30 IU/ml) alone, BrHPP (200 nM) + rhIL2 or  $\alpha$ CD3/CD28 (1  $\mu$ g) + rhIL2 for 12 days in 24 well plate at 37 °C in 5% CO<sub>2</sub> incubator. BrHPP is a synthetic analogue of isopentenyl pyrophosphate (Espinosa et al., 2001) and is used as antigen for  $\gamma\delta$  T cells. The cells were fed with rhIL2 every 3rd day and subcultured on the 6th day. PBMCs without any stimulation (Day 0) were kept as control. On termination of culture on the 12th day, expanded PBMCs were collected, washed in cold  $1 \times$  PBS (4 °C) and resuspended in cold FACS buffer ( $1 \times$  PBS, 2% FCS, 0.01% sodium azide) as  $1 \times 10^6$  PBMCs/50  $\mu$ l. These PBMCs were then stained for markers like  $\gamma\delta$ -TCR-APC (BD Pharmingen, USA), CD25-PECy7 (BD Pharmingen, USA), CD69-FITC (BD Pharmingen, USA), RANKL-PE (Biolegend, USA) at 4 °C for 30 min in dark, followed by fixing in 1% paraformaldehyde at 4 °C for 15 min in dark. For "freshly isolated"  $\gamma\delta$  T cells (directly separated from PBMCs on Day 0), purified  $\gamma\delta$  T cells were rested overnight and then were stimulated either with rhIL2 (0.5 IU) alone,  $\alpha$ CD3/CD28 (1  $\mu$ g) + rhIL2, BrHPP (200 nM) + rhIL2 or left unstimulated (control) for 24 h at 37 °C in 5% CO<sub>2</sub> incubator. 50,000 events were acquired on a FACS Aria (Becton Dickinson, USA) flow cytometer and data was analysed using FlowJo Software (Version 10, Tree Star, USA). For the above experiments, lymphocytes were gated on the basis of their forward and side scatter. Further  $\gamma\delta$  T cells were gated on the basis of the fluorescence intensity versus forward scatter and expression of CD69, CD25 and RANKL was analysed on the gated  $\gamma\delta$  T cell population.

### 2.4. Cytometric bead array

"Activated" (separated from  $\alpha$ CD3/CD28 expanded PBMCs) and "freshly isolated"  $\gamma\delta$  T cells ( $5 \times 10^4$ ), resuspended in cRPMI (200  $\mu$ l), were stimulated with rhIL2 (0.5 IU) alone or BrHPP (200 nM) + rhIL2 (0.5 IU), in round bottom 96 well plate (Nunc) for 24 h at 37 °C in 5% CO<sub>2</sub> incubator. The experimental groups were as represented below (Groups 1 to 4).



After 24 h, cells free supernatants from Groups 1–4 were collected and were stored at  $-80$  °C. As experimental controls, cell free supernatants were collected from "activated" and "freshly isolated"  $\gamma\delta$  T cells incubated in medium only (unstimulated) for 24 h. Th1/Th2/Th17 cytokines (IL2, IL4, IL6, IL10, TNF $\alpha$ , IFN $\gamma$  and IL17A) in these supernatants were analysed using cytometric bead array kit (BD Biosciences) as per the kit instructions. In brief, 50  $\mu$ l test samples and PE detection antibody were incubated with capture bead reagent for 3 h at room temperature in dark, followed by washing with wash buffer. Samples were acquired on BD FACS Aria cytometer (BD Bioscience, San Jose, CA, USA) and data was analyzed using FCAP Array software version 1.0 (BD Biosciences).

### 2.5. Generation of human osteoclasts

Human osteoclasts were generated from CD14<sup>+</sup> cells (monocytes) in the presence of rhMCSF and rhRANKL (Gupta et al., 2010). In brief,

CD14<sup>+</sup> cells were separated from healthy PBMCs by positive selection method using CD14 MACS kit (Milteney Biotech). CD14<sup>+</sup> cells ( $1 \times 10^5$ ) were cultured on thermax coverslips (Nunc) in flat bottom 96 well plate containing 200  $\mu$ l  $\alpha$ MEM (10% heat inactivated FCS (Invitrogen Life Technologies, USA), 2 mM L-glutamine and antibiotics) at 37 °C in 5% CO<sub>2</sub> incubator. These cultures were supplemented with 30 ng/ml rhMCSF (R&D Systems) and 40 ng/ml rhRANKL (R&D Systems) and were replenished on every 3rd day. On the 21st day, mature multinucleated osteoclasts were characterized by staining for expression of vitronectin receptor ( $\alpha$ V $\beta$ 3 integrin) using monoclonal antibody for CD51/61 (Biogend), also known as 23c6 antibody along with nuclear stain DAPI. 23c6<sup>+</sup> cells having >3 nuclei were considered as mature osteoclasts (Supplementary Fig. 1A).

## 2.6. CD14<sup>+</sup> and $\gamma\delta$ T cell coculture assay

Effect of  $\gamma\delta$  T cells on osteoclastogenesis was analysed by generating osteoclasts from CD14<sup>+</sup> cells in the presence of autologous “activated” (ACT) or “freshly isolated” (FI)  $\gamma\delta$  T cells. On Day 0, CD14<sup>+</sup> cells ( $1 \times 10^5$ ) were cocultured with unstimulated “activated” or “freshly isolated”  $\gamma\delta$  T cells ( $1 \times 10^4$ ) in  $\alpha$ MEM (200  $\mu$ l) containing rhMCSF (30 ng/ml), rhRANKL (40 ng/ml) and rhIL2 (0.5 IU). As a positive control, osteoclasts were generated from CD14<sup>+</sup> cells in the presence of rhMCSF and rhRANKL only. To analyse the effect of phosphoantigen stimulated “freshly isolated”  $\gamma\delta$  T cells on osteoclastogenesis, BrHPP (200 nM) was also added to cocultures along with rhMCSF and rhRANKL on Day 0. For 21 days, CD14:  $\gamma\delta$  T cells (10:1) were nourished with rhMCSF, rhRANKL and rhIL2 on every 3rd day. On the 21st day, multinucleated (>3 nuclei) 23c6<sup>+</sup> osteoclasts generated in an entire well were quantified and the effect of  $\gamma\delta$  T cells on osteoclastogenesis was determined by analysing increase or decrease in the total number of osteoclast generated per well as compared to control wells.

## 2.7. In vitro bone resorption assay using osteoclast activity assay substrate (OAAS)

“Activated” and “freshly isolated”  $\gamma\delta$  T cells were stimulated with rhIL2 or BrHPP + rhIL2 for 24 h and cell free supernatants were collected (Groups 1–4) as described above (Section 2.4). Osteoclast precursor cells were generated from CD14<sup>+</sup> cells ( $1 \times 10^5$ ) in the presence of rhMCSF and rhRANKL for 12 days in OAAS module (BD Biosciences). OAAS is a 16 well module, with thin calcium-phosphate coated wells and is used to evaluate functional activity of osteoclasts in vitro. From the 12th day onwards, every 3rd day, the cultures were supplemented with pretitrated volumes of cell free supernatant (50  $\mu$ l) from Groups 1–4 along with rhMCSF and rhRANKL. Osteoclasts generated in the presence of rhMCSF and rhRANKL were kept as positive control. On the 21st day, the cells were bleached out using 6% sodium hypochlorite and 5.2% sodium chloride. OAAS plates were air dried and resorption area/pits generated by mature resorbing osteoclasts were imaged using light microscopy (Supplementary Fig. 1B). The resorptive ability of osteoclasts was assessed by quantifying the total resorbed area generated in OAAS well using ImageJ Software.

## 2.8. Cytokine neutralization

Osteoclast precursor cells were generated from CD14<sup>+</sup> cells ( $1 \times 10^5$ ) in the presence of rhMCSF and rhRANKL for 12 days in OAAS module as described above. From the 12th day onwards, every 3rd day, along with rhMCSF and rhRANKL, the cultures were supplemented with 50  $\mu$ l cell free supernatant of unstimulated “activated” and “freshly isolated”  $\gamma\delta$  T cells, with or without monoclonal mouse antihuman  $\alpha$ IFN $\gamma$  or  $\alpha$ IL6 neutralization antibody (10  $\mu$ g/ml/well) respectively. Osteoclasts generated in the presence of rhMCSF and rhRANKL were kept as positive control. On the 21st day, the cultures were terminated and resorption area was calculated using ImageJ software.

## 2.9. Statistical analysis

The data was analysed using student's unpaired t test. p values  $\leq 0.05$  were considered statistically significant [ $p < 0.05$  (\*),  $p < 0.005$  (\*\*),  $p < 0.0005$  (\*\*\*)]. Error bar indicates mean  $\pm$  SEM.

## 3. Results

### 3.1. Expression of activation markers (CD69, CD25 and RANKL) on $\gamma\delta$ T cells

Expression of activation markers (CD69, CD25 and RANKL) on  $\gamma\delta$  T cells were analysed by stimulating these cells with two distinct TCR signals ( $\alpha$ CD3/CD28 or BrHPP) in the presence of rhIL2.

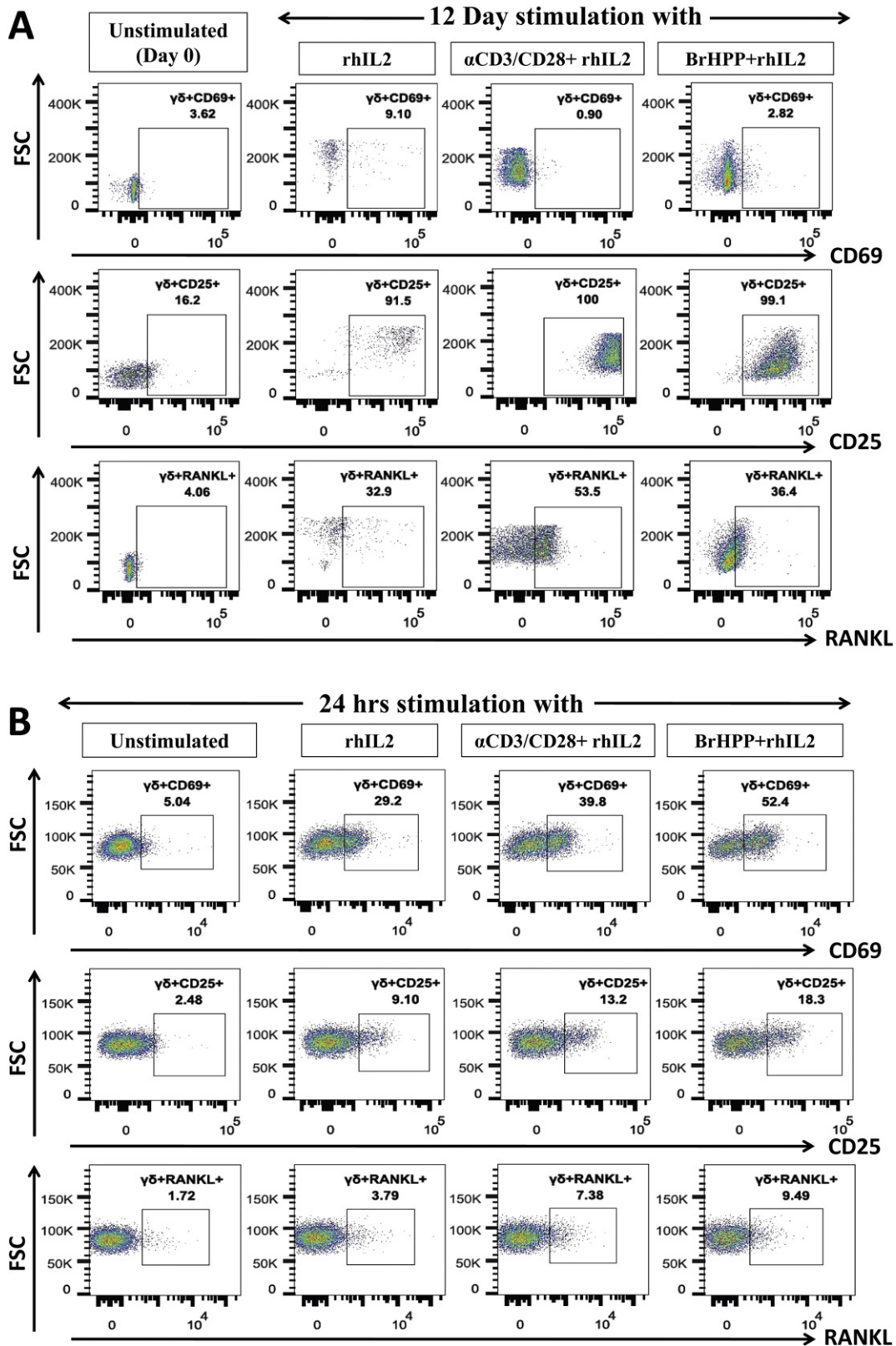
As shown in Fig. 1A, unstimulated PBMCs (Day 0) showed lower expression levels of CD69 (3.62% [percent positive], MFI = 72.5 [mean fluorescent intensity]), CD25 (16.2%, MFI = 405) and RANKL (4.06%, MFI = 115) on gated  $\gamma\delta$  T cells. However, a marked increase in expression of CD25 and RANKL was observed on  $\gamma\delta$  T cells upon stimulation of PBMCs with  $\alpha$ CD3/CD28 + rhIL2 (100%, MFI = 134,379 and 53.5%, MFI = 641 respectively) or BrHPP + rhIL2 (99.1%, MFI = 17,740 and 36.4%, MFI = 654 respectively) for 12 days. Almost all  $\gamma\delta$  T cells were positive for CD25 expression after stimulation, indicating that these cells were in highly activated state. Expression of CD69 (early activation marker) on  $\gamma\delta$  T cells was found marginal changed upon stimulation of PBMCs with  $\alpha$ CD3/CD28 + rhIL2 (0.9%, MFI = -714) and BrHPP + rhIL2 (2.82%, MFI = 211) for 12 days compared to unstimulated PBMCs (3.62%, MFI = 72.5).

Expression of these activation markers were also analysed on immunomagnetically purified  $\gamma\delta$  T cells (Fig. 1B). Purified (“freshly isolated”)  $\gamma\delta$  T cells were either left unstimulated (control) or were stimulated with rhIL2 alone or a combination of  $\alpha$ CD3/CD28 + rhIL2 or BrHPP + rhIL2 for 24 h. As shown in Fig. 1B, in an unstimulated state, “freshly isolated”  $\gamma\delta$  T cells showed lower levels of CD69 (5.04%, MFI = 126), CD25 (2.48%, MFI = 103) and RANKL (1.72%, MFI = 41.4). Upon stimulation with rhIL2 for 24 h, increase in the expression of CD69 (29.2%, MFI = 295), CD25 (9.10% MFI = 354) and RANKL (3.79% MFI = 81.3) was observed. “Freshly isolated”  $\gamma\delta$  T cells stimulated with  $\alpha$ CD3/CD28 + rhIL2 or BrHPP + rhIL2 showed markedly higher levels of CD69 (39.8%, MFI = 344 and 52.4%, MFI = 335 respectively), CD25 (13.2%, MFI = 512 and 18.3%, MFI = 677 respectively) and RANKL (7.38%, MFI = 106 and 9.49%, MFI = 77.6 respectively) as compared to unstimulated and rhIL2 stimulated  $\gamma\delta$  T cells. Thus on the basis of expression of activation markers (CD69, CD25 and RANKL)  $\gamma\delta$  T cells were considered as “non-activated” (freshly isolated) and “activated” (isolated from  $\alpha$ CD3/CD28 + rhIL2 stimulated PBMCs)  $\gamma\delta$  T cells.

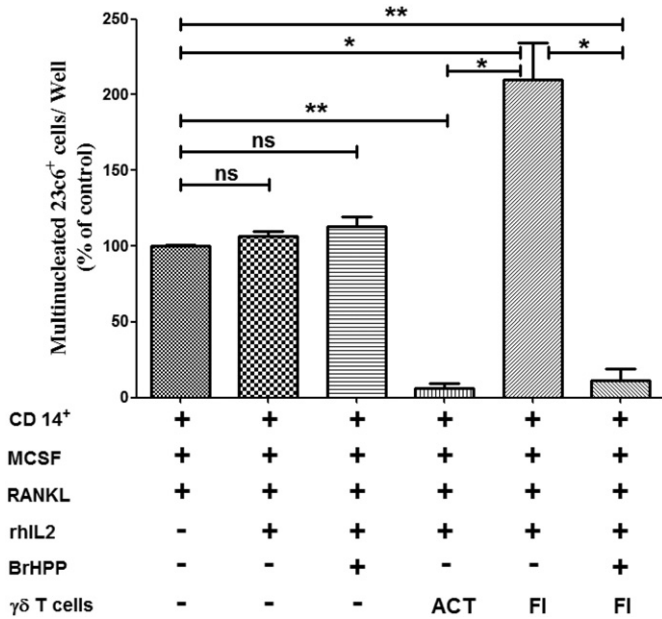
### 3.2. Direct effect of $\gamma\delta$ T cells on generation of osteoclast

CD14<sup>+</sup> cells ( $1 \times 10^5$ /well, osteoclast precursor cells) were cocultured with “activated” or “freshly isolated”  $\gamma\delta$  T cells ( $1 \times 10^4$ /well, 10:1) in the presence of rhMCSF, rhRANKL and rhIL2 for 21 days (described in material and methods Section 2.6), with intermediate feedings on every 3rd day. Osteoclasts generated in the presence of rhMCSF and rhRANKL served as a positive control. Osteoclasts showing multinucleation ( $\geq 3$  nuclei) and 23c6 positivity (vitronectin receptor) were considered as mature osteoclasts. Number of osteoclasts generated ( $25 \pm 4$  osteoclasts/well) in the presence of rhMCSF and rhRANKL (positive control) were normalized to 100% and data has been represented as relative increase or decrease in number of osteoclasts generated per well compared to positive control. Coculture of “activated”  $\gamma\delta$  T cells with CD14<sup>+</sup> cells in the presence of rhMCSF and rhRANKL showed significant reduction ( $1.5 \pm 0.5$  osteoclasts/well, 93.6% reduction,  $p = 0.0012$ ) in 23c6<sup>+</sup> multinucleated osteoclasts compared to positive control (Fig. 2). In contrast, generation of osteoclasts in the presence of “freshly isolated”  $\gamma\delta$  T cells showed marked increase in the number of 23c6<sup>+</sup> multinucleated osteoclasts ( $53.5 \pm 14.5$  osteoclasts/well, 110%





**Fig. 1.** Analysis of activation markers (CD69, CD25, and RANKL) on  $\gamma\delta$  T cells. **A)** Expression of activation markers (CD69, CD25, and RANKL) was analysed on  $\gamma\delta$  T cells in unstimulated PBMCs ( $1 \times 10^6$ /ml/well) or PBMCs expanded with rhIL2 (30 IU/ml) alone,  $\alpha$ CD3/CD28 + rhIL2 or BrHPP (200 nM) + rhIL2 for 12 days. After the 12th day, PBMCs were stained using  $\gamma\delta$ -APC, CD69-FITC, CD25-PeCy7, and RANKL-PE. Baseline expression of these markers on  $\gamma\delta$  T cells was analysed by staining unstimulated PBMCs (Day 0, control). The gating strategy used was as follows: Lymphocytes were gated based on their forward and side scatter. Depending on the fluorescence intensity of  $\gamma\delta$ -APC,  $\gamma\delta$  T cells were gated. Further percentages of  $\gamma\delta$  T cells expressing CD69, CD25 and RANKL were analysed on gated  $\gamma\delta$  T cells. The gates have been set according to only cell controls for respective stimulation. **B)** Purified  $\gamma\delta$  T cells were also stimulated with rhIL2 alone,  $\alpha$ CD3/CD28 + rhIL2 or BrHPP + rhIL2 for 24 h or left unstimulated (medium alone) and were analysed for the expression of CD69, CD25 and RANKL. The gating strategy used was as described above. (Three independent experiments were carried out and the representative figure is given.)



**Fig. 2.** Effect of “activated” and “freshly isolated”  $\gamma\delta$  T cells on generation of osteoclasts. CD14<sup>+</sup> cells ( $1 \times 10^5$ /well) were co-cultured with “activated” (ACT) or “freshly isolated” (FI)  $\gamma\delta$  T cells ( $1 \times 10^4$ ) in  $\alpha$ MEM supplemented with rhMCSF (30 ng/ml), rhRANKL (40 ng/ml) and rhIL2 (0.5 IU/well) on thermanox coverslips (Nunc) in flat bottom 96 well plate. Osteoclasts generated in the presence of rhMCSF and rhRANKL were kept as positive control. “Freshly isolated”  $\gamma\delta$  T cells were stimulated with phosphoantigen by supplementing the cultures with BrHPP (200 nM) along with rhMCSF, rhRANKL and rhIL2 on Day 0. The CD14<sup>+</sup>:  $\gamma\delta$  T cell cocultures were fed with rhMCSF, rhRANKL, rhIL2 every 3rd day for 21 days. On the 21st day, osteoclasts were characterized by staining them for vitronectin receptor (anti-human-CD51/CD61-AF488) and total number of osteoclasts (multinucleated 23c6<sup>+</sup> cells) generated per well were quantitated. Number of osteoclasts generated in the presence of rhMCSF and rhRANKL (positive control) were normalized to 100% and data has been represented as relative increase or decrease in number of osteoclasts generated per well compared to positive control. Three independent experiments were carried out and the data is represented as mean  $\pm$  SEM. [ $p < 0.05$  (\*),  $p < 0.005$  (\*\*),  $p < 0.0005$  (\*\*\*)].

increase,  $p = 0.0458$ ). These results indicated that “activated”  $\gamma\delta$  T cells inhibit osteoclastogenesis, while “freshly isolated”  $\gamma\delta$  T cells tend to increase the number of osteoclasts upon coculture. In order to confirm this observation “freshly isolated”  $\gamma\delta$  T cells were cocultured with CD14<sup>+</sup> cells in the presence of phosphoantigen BrHPP along with rhMCSF, rhRANKL and rhIL2. It was interesting to observe that generation of osteoclasts in the presence of “freshly isolated”  $\gamma\delta$  T cells that were stimulated with BrHPP showed significant reduction ( $2.5 \pm 1.5$  osteoclasts/well, 88.8% reduction,  $p = 0.0078$ ) in the total number of 23c6<sup>+</sup> osteoclasts, confirming that “activation status” of  $\gamma\delta$  T cells govern their function to regulate osteoclastogenesis. CD14<sup>+</sup> cells cultured in the presence of rhMCSF and rhRANKL in the presence of rhIL2 ( $26.5 \pm 3.5$  osteoclasts/well) or BrHPP + rhIL2 ( $28 \pm 3$  osteoclasts/well) showed osteoclast numbers that were comparable to that observed in positive control ( $25 \pm 4$  osteoclasts/well), indicating that rhIL2 or BrHPP + rhIL2 had no effect on osteoclast generation.

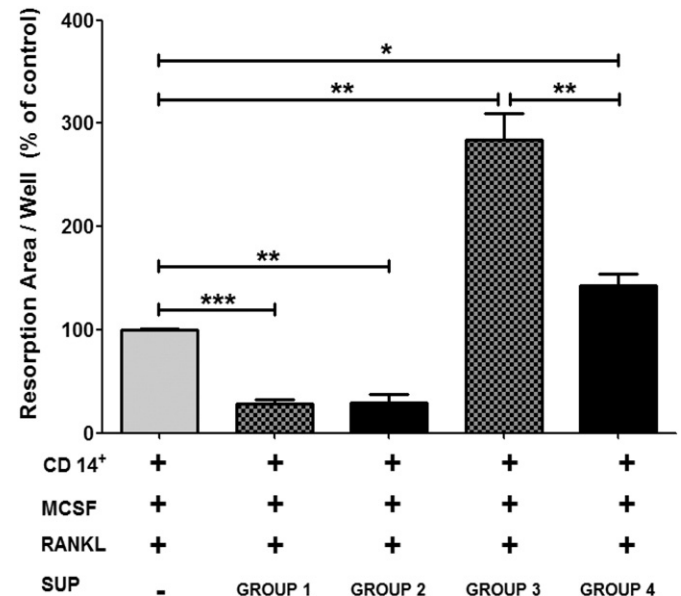
### 3.3. Effect of soluble factors secreted by phosphoantigen stimulated $\gamma\delta$ T cells on function of osteoclasts

In order to investigate the indirect effect of  $\gamma\delta$  T cells on osteoclast function mediated through secretion of soluble factors upon phosphoantigenic stimuli, osteoclast precursor cells (OPCs, generated on OAAS using rhMCSF and rhRANKL for 12 days) were cultured in the presence of pretitrated volumes of cell free supernatants of “activated” and “freshly isolated”  $\gamma\delta$  T cells, stimulated with rhIL2 or BrHPP + rhIL2 (Groups 1–4), along with rhMCSF and rhRANKL. Osteoclasts cultured in the presence of rhMCSF and rhRANKL were kept as positive control. Total resorbed area generated by resorbing osteoclasts

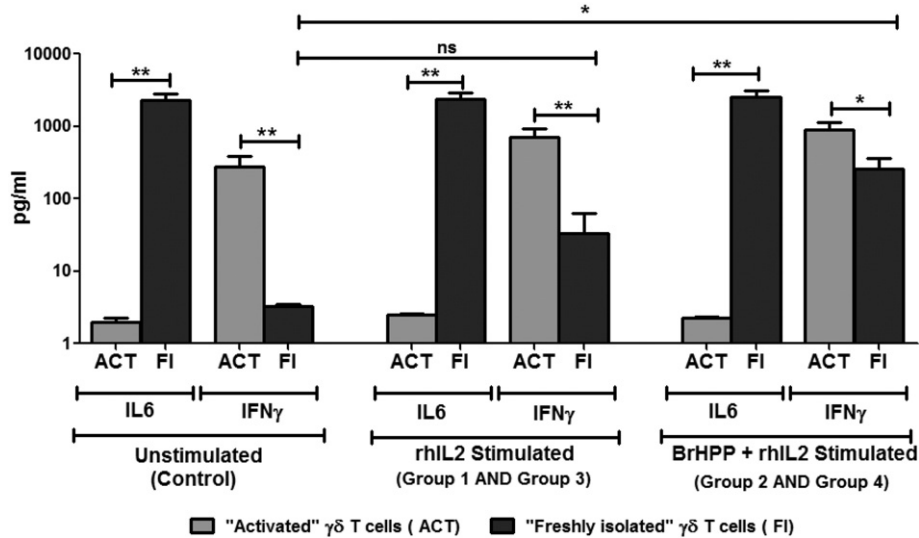
in OAAS well was used as end point measurement of assay. Total resorption area generated per well by osteoclasts cultured in the presence of rhMCSF and rhRANKL (positive control) was normalized to 100% and data has been represented as relative increase or decrease in resorption area compared to positive control. As shown in Fig. 3, OPCs, supplemented with supernatants of “activated”  $\gamma\delta$  T cells which were stimulated with rhIL2 alone (Group 1) or BrHPP + rhIL2 (Group 2) showed 72% and 70.4% reduction ( $p = 0.0001$  and  $p = 0.0011$  respectively) in the total resorbed area as compared to positive control. Similarly, effect of soluble factors secreted by “freshly isolated”  $\gamma\delta$  T cells which were stimulated with rhIL2 alone (Group 3) or BrHPP + rhIL2 (Group 4) on osteoclast function showed significant increase (184.1%,  $p = 0.002$  and 42.6%,  $p = 0.0192$  respectively) in total resorbed area as compared to positive control. The results clearly indicated that soluble factors secreted by “activated”  $\gamma\delta$  T cells potentially suppress osteoclast mediated bone resorption, whereas soluble factors secreted by “freshly isolated”  $\gamma\delta$  T cells increase bone resorption.

### 3.4. Cytokine profiling of supernatants of antigen activated $\gamma\delta$ T cells

Cell free supernatants were collected from “activated” (ACT) and “freshly isolated” (FI)  $\gamma\delta$  T cells. The various groups are described in materials and methods, Section 2.4. “Activated” and “freshly isolated”  $\gamma\delta$  T cells were either left unstimulated (medium only control) or were stimulated with rhIL2 alone (Group 1, Group 3), BrHPP + rhIL2 (Group 2, Group 4). Cell free supernatants of these cells were collected and analysed for Th1/Th2/Th17 cytokines by CBA, as represented in Supplementary data, Table 1. Analysis of cell free supernatants of “activated” and “freshly isolated”  $\gamma\delta$  T cells in the unstimulated control group interestingly showed significant differences in levels of two cytokines- IL6 (pro-osteoclastogenic) and IFN $\gamma$  (anti-osteoclastogenic) compared to other cytokines (Supplementary data, Table 1). As shown in Fig. 4, in



**Fig. 3.** Effect of supernatants of  $\gamma\delta$  T cells on resorptive ability of osteoclasts. Osteoclasts were generated from CD14<sup>+</sup> cells ( $1 \times 10^5$ /well) in the presence of rhMCSF (30 ng/ml) and rhRANKL (40 ng/ml) for 12 days on osteoclast activity assay substrate. From the 12th day onwards, the cultures were fed with cell free supernatants of “activated” and “freshly isolated”  $\gamma\delta$  T cells, stimulated with rhIL2 (Group 1 and Group 3) or BrHPP + rhIL2 (Group 2 and Group 4) respectively, along with rhMCSF and rhRANKL on every 3rd day. Osteoclasts generated in the presence of rhMCSF and rhRANKL were kept as positive control. The cultures were terminated on the 21st day and the area resorbed by mature osteoclasts was calculated using ImageJ software. Total resorption area generated per well by osteoclasts cultured in the presence of rhMCSF and rhRANKL (positive control) was normalized to 100% and data has been represented as relative increase or decrease in resorption area compared to positive control. The data shown is mean  $\pm$  SEM of three independent experiments. [ $p < 0.05$  (\*),  $p < 0.005$  (\*\*),  $p < 0.0005$  (\*\*\*)].



**Fig. 4.** Estimation of IL6 and IFN $\gamma$  levels in cell free supernatants of “activated” (ACT) and “freshly isolated” (FI)  $\gamma\delta$  T cells. “Activated” (purified from  $\alpha$ CD3/CD28 + rhIL2 expanded PBMCs) or “freshly isolated” (directly purified from PBMCs on Day 0)  $\gamma\delta$  T cells ( $5 \times 10^4$ /well) were stimulated with rhIL2 (0.5 IU/well) or BrHPP (200 nM) + rhIL2 for 24 h at 37 °C in CO $_2$  incubator. Both “activated” and “freshly isolated”  $\gamma\delta$  T cells, which were unstimulated (incubated in culture medium alone), were kept as control. After 24 h, the cell free supernatants were collected. Th1/Th2/Th17 cytokines of these cell free supernatants were quantified by cytometric bead array (CBA). In an unstimulated state, supernatants of “activated” and “freshly isolated”  $\gamma\delta$  T cells showed marked differences in IL6 and IFN $\gamma$  levels. Stimulation of “activated” and “freshly isolated”  $\gamma\delta$  T cells with rhIL2 (Group 1, Group 3 respectively) or BrHPP + rhIL2 (Group 2, Group 4 respectively) showed enhanced IFN $\gamma$  secretion. Levels of IL6 remained higher in cell free supernatants of “freshly isolated”  $\gamma\delta$  T cells. (“Activated”  $\gamma\delta$  T cells (n = 5), “freshly isolated”  $\gamma\delta$  T cells (n = 7)). [p < 0.05 (\*), p < 0.005 (\*\*), p < 0.0005 (\*\*\*)].

an unstimulated state, “freshly isolated”  $\gamma\delta$  T cells showed higher levels of IL6 (Mean  $\pm$  SEM 2318  $\pm$  471 pg/ml), while “activated”  $\gamma\delta$  T cells showed significantly (p = 0.005) lower levels of IL6 (1.9  $\pm$  0.3 pg/ml). Comparison of IFN $\gamma$  levels showed that, “freshly isolated”  $\gamma\delta$  T cells secreted very low (3.2  $\pm$  0.27 pg/ml) levels of IFN $\gamma$ , while “activated”  $\gamma\delta$  T cells showed significantly (p = 0.008) higher levels of IFN $\gamma$  (274.1  $\pm$  111.2 pg/ml).

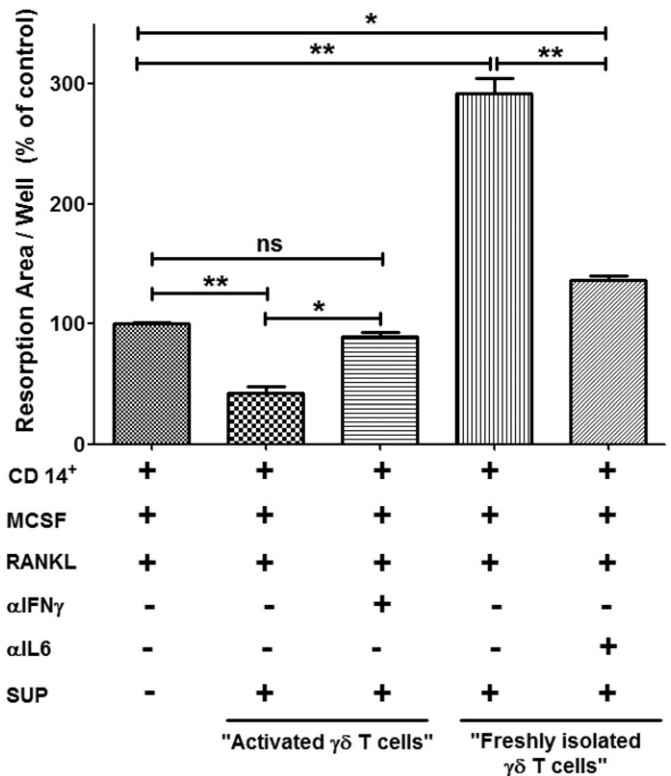
Upon stimulation with rhIL2, supernatant of “activated”  $\gamma\delta$  T cells (Group 1) showed significantly higher levels of IFN $\gamma$  (706.5  $\pm$  211.8 pg/ml, p = 0.0044) compared to “freshly isolated”  $\gamma\delta$  T cells (33.5  $\pm$  29.1 pg/ml, Group 3). Similarly, upon stimulation with BrHPP + rhIL2, “activated”  $\gamma\delta$  T cells (Group 2) showed significantly higher levels of IFN $\gamma$  (879.7  $\pm$  240 pg/ml, p = 0.0264) compared to “freshly isolated”  $\gamma\delta$  T cells (260.7  $\pm$  101.2 pg/ml Group 4). Freshly isolated  $\gamma\delta$  T cells upon stimulation with rhIL2 showed a progressive increase in IFN $\gamma$  production that further enhanced with BrHPP + rhIL2 stimulation (p = 0.01) compared to unstimulated  $\gamma\delta$  T cells.

The levels of IL6 secreted by “activated” (Group 1 and Group 3) and “freshly isolated” (Group 2 and Group 4)  $\gamma\delta$  T cells were not significantly different compared to supernatants of unstimulated  $\gamma\delta$  T cells (control).

Results indicate that “freshly isolated”  $\gamma\delta$  T cells secrete higher levels of IL6, thus have pro-osteoclastogenic effect, while “activated”  $\gamma\delta$  T cells maintain higher levels of IFN $\gamma$  playing an anti-osteoclastogenic role.

### 3.5. Effect of IFN $\gamma$ or IL6 neutralization in cell free supernatants of $\gamma\delta$ T cells on osteoclast function

To validate that the effect of  $\gamma\delta$  T cells on osteoclastogenesis is mediated through cytokines (IFN $\gamma$  and IL6), functional assays were carried out using neutralizing antibodies to these cytokines. Osteoclasts were generated in the presence of cell free supernatants of unstimulated “activated” and “freshly isolated”  $\gamma\delta$  T cells in the presence or absence of  $\alpha$ IFN $\gamma$  or  $\alpha$ IL6 antibody. As shown in Fig. 5, osteoclasts generated in presence of cell free supernatants of “activated”  $\gamma\delta$  T cells showed significant (57.4%) reduction in resorption area (10.78  $\pm$  1  $\mu$ m $^2$ ) compared to positive control (25.4  $\pm$  0.5  $\mu$ m $^2$ , 100%). After addition of  $\alpha$ IFN $\gamma$  antibody to these cultures the inhibition was reversed and brought to 89.7% (22.7  $\pm$  0.3  $\mu$ m $^2$ ), which was comparable to that observed in positive



**Fig. 5.** Effect of IFN $\gamma$  and IL6 neutralization in cell free supernatants of “activated” (ACT) and “freshly isolated” (FI)  $\gamma\delta$  T cells on osteoclast function. CD14 $^+$  cells ( $1 \times 10^5$ ) were cultured in the presence of rhMCSF and rhRANKL for 12 days in OAS module to generate OPCs. After which, every 3rd day, the cultures were supplemented with 50  $\mu$ l supernatants of unstimulated “activated” and “freshly isolated”  $\gamma\delta$  T cells, rhMCSF, and rhRANKL with or without monoclonal mouse  $\alpha$ IFN $\gamma$  or  $\alpha$ IL6 neutralization antibody (10  $\mu$ g/ml/well). Osteoclasts generated in the presence of rhMCSF and rhRANKL were kept as positive control. On the 21st day, the cultures were terminated and resorption area was calculated. Resorption area generated in positive control was normalized to 100% and data has been represented as relative increase or decrease in resorption area compared to positive control. The data shown is mean  $\pm$  SEM of two independent experiments. [p < 0.05 (\*), p < 0.005 (\*\*), p < 0.0005 (\*\*\*)].



control, confirming our observation that IFN $\gamma$  is a major cytokine generated by “activated”  $\gamma\delta$  T cells that inhibits osteoclastogenesis.

Similarly, osteoclasts generated in the presence of cell free supernatants of “freshly isolated”  $\gamma\delta$  T cells showed increase in resorption area by 191.9% ( $74.1 \pm 1.6 \mu\text{m}^2$ ) over positive control (100%). Upon addition of  $\alpha$ IL6 antibody to the cultures, there was a marked reduction in the resorption area ( $34.5 \pm 0.2 \mu\text{m}^2$ ,  $p = 0.0068$ ) reducing from 191.9% to 36.1% over positive control (100%). Our results suggests that the dynamics of IFN $\gamma$  and IL6 play a major role in mediating the pro and anti-osteoclastogenic effects of “activated”  $\gamma\delta$  T cells and “freshly isolated”  $\gamma\delta$  T cells respectively.

#### 4. Discussion

Bone is a common site of metastasis in breast cancer (Suva et al., 2009), prostate cancer and multiple myeloma (Roodman, 2010). Metastatic tumour cells disturb the bone metabolism by releasing factors which induce differentiation and activation of osteoclasts (Roodman, 2001). It has also been reported that cytokines secreted by lymphocytes present in the bone microenvironment affect bone metabolism (Gillespie, 2007). Investigations are focused on understanding how T lymphocytes interact with osteoclasts and influence their function. Activated CD4<sup>+</sup> T cells express RANKL and secrete cytokines such as IL1 $\beta$ , IL6, IL17, TNF $\alpha$  and RANKL that support osteoclastogenesis (Horwood et al., 1999; Kotake et al., 2001; Lee et al., 2011b; Kong et al., 1999). Interestingly, it has also been reported that activated T lymphocytes secrete cytokines such as IFN $\gamma$ , IL4, IL10 and GM-CSF that inhibit osteoclastogenesis (Walsh et al., 2006; Sato and Takayanagi, 2006). It is still not understood what dictates the pro and anti-osteoclastogenic behaviour of the lymphocytes. Although the role of CD4<sup>+</sup>  $\alpha\beta$  T cells in osteoclastogenesis has been investigated, the role of  $\gamma\delta$  T cells is not well understood. In the present study, we have made an attempt to analyze the effect of “activated” and “freshly isolated”  $\gamma\delta$  T cells on osteoclast generation and function.

We used  $\gamma\delta$  T cells from two sources; those that were isolated directly from PBMCs (“freshly isolated”  $\gamma\delta$  T cells) and those that were isolated from PBMCs stimulated with  $\alpha$ CD3/CD28 + rhIL2 (“activated”  $\gamma\delta$  T cells). Coculture of “freshly isolated”  $\gamma\delta$  T cells with CD14<sup>+</sup> cells enhanced the generation of osteoclasts and stimulated their resorptive ability. On the contrary, “activated”  $\gamma\delta$  T cells showed inhibitory effect on generation and function of osteoclasts. The process of osteoclastogenesis is majorly influenced by cytokine milieu in the microenvironment, where IL6, IL17, TNF $\alpha$ , TGF $\beta$ , IFN $\gamma$  and RANKL play indispensable role. Analysis of cell free supernatants of “freshly isolated”  $\gamma\delta$  T cells at baseline levels (unstimulated) showed marked difference in their IL6 and IFN $\gamma$  levels. In an unstimulated state, “freshly isolated”  $\gamma\delta$  T cells were major producers of IL6, with negligible levels of IFN $\gamma$ . On the contrary, “activated”  $\gamma\delta$  T cells showed higher IFN $\gamma$  levels with low IL6 production.

IL6 is a potent pro-osteoclastogenic cytokine, with the capacity to induce osteoclastogenesis in RANKL independent manner (Kudo et al., 2003) and it also acts in synergistic manner with TNF $\alpha$  to induce osteoclastogenesis (Ragab et al., 2002). IL6 has been reported to stimulate formation of osteoclast like multinucleated cells in long term cultures of human bone marrow through induction of IL1 $\beta$  (Kurihara et al., 1990). T lymphocytes from IL6<sup>-/-</sup> mice have shown lesser production of IL17 (pro-osteoclastogenic) and also affected RANKL/OPG production by T lymphocytes (Wong et al., 2006). Cultures of osteoclasts from bone marrow cells of IL6<sup>-/-</sup> mice in the presence of MCSF and RANKL produced ~50% lesser osteoclasts as compared to wild type mice (Wong et al., 2006). On the contrary, IFN $\gamma$  has been appreciated for its protective role in osteoclastogenesis by inhibiting osteoclast formation and bone resorption in vitro (Takayanagi et al., 2000; Kamolmatyakul et al., 2001). Mice lacking one of the components of IFN $\gamma$  receptor (IFN $\gamma$ R1) showed enhanced osteoclastogenesis from bone marrow derived monocyte/macrophage precursor cells (BMMs) and bone loss

(Takayanagi et al., 2000). Also, IFN $\gamma$  producing activated T cells showed osteoclast generation from BMMs in the absence of RANKL in IFN $\gamma$ R<sup>-/-</sup> mice (Takayanagi et al., 2000). IFN $\gamma$  either downregulates TRAF6 (TNF receptor associated factor 6) expression or activates/enhances ubiquitin dependant proteosomal systems whose direct target is TRAF6 (Takayanagi et al., 2000; Takayanagi et al., 2005), which results in disruption of RANK–RANKL signalling.

In order to confirm that IL6 and IFN $\gamma$  are the key cytokines involved in the pro and anti-osteoclastogenic effects mediated by “freshly isolated” and “activated”  $\gamma\delta$  T cells respectively, neutralization experiment was carried out using  $\alpha$ IL6 and  $\alpha$ IFN $\gamma$  antibodies. Addition of  $\alpha$ IL6 antibody to the supernatants of “freshly isolated”  $\gamma\delta$  T cells showed a marked reduction in the resorption area. However, the resorption area remained marginally above that was observed with positive control (rhM-CSF + rhRANKL). The data indicates that other cytokines like TNF $\alpha$  and IL17 may also contribute to the pro-osteoclastogenic effect, which may be present in the supernatants after blocking IL6 (Supplementary Table 1). Similarly blocking of IFN $\gamma$  significantly reversed (89.74%) the anti-osteoclastogenic effect, indicating that IFN $\gamma$  is the dominant cytokine inhibiting osteoclastogenesis.

Human  $\gamma\delta$  T cells are Th1 type cells which produce copious amounts of IFN $\gamma$  upon stimulation (Haas et al., 1993; Wang et al., 2001; Garcia et al., 1997). BrHPP, an analogue of IPP, known to be a potent antigen of  $\gamma\delta$  T cells was used to stimulate “freshly isolated”  $\gamma\delta$  T cells in the presence of rhIL2. Upon stimulation with BrHPP + rhIL2, the activation markers (CD69, CD25 and RANKL) on these cells were found to be increased compared to unstimulated  $\gamma\delta$  T cells. CD69, an early activation marker, is expressed on T cells upon triggering through TCR complex (Ziegler et al., 1994). T cell activation through CD69 results in upregulation of CD25 and induction of Th1 cytokine (IL2 and IFN $\gamma$ ), ultimately triggering proliferation of the lymphocytes (Rutella et al., 1999). As expected, higher levels of IFN $\gamma$  along with increased expression of activation markers (CD25 and RANKL) were observed on “activated”  $\gamma\delta$  T cells (which were expanded using  $\alpha$ CD3/CD28 + rhIL2), while no significant change was observed in early activation marker CD69. “Freshly isolated”  $\gamma\delta$  T cells upon stimulation with BrHPP + rhIL2 showed increase in IFN $\gamma$  production, along with significant increase in early activation markers like CD69 and RANKL. Compared to rhIL2 stimulated, “freshly isolated”  $\gamma\delta$  T cells stimulated with BrHPP + rhIL2, irrespective of short or long term activation, showed inhibitory effect on generation (vitronectin receptor expression) and resorptive ability (resorption area on OAS plates) of osteoclasts. Thus, “freshly isolated”  $\gamma\delta$  T cells stimulated with phosphoantigen (BrHPP + rhIL2) exhibited the characteristics of “activated”  $\gamma\delta$  T cells to suppress osteoclast generation and function.

To best of our knowledge, this is the first study to show that activation status and the cytokines released by  $\gamma\delta$  T cells dictates their effect on osteoclastogenesis. Aminobisphosphonates, the new generation bisphosphonates, are used as standard treatment modality in patients with breast cancer, prostate cancer and multiple myeloma patients exhibiting skeletal metastasis (Dhar and Chiplunkar, 2010; Roodman and Dougall, 2008). Aminobisphosphonates inhibit activity and survival of osteoclasts (Caraglia et al., 2006). Besides their antitumor and anti-resorptive ability, aminobisphosphonate (Zoledronic acid) are potent activators of  $\gamma\delta$  T cells. IL6, dominantly secreted by “freshly isolated”  $\gamma\delta$  T cells (low CD25, CD69 expression) promote osteoclastogenesis, while antigen (BrHPP) activated  $\gamma\delta$  T cells (high CD25, CD69 expression) inhibit osteoclastogenesis through increased IFN $\gamma$  production. This data adds a new dimension towards understanding the mechanism of action of aminobisphosphonate in patients receiving this treatment for skeletal metastasis. Although antitumor and anti-resorptive roles of aminobisphosphonates are reported, the present study further demonstrates the crosstalk of immune cells and osteoclasts mediated by aminobisphosphonates. Future immunotherapeutic approaches can utilize aminobisphosphonates activated  $\gamma\delta$  T cells for treatment of skeletal metastasis.

## 5. Conclusion

Effect of  $\gamma\delta$  T cells on osteoclastogenesis depends on their activation status. Phosphoantigen “activated”  $\gamma\delta$  T cells express higher levels of activation markers (CD25 and RANKL expression) and secrete higher levels of IFN $\gamma$  (anti-osteoclastogenic), thus inhibiting osteoclast generation and function. On the contrary, “freshly isolated”  $\gamma\delta$  T cells, in an unstimulated state (low CD25, CD69 and RANKL expression) secrete higher levels of IL6 (pro-osteoclastogenic) and enhance osteoclast generation and function. “Freshly isolated”  $\gamma\delta$  T cells, when stimulated with phosphoantigen (BrHPP) showed increased IFN $\gamma$  secretion and attained anti-osteoclastogenic potential. Neutralization of IFN $\gamma$  and IL6 using blocking antibodies majorly diminished the “inhibitory” or “stimulatory” effect of “freshly isolated” and “activated”  $\gamma\delta$  T cells respectively on osteoclastogenesis. In conclusion, activation status and dynamics of IL6/IFN $\gamma$  dictates pro or anti-osteoclastogenic role of  $\gamma\delta$  T cells.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bonr.2015.10.004>.

## Conflict of interest statement

The author (s) declare(s) that there is no conflict of interest.

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## References

- Beetz, S., Marischen, L., Kabelitz, D., Wesch, D., 2007. Human gamma delta T cells: candidates for the development of immunotherapeutic strategies. *Immunol. Res.* 37, 97–111.
- Born, W.K., Reardon, C.L., O'Brien, R.L., 2006. The function of gammadelta T cells in innate immunity. *Curr. Opin. Immunol.* 18, 31–38.
- Buccheri, S., Guggino, G., Caccamo, N., Li Donni, P., Dieli, F., 2014. Efficacy and safety of gammadelta T cell-based tumor immunotherapy: a meta-analysis. *J. Biol. Regul. Homeost. Agents* 28, 81–90.
- Caccamo, N., La Mendola, C., Orlando, V., Meraviglia, S., Todaro, M., Stassi, G., Sireci, G., Fournie, J.J., Dieli, F., 2011. Differentiation, phenotype, and function of interleukin-17-producing human Vgamma9Vdelta2 T cells. *Blood* 118, 129–138.
- Caccamo, N., Todaro, M., Sireci, G., Meraviglia, S., Stassi, G., Dieli, F., 2013. Mechanisms underlying lineage commitment and plasticity of human gammadelta T cells. *Cell. Mol. Immunol.* 10, 30–34.
- Caraglia, M., Santini, D., Marra, M., Vincenzi, B., Tonini, G., Budillon, A., 2006. Emerging anti-cancer molecular mechanisms of aminobisphosphonates. *Endocr. Relat. Cancer* 13, 7–26.
- Casetti, R., Agrati, C., Wallace, M., Sacchi, A., Martini, F., Martino, A., Rinaldi, A., Malkovsky, M., 2009. Cutting edge: TGF-beta1 and IL-15 induce FOXP3+ gammadelta regulatory T cells in the presence of antigen stimulation. *J. Immunol.* 183, 3574–3577.
- Chiplunkar, S., Dhar, S., Wesch, D., Kabelitz, D., 2009. Gammadelta T cells in cancer immunotherapy: current status and future prospects. *Immunotherapy* 1, 663–678.
- Dhar, S., Chiplunkar, S.V., 2010. Lysis of aminobisphosphonate-sensitized MCF-7 breast tumor cells by Vgamma9Vdelta2 T cells. *Cancer Immunol.* 10, 10.
- Espinosa, E., Belmont, C., Pont, F., Luciani, B., Poupot, R., Romagne, F., Brailly, H., Bonneville, M., Fournie, J.J., 2001. Chemical synthesis and biological activity of bromohydrin pyrophosphate, a potent stimulator of human gamma delta T cells. *J. Biol. Chem.* 276, 18337–18344.
- Garcia, V.E., Sieling, P.A., Gong, J., Barnes, P.F., Uyemura, K., Tanaka, Y., Bloom, B.R., Morita, C.T., Modlin, R.L., 1997. Single-cell cytokine analysis of gamma delta T cell responses to nonpeptide mycobacterial antigens. *J. Immunol.* 159, 1328–1335.
- Gertner-Dardenne, J., Castellano, R., Mamessier, E., Garbit, S., Kochbati, E., Etienne, A., Charbonnier, A., Collette, Y., Vey, N., Olive, D., 2012. Human Vgamma9Vdelta2 T cells specifically recognize and kill acute myeloid leukemic blasts. *J. Immunol.* 188, 4701–4708.
- Gillespie, M.T., 2007. Impact of cytokines and T lymphocytes upon osteoclast differentiation and function. *Arthritis Res. Ther.* 9, 103.
- Gomes, A.Q., Correia, D.V., Grosso, A.R., Lanca, T., Ferreira, C., Lacerda, J.F., Barata, J.T., Silva, M.G., Silva-Santos, B., 2010. Identification of a panel of ten cell surface protein antigens associated with immunotargeting of leukemias and lymphomas by peripheral blood gammadelta T cells. *Haematologica* 95, 1397–1404.
- Gupta, N., Barhanpurkar, A.P., Tomar, G.B., Srivastava, R.K., Kour, S., Pote, S.T., Mishra, G.C., Wani, M.R., 2010. IL-3 inhibits human osteoclastogenesis and bone resorption through downregulation of c-Fms and diverts the cells to dendritic cell lineage. *J. Immunol.* 185, 2261–2272.
- Haas, W., Pereira, P., Tonegawa, S., 1993. Gamma/delta cells. *Annu. Rev. Immunol.* 11, 637–685.
- Havran, W.L., Jameson, J.M., Witherden, D.A., 2005. Epithelial cells and their neighbors. III. Interactions between intraepithelial lymphocytes and neighboring epithelial cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* 289, G627–G630.
- Hayday, A.C., 2000. [Gamma][delta] cells: a right time and a right place for a conserved third way of protection. *Annu. Rev. Immunol.* 18, 975–1026.
- Hayday, A.C., 2009. Gammadelta T cells and the lymphoid stress-surveillance response. *Immunity* 31, 184–196.
- Horwood, N.J., Kartsogiannis, V., Quinn, J.M., Romas, E., Martin, T.J., Gillespie, M.T., 1999. Activated T lymphocytes support osteoclast formation in vitro. *Biochem. Biophys. Res. Commun.* 265, 144–150.
- Jameson, J.M., Cauvi, G., Sharp, L.L., Witherden, D.A., Havran, W.L., 2005. Gammadelta T cell-induced hyaluronan production by epithelial cells regulates inflammation. *J. Exp. Med.* 201, 1269–1279.
- Kabelitz, D., Kalyan, S., Oberg, H.H., Wesch, D., 2013. Human Vdelta2 versus non-Vdelta2 gammadelta T cells in antitumor immunity. *Oncimmunology* 2, e23304.
- Kamolmatyakul, S., Chen, W., Li, Y.P., 2001. Interferon-gamma down-regulates gene expression of cathepsin K in osteoclasts and inhibits osteoclast formation. *J. Dent. Res.* 80, 351–355.
- Keystone, E.C., Rittershaus, C., Wood, N., Snow, K.M., Flatow, J., Purvis, J.C., Poplonski, L., Kung, P.C., 1991. Elevation of a gamma delta T cell subset in peripheral blood and synovial fluid of patients with rheumatoid arthritis. *Clin. Exp. Immunol.* 84, 78–82.
- Kong, Y.Y., Feige, U., Sarosi, I., Bolon, B., Tafuri, A., Morony, S., Capparelli, C., Li, J., Elliott, R., McCabe, S., Wong, T., Campagnuolo, G., Moran, E., Bogoch, E.R., Van, G., Nguyen, L.T., Ohashi, P.S., Lacey, D.L., Fish, E., Boyle, W.J., Penninger, J.M., 1999. Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. *Nature* 402, 304–309.
- Konigshofer, Y., Chien, Y.H., 2006. Gammadelta T cells –innate immune lymphocytes? *Curr. Opin. Immunol.* 18, 527–533.
- Kotake, S., Udagawa, N., Hakoda, M., Mogi, M., Yano, K., Tsuda, E., Takahashi, K., Furuya, T., Ishiyama, S., Kim, K.J., Saito, S., Nishikawa, T., Takahashi, N., Togari, A., Tomatsu, T., Suda, T., Kamatani, N., 2001. Activated human T cells directly induce osteoclastogenesis from human monocytes: possible role of T cells in bone destruction in rheumatoid arthritis patients. *Arthritis Rheum.* 44, 1003–1012.
- Kudo, O., Sabokbar, A., Pocock, A., Itonaga, I., Fujikawa, Y., Athanasou, N.A., 2003. Interleukin-6 and interleukin-11 support human osteoclast formation by a RANKL-independent mechanism. *Bone* 32, 1–7.
- Kurihara, N., Bertolini, D., Suda, T., Akiyama, Y., Roodman, G.D., 1990. IL-6 stimulates osteoclast-like multinucleated cell formation in long term human marrow cultures by inducing IL-1 release. *J. Immunol.* 144, 4226–4230.
- Laad, A.D., Thomas, M.L., Fakhri, A.R., Chiplunkar, S.V., 1999. Human gamma delta T cells recognize heat shock protein-60 on oral tumor cells. *Int. J. Cancer* 80, 709–714.
- Lam, J., Takahashi, S., Barker, J.E., Kanagawa, O., Ross, F.P., Teitelbaum, S.L., 2000. TNF-alpha induces osteoclastogenesis by direct stimulation of macrophages exposed to permissive levels of RANK ligand. *J. Clin. Invest.* 106, 1481–1488.
- Lee, R.J., Saylor, P.J., Smith, M.R., 2011a. Treatment and prevention of bone complications from prostate cancer. *Bone* 48, 88–95.
- Lee, S.J., Nam, K.I., Jin, H.M., Cho, Y.N., Lee, S.E., Kim, T.J., Lee, S.S., Kee, S.J., Lee, K.B., Kim, N., Park, Y.W., 2011b. Bone destruction by receptor activator of nuclear factor kappaB ligand-expressing T cells in chronic gouty arthritis. *Arthritis Res. Ther.* 13, R164.
- Lipton, A., Goessl, C., 2011. Clinical development of anti-RANKL therapies for treatment and prevention of bone metastasis. *Bone* 48, 96–99.
- Ness-Schwickerath, K.J., Jin, C., Morita, C.T., 2010. Cytokine requirements for the differentiation and expansion of IL-17A- and IL-22-producing human Vgamma2Vdelta2 T cells. *J. Immunol.* 184, 7268–7280.
- Neville-Webbe, H.L., Coleman, R.E., 2010. Bisphosphonates and RANK ligand inhibitors for the treatment and prevention of metastatic bone disease. *Eur. J. Cancer* 46, 1211–1222.
- Pappalardo, A., Thompson, K., 2013. Activated gammadelta T cells inhibit osteoclast differentiation and resorptive activity in vitro. *Clin. Exp. Immunol.* 174, 281–291.
- Pollinger, B., Jung, T., Smetzer, B., Walker, U.A., Tyndall, A., Allard, C., Bay, S., Keller, R., Raulf, F., Di Padova, F., O'Reilly, T., Horwood, N.J., Patel, D.D., Littlewood-Evans, A., 2011. Th17 cells, not IL-17+ gammadelta T cells, drive arthritic bone destruction in mice and humans. *J. Immunol.* 186, 2602–2612.
- Ragab, A.A., Nalepka, J.L., Bi, Y., Greenfield, E.M., 2002. Cytokines synergistically induce osteoclast differentiation: support by immortalized or normal calvarial cells. *Am. J. Physiol. Cell Physiol.* 283, C679–C687.
- Roodman, G.D., 2001. Biology of osteoclast activation in cancer. *J. Clin. Oncol.* 19, 3562–3571.
- Roodman, G.D., 2010. Pathogenesis of myeloma bone disease. *J. Cell. Biochem.* 109, 283–291.
- Roodman, G.D., Dougall, W.C., 2008. RANK ligand as a therapeutic target for bone metastases and multiple myeloma. *Cancer Treat. Rev.* 34, 92–101.
- Rutella, S., Rumi, C., Lucia, M.B., Barberi, T., Puggioni, P.L., Lai, M., Romano, A., Cuda, R., Leone, G., 1999. Induction of CD69 antigen on normal CD4+ and CD8+ lymphocyte subsets and its relationship with the phenotype of responding T-cells. *Cytometry* 38, 95–101.
- Santini, D., Martini, F., Fratto, M.E., Galluzzo, S., Vincenzi, B., Agrati, C., Turchi, F., Piacentini, P., Rocci, L., Manavalan, J.S., Tonini, G., Poccia, F., 2009. In vivo effects of zoledronic acid on peripheral gammadelta T lymphocytes in early breast cancer patients. *Cancer Immunol. Immunother.* 58, 31–38.
- Sato, K., Takayanagi, H., 2006. Osteoclasts, rheumatoid arthritis, and osteoimmunology. *Curr. Opin. Rheumatol.* 18, 419–426.



- Sato, K., Suematsu, A., Okamoto, K., Yamaguchi, A., Morishita, Y., Kadono, Y., Tanaka, S., Kodama, T., Akira, S., Iwakura, Y., Cua, D.J., Takayanagi, H., 2006. Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction. *J. Exp. Med.* 203, 2673–2682.
- Sharp, L.L., Jameson, J.M., Cauvi, G., Havran, W.L., 2005. Dendritic epidermal T cells regulate skin homeostasis through local production of insulin-like growth factor 1. *Nat. Immunol.* 6, 73–79.
- Sireci, G., Champagne, E., Fournie, J.J., Dieli, F., Salerno, A., 1997. Patterns of phosphoantigen stimulation of human Vgamma9/Vdelta2 T cell clones include Th0 cytokines. *Hum. Immunol.* 58, 70–82.
- Soysa, N.S., Alles, N., Aoki, K., Ohya, K., 2012. Osteoclast formation and differentiation: an overview. *J. Med. Dent. Sci.* 59, 65–74.
- Suva, L.J., Griffin, R.J., Makhoul, I., 2009. Mechanisms of bone metastases of breast cancer. *Endocr. Relat. Cancer* 16, 703–713.
- Takayanagi, H., 2007. Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems. *Nat. Rev. Immunol.* 7, 292–304.
- Takayanagi, H., Ogasawara, K., Hida, S., Chiba, T., Murata, S., Sato, K., Takaoka, A., Yokochi, T., Oda, H., Tanaka, K., Nakamura, K., Taniguchi, T., 2000. T-cell-mediated regulation of osteoclastogenesis by signalling cross-talk between RANKL and IFN-gamma. *Nature* 408, 600–605.
- Takayanagi, H., Sato, K., Takaoka, A., Taniguchi, T., 2005. Interplay between interferon and other cytokine systems in bone metabolism. *Immunol. Rev.* 208, 181–193.
- Teitelbaum, S.L., 2007. Osteoclasts: what do they do and how do they do it? *Am. J. Pathol.* 170, 427–435.
- Thomas, M.L., Samant, U.C., Deshpande, R.K., Chiplunkar, S.V., 2000. Gammadelta T cells lyse autologous and allogenic oesophageal tumours: involvement of heat-shock proteins in the tumour cell lysis. *Cancer Immunol. Immunother.* 48, 653–659.
- Todaro, M., D'Asaro, M., Caccamo, N., Iovino, F., Francipane, M.G., Meraviglia, S., Orlando, V., La Mendola, C., Gulotta, G., Salerno, A., Dieli, F., Stassi, G., 2009. Efficient killing of human colon cancer stem cells by gammadelta T lymphocytes. *J. Immunol.* 182, 7287–7296.
- Walsh, M.C., Kim, N., Kadono, Y., Rho, J., Lee, S.Y., Lorenzo, J., Choi, Y., 2006. Osteoimmunology: interplay between the immune system and bone metabolism. *Annu. Rev. Immunol.* 24, 33–63.
- Wang, L., Kamath, A., Das, H., Li, L., Bukowski, J.F., 2001. Antibacterial effect of human V gamma 2V delta 2 T cells in vivo. *J. Clin. Invest.* 108, 1349–1357.
- Weitzmann, M.N., Cenci, S., Rifas, L., Haug, J., Dipersio, J., Pacifici, R., 2001. T cell activation induces human osteoclast formation via receptor activator of nuclear factor kappaB ligand-dependent and -independent mechanisms. *J. Bone Miner. Res.* 16, 328–337.
- Wesch, D., Glatzel, A., Kabelitz, D., 2001. Differentiation of resting human peripheral blood gamma delta T cells toward Th1- or Th2-phenotype. *Cell. Immunol.* 212, 110–117.
- Wong, P.K., Quinn, J.M., Sims, N.A., van Nieuwenhuijze, A., Campbell, I.K., Wicks, I.P., 2006. Interleukin-6 modulates production of T lymphocyte-derived cytokines in antigen-induced arthritis and drives inflammation-induced osteoclastogenesis. *Arthritis Rheum.* 54, 158–168.
- Wrobel, P., Shojaei, H., Schitteck, B., Gieseler, F., Wollenberg, B., Kalthoff, H., Kabelitz, D., Wesch, D., 2007. Lysis of a broad range of epithelial tumour cells by human gamma delta T cells: involvement of NKG2D ligands and T-cell receptor- versus NKG2D-dependent recognition. *Scand. J. Immunol.* 66, 320–328.
- Wu, Y.L., Ding, Y.P., Tanaka, Y., Shen, L.W., Wei, C.H., Minato, N., Zhang, W., 2014. Gammadelta T cells and their potential for immunotherapy. *Int. J. Biol. Sci.* 10, 119–135.
- Ziegler, S.F., Ramsdell, F., Alderson, M.R., 1994. The activation antigen CD69. *Stem Cells* 12, 456–465.