Vγ9Vδ2 T cells inhibit immature dendritic cell transdifferentiation into osteoclasts through downregulation of RANK, c-Fos and ATP6V0D2

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Abstract. Osteoimmunological studies have revealed that T cells exert a powerful impact on the formation and activity of osteoclasts and bone remodeling. Evidence demonstrates that immature dendritic cells (iDCs) are more efficient transdifferentiating into osteoclasts (OCs) than monocytes. However, whether Vγ9Vδ2 T (γδ T) cells stimulate or inhibit iDC transdifferentiation into OCs has never been reported. The aim of the present study was to investigate the effects of γδ T cells on this transdifferentiation process. γδ T cells and iDCs were isolated from the peripheral blood of healthy volunteers separately and were co-cultured with Transwelll inserts, with γδ T cells in the upper chamber and iDCs in the lower chamber. IDCs were treated with macrophage-colony stimulating factor and receptor activator of nuclear factor-κB (RANK) ligand. Tartrate resistant acid phosphatase (TRAP) assay and dentine resorption assay were performed to detect OC formation and their resorption capacity, respectively. The mRNA expression of OCs was examined using a microarray and real time-quantitative polymerase chain reaction to trace the changes during iDC transdifferentiation into OCs. The results demonstrated that γδ T cells significantly inhibited the generation of the TRAP-positive OCs from iDCs and their resorption capacity. The microarray analysis identified decreased expression level of Fos proto-oncogene AP-1 transcription factor subunit (c-Fos), ATPase H^+ transporting V0 subunit d (ATP6V0D2) and cathepsin K when iDCs were co-cultured with γδ T cells. These genes are associated with OC differentiation, indicating that γδ T cells suppressed iDCs osteoclastogenesis by downregulation of the RANK/c-Fos/ATP6V0D2 signaling pathway. The present findings provide novel insights into the interactions between human γδ T cells and iDCs, and demonstrate that γδ T cells are capable of inhibiting OC formation and their activity via downregulation of genes associated with OC differentiation.

Introduction

The study of the interface between bone and the immune system (termed osteoimmunology) focuses on shared niches, mechanistic receptors and cytokines in physiological and pathological conditions (1,2). In recent years, intensive studies suggest that there is a close relationship between the abnormal activation of immune cells and the bone absorption function of osteoclasts (OCs) (3). OCs are stimulated to differentiate and become activated by cytokines that are produced by immune cells, such as dendritic cells and T cells, *in vitro* or *in vivo* in experimental models (4-7).

Bone homeostasis is continuously regulated by two processes: Bone formation and bone resorption by osteoblasts and OCs, respectively (8,9). OCs are multinuclear cells that are essential for bone remodeling, and their activity depends on the high local concentration of the receptor activator of nuclear factor-κB ligand (RANKL) and macrophage-colony stimulating factor (M-CSF) (10). RANKL, a member of the tumor necrosis factor (TNF) superfamily, interacts with receptor activator of nuclear factor-κB (RANK) that is expressed on OC progenitors, and then binds to TNF receptor-associated factor 6 (TRAF6) to activate downstream signaling cascades (11), such as the mitogen-activated protein

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Abbreviations: α-MEM, α-inimal essential medium; FBS, fetal bovine serum; PBMNCs, peripheral blood mononuclear cells; iDCs, immature dendritic cells; OC, osteoclast; rh-IL-2, recombinant human interleukin-2; rh-IL-4, recombinant human interleukin-4; GM-CSF, granulocyte macrophage-colony stimulating factor; M-CSF, macrophage-colony stimulating factor; RANK, receptor activator of nuclear factor-κB; RANKL, receptor activator of nuclear factor-κB ligand

Key words: Vγ9Vδ2 T cells, immature dendritic cells, osteoclast, receptor activator of nuclear factor-κB, Fos proto-oncogene AP-1 transcription factor subunit, ATPase H^+ transporting V0 subunit d2

kinase (MAPK)/nuclear factor-κB (NF-κB) signal pathway, to promote OC formation and bone resorption (12). Most studies have focused on osteoclastogenesis from the monocytes. However, immature dendritic cells (iDCs) derived from hematopoietic progenitors are also crucial in osteoclastogenesis, especially in pathologies, such as rheumatoid arthritis and lytic bone metastases of malignancies (4,13,14). iDCs are alternate OC precursors and have been reported to be more efficient in OC transdifferentiation than monocytes (15). Suppressing iDC transdifferentiation into OCs might be a novel strategy for the treatment of bone disease, such as myeloma bone disease. γδ T cells are a subset of T cells and serve an important role in antitumor immunity. The effect of γδ T cells on osteoclastogenesis has rarely been studied. The present study aimed to explore the role of human γδ T cells in the process of iDC transdifferentiation into OCs.

Materials and methods

Human γδ T cells preparation. Peripheral blood mononuclear cells (PBMNCs) from ten healthy male adult volunteers were isolated by Ficoll Paque (Pharmacia Biotech, Piscataway, NJ, USA). The protocols were approved by the Medical Ethics Committee of The First Affiliated Hospital of Fujian Medical University, with the approval reference number 2016[016]. The PBMNCs were stimulated with 1 μM zoledronate acid (ZOL) and 100 U/ml recombinant human interleukin-2 (rh-IL-2; Sigma-Alrdich; Merck KGaA, Darmstadt, Germany) in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at a concentration of $1x10^6$/ml and were incubated at 37˚C with 5% CO_2 for 10 days. The medium was half replaced with fresh medium containing 100 U/ml rh-IL-2 every three days. At day 7, the cells were stained with anti-TCR Vγ9-fluorescein isothiocyanate (cat. no. 130-107-487; 1:50: Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and TCR Vδ2-phycoerythrin (cat. no. 130-111-010; 1:50: Miltenyi Biotec GmbH), or isotype controls, and analyzed by flow cytometry for enrichment of Vγ9 and Vδ2-double positive cells. At day 10, the cells were positively purified using magnetic-activated cell sorting with anti-γδ TCR magnetic beads kit (Miltenyi Biotec GmbH), as previously described (16).

$CD14^+$ monocyte purification and iDC culture. PBMNCs were purified using CD14 MicroBeads human monocyte kit (Miltenyi Biotec GmbH). The phenotype of the monocytes displayed >95% purity, as examined by flow cytometry analysis for CD14 expression. To obtain iDCs, these $CD14^+$ monocytes were suspended at $5x10^5$ cells/ml in RPMI 1640 with 10% FBS (both Gibco; Thermo Fisher Scientific, Inc.), 100 ng/ml rh-GM-CSF (PeproTech, Inc., Rocky Hill, NJ, USA) and 10 ng/ml rh-IL-4 (PeproTech, Inc.) for 6 days. The membrane marker CD1a and isotype were assessed by flow cytometric analysis. Immunolabeling of cell suspensions was performed in 1% bovine serum albumin (BSA) and 3% human serum/PBS (both Gibco; Thermo Fisher Scientific, Inc.). The purity of the isolated cell subsets was assessed by flow cytometry on a FACSCalibur (BD Biosciences, San Jose, CA, USA) and the data were analyzed using the Cell Quest program version 6.0 (FACScan; BD Biosciences).

Co-culture of γδ T cells and iDCs. γδ T cells and autologous iDCs from the same healthy volunteer were co-cultured at different ratios in Transwell inserts. The γδ T cells were added in the upper and the iDCs were added in the lower compartment of the Transwells, with the medium containing 10% FBS, 100 ng/ml rh-RANKL (PeproTech, Inc.) and 25 ng/ml rh-M-CSF (PeproTech, Inc.). To further confirm if the stage of OC differentiation was important for the inhibitory effect of γδ T cells, γδ T cells were co-cultured with iDCs at the ratio of 1:1 and iDCs were collected at different time-points of culture. The day 0-1 group, day 3-4 group and the day 0-4 group were treated with γδ T cells during days 0-1, day 3-4 and day 0-4, respectively.

Tartrate resistant acid phosphatase (TRAP) staining assay. At day 9 of co-culture, iDCs were collected, fixed with 5% paraformaldehyde for 10 min and treated with acetone/ethanol for 30 sec. Then the cells were washed with PBS, plated on the slides and stained using the Leukocyte Acid Phosphatase kit 387-A (Sigma-Aldrich; Merck KGaA) to TRAP activity. Multinucleated cells with >3 nuclei were counted under microscopy in ten different fields/well.

Bone resorption capacity assay. To assess the OC resorption capacity, iDCs from the lower compartment of Transwelll inserts were collected and resuspended in 96-well plates with a dentine disc at the bottom, at a density of $2x10^4$ cells/ml. After 24 h incubation so that the cells attach, the culture was maintained in the medium containing 25 ng/ml rh-M-CSF and 100 ng/ml rh-RANKL for 14 days. Cultures had half of the medium changed with fresh cytokines and conditioned medium every 2-3 days. Then, dentine was stained with 1% toluidine. Images of the resorption pit were captured using reflected light microscopy and the pit area of dentine was measured by Image-pro plus software version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Affymetrix GeneChip assays. IDCs were co-cultured with γδ T cells (1:1 ratio) in the presence of rh-RANKL and rh-M-CSF. As a control, iDCs (without γδ T cells) were cultured alone. Four samples were prepared from two unrelated healthy volunteer donors. On day 9 of co-culture, total RNA was extracted from the iDCs, using TRIzol reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. The samples were then purified with the RNeasy MinElute cleanup kit (Qiagen GmbH, Hilden, Germany). Affymetrix Human Genome U133 Plus 2.0 Arrays were used in the microarray analysis and the data were analyzed using Affymetrix GeneChip Command Console Software (TGCC; Affymetrix; Thermo Fisher Scientific, Inc.). Afterwards, genes with 'absent' scores were filtered out and the remaining genes were analyzed. Microarray data were normalized using the Robust Multiarray Average method. Significance Analysis of Microarrays (17) was used to identify genes that are differentially expressed. Furthermore, fold-change analysis, which calculated the ratios of geometric means of expression intensities, was performed. To select the differentially expressed genes (DEGs), the criteria were: Threshold values of ≥2 and ≤-2-fold change; and significance level <5%. Differentially expressed genes were subjected to gene ontology (GO) and

Table I. Sequences of primers used in reverse transcription-quantitative polymerase chain reaction.

Gene	Primer	Sequence (5'-3')
Cathepsin K	Forward	GGAAGAAGACCCACAGGAAGCAATA
	Reverse	GAGAAGCCTCAAGGTTATGGATGGA
ATP6V0D2	Forward	GTCCCATTCTTGAGTTTGAGG
	Reverse	GGATAGAGTTTGCCGAAGGTT
RANK	Forward	CAGTGAGAAGCATTATGAGCATC
	Reverse	ATTCCAGCTATCCAAGTATTCATCC
c-Fos	Forward	TTGCTGCATAAAGTTTGTGATACAG
	Reverse	AGGAAAAGGCATCAGAGAAGTAGC
GAPDH	Forward	CCAGCAAGAGCACAAGAGGAAGAG
	Reverse	GGTCTACATGGCAACTGTGAGGAG

ATP6V0D2, ATPase H^+ transporting V0 subunit d; RANK, receptor activator of nuclear factor-κB; c-Fos, Fos proto-oncogene AP-1 transcription factor subunit.

Kyoto Encyclopedia of Genes Genomes (KEGG) functional analysis for biological processes using Ingenuity Pathway Analysis (Qiagen GmbH).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. RNA was extracted using RNeasy Mini kit (Qiagen GmbH) from each group of iDCs, converted to cDNA using a PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China), and qPCR analysis was performed using the SYBR PreMix Ex Taq II kit (Takara Bio, Inc., Otsu, Japan). The sequences of primers used in this study were listed in Table I. qPCR reactions were run on an ABI 7500 Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The qPCR reaction was performed at 95˚C for 5 min, followed by 40 cycles of 95˚C for 30 sec, 60˚C for 30 sec, and 72˚C for 30 sec. The data were analyzed using the $2^{-\Delta\Delta Cq}$ relative expression method (18). qPCR reactions were performed in triplicate.

Western blot analysis. IDCs were seeded at $1.0x10^6$ cells/well in 6-well plates with 100 ng/ml rh-RANKL and 25 ng/ml rh-M-CSF and co-cultured with $1x10^5$ γδ T cells for 12 h. As a control, iDCs cultured alone (without γδ T cells) were used. At day 9, the cells were lysed with RIPA buffer. The protein concentrations were determined using a Bio-Rad protein assay kit. Then 20 μg of protein from each sample were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (BD Biosciences). The membranes were blocked with 5% non-fat milk in TBST buffer. Membranes were incubated with the following primary antibodies: β-actin (cat. no. bs-0061R; BIOSS, Beijing, China), RANK (cat.no. 4845; Cell Signaling Technology, Inc., Danvers, MA, USA), c-Fos (cat. no. 4384; Cell Signaling Technology, Inc.) and ATP6V0D2 (cat. no. ab194557; Abcam, Cambridge, MA, USA) at a dilution of 1:1,000 at 4˚C overnight. The immunoreactive bands were then incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (cat. no. A0208; 1:1,000; Beyotime Institute of Biotechnology, Haimen, China) for 1 h at room temperature. The bands were visualized using an enhanced chemiluminescence detection system (Amersham; GE Healthcare Life Sciences, Piscataway, NJ, USA). Densitometric analysis was performed using Quantity One software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Statistical analysis was conducted using SPSS 20.0 (IBM Corp., Armonk, NY, USA). All quantitative data were expressed as mean ± standard deviation. Statistical differences were analyzed by one-way analysis of variance, followed by Tukey post-hoc test for multiple-group comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

γδ T cells inhibit OC differentiation from iDCs in vitro. At day 10 of culture of the PBMNCs in the medium containing ZOL and rh-IL-2, the cells were collected and analyzed with flow cytometry. The results demonstrated that the ratio of Vγ9 and Vδ2-double positive cells was 86.5% of total (Fig. 1A). $CD14^+$ PBMNCs were cultured with rh-GM-CSF and rh-IL-4 in RPMI 1640 medium. After 6 days of incubation, these round mononuclear cells grew branched projections. Flow cytometric analysis revealed that the CD1a-positive rate was up to 86%, and therefore, these cells could be regarded as iDCs (Fig. 1B). After iDCs were co-cultured with γδ T cells, maintaining culture of these iDCs in the presence of RANKL and M-CSF produced significantly fewer $TRAP^+$ OCs compared with the control iDCs (without γδ T cell exposure; $P<0.001$). The higher the radio of γδ T: iDCs in the culture, the fewer TRAP-positive OCs were observed (Fig. 2A). The difference between each adjacent ratio group was highly significant ($P<0.001$). Furthermore, γδ T cells were added at different time-points of OC differentiation. The number of $TRAP^+$ OCs in the day 0-1 group was significantly lower compared with the control group ($P<0.001$) and also significantly lower compared with the day 3-4 group ($P<0.001$; Fig. 2B). No significant

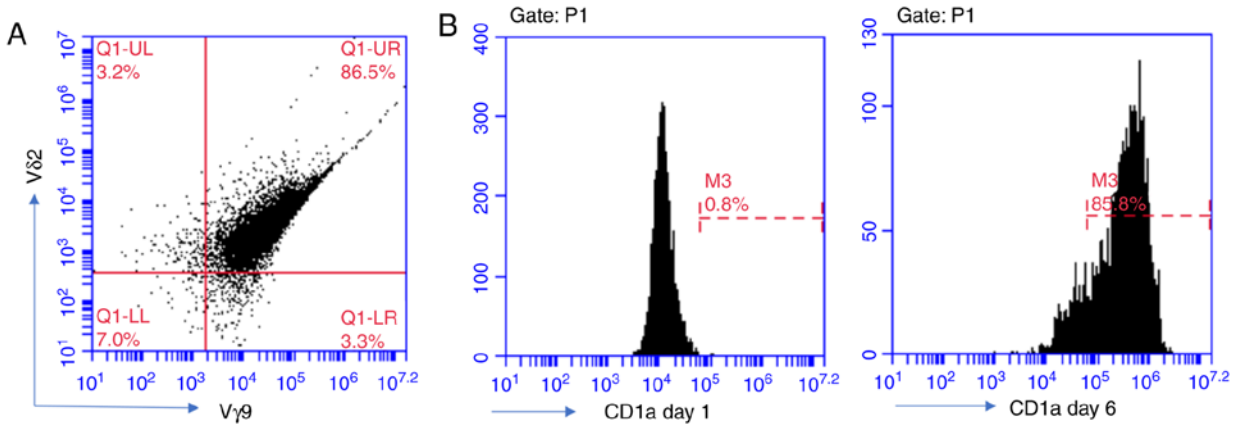

Figure 1. Flow cytometry analysis of cultured cells. (A) PBMNCs were cultured in 24-well plates at a density of $1x10^6$/well in medium containing ZOL and rh-IL-2. The cultured cells were collected on day 7, stained with anti-TCR Vγ9-FITC and TCR Vδ2-PE and analyzed by flow cytometry. (B) $CD14^+$ PBMNCs were cultured in medium containing rh-GM-CSF and rh-IL-4 for 6 days. The CD1a phenotype was analyzed by flow cytometry. Left: the expression of CD1a at day 1. Right, the expression of CD1a at day 6. Data shown are from one experiment and are representative of two further experiments from independent donors. PBMNCs, peripheral blood mononuclear cells; ZOL, zoledronate acid; rh, recombinant human; IL, interleukin; FITC, fluorescein isothiocyanate; PE, phycoerythrin; GM-CSF, granulocyte macrophage-colony stimulating factor.

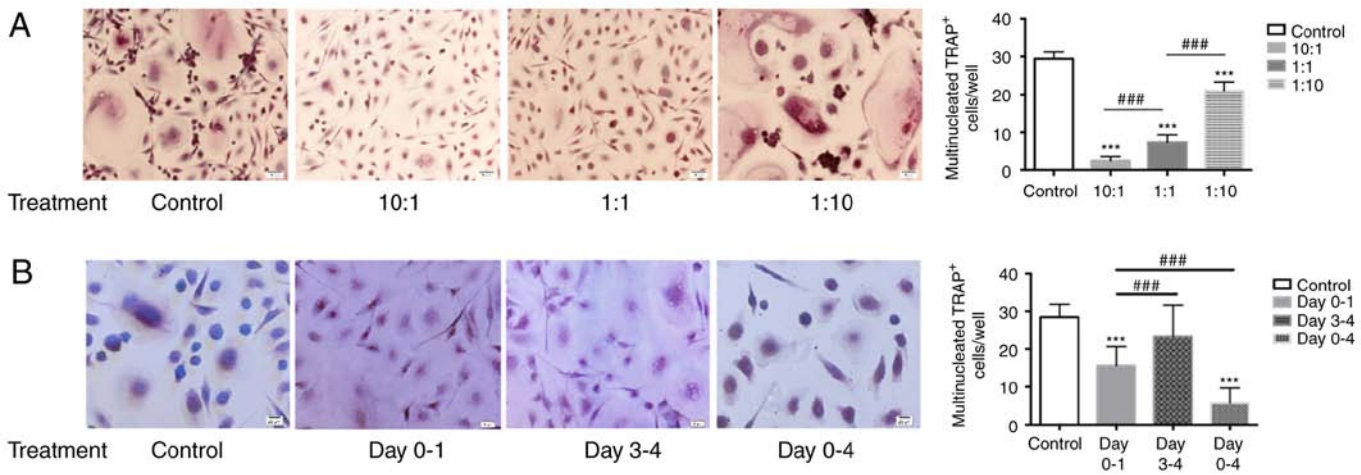

Figure 2. TRAP-positive OCs generated from iDCs following co-culture with γδ T cells. iDCs were collected following co-culture, maintained in α-MEM supplemented with rh-RANKL and rh-M-CSF for 9 days and assayed for TRAP. (A) γδ T cells were co-cultured with iDCs in the Transwell insert at the ratio of 10:1, 1:1 and 1:10 for 24 h. (B) γδ T cells were co-cultured with iDCs in the Transwell insert at the ratio of 1:1 and iDCs were collected at different time-points of culture (duration of treatment, days 0-1, 3-4 or 0-4). Representative images of the staining (magnification, x200) and quantification of $TRAP^+$ cells/well is shown. Data are presented as the mean ± standard deviation from three experiments from independent donors. $^{***}P<0.001$ compared with control; $^{###}P<0.001$ with comparisons indicated by lines. TRAP, tartrate resistant acid phosphatase; OCs, osteoclasts; iDCs, immature dendritic cells; rh, recombinant human; RANKL, receptor activator of nuclear factor-κB ligand; M-CSF, macrophage-colony stimulating factor.

difference was observed between the day 3-4 group and control (Fig. 2B). Thus, only the cells at the early stage of OC differentiation (treatment during the 0-1 days) were sensitive to the inhibitory effect of γδ T cells.

γδ T cells inhibit OC resorption capacity in vitro. Toluidine blue staining revealed that the lacunar resorption area on dentine slices of the co-culture group was significantly decreased compared with the control group (P<0.001; Fig. 3). The higher the radio of γδT: iDCs, the smaller the lacunar resorption area. There was a significant difference between the adjacent ratio groups (P<0.001; Fig. 3).

γδ T cells negatively regulate the RANK/c-Fos/ATP6V0D2 pathway. Fig. 4A illustrates the changed in mRNA expression patterns between the co-culture of γδ T cells and iDCs and the individual culture of iDCs. A total of 293 mRNAs, whose expression change was >2-fold, were identified. Among them, 123 mRNAs were upregulated and 170 mRNAs were downregulated in iDCs co-cultured with γδ T cells, compared with iCDs cultured alone (Fig. 4A). The DEGs, identified from the microarray analysis as associated with OC differentiation are shown in Fig. 4B. RT-qPCR was then used to confirm the changes in mRNA expression for several of these genes. The mRNA expression levels of RANK, c-Fos, ATP6V0D2, cathepsin K and CTR were decreased in iDCs co-cultured with γδ T cells, compared with iDCs alone, and these changes were consistent with the microarray results (Fig. 4C). The significantly differentially expressed genes were examined further by GO and KEGG functional analysis. The GO enrichment

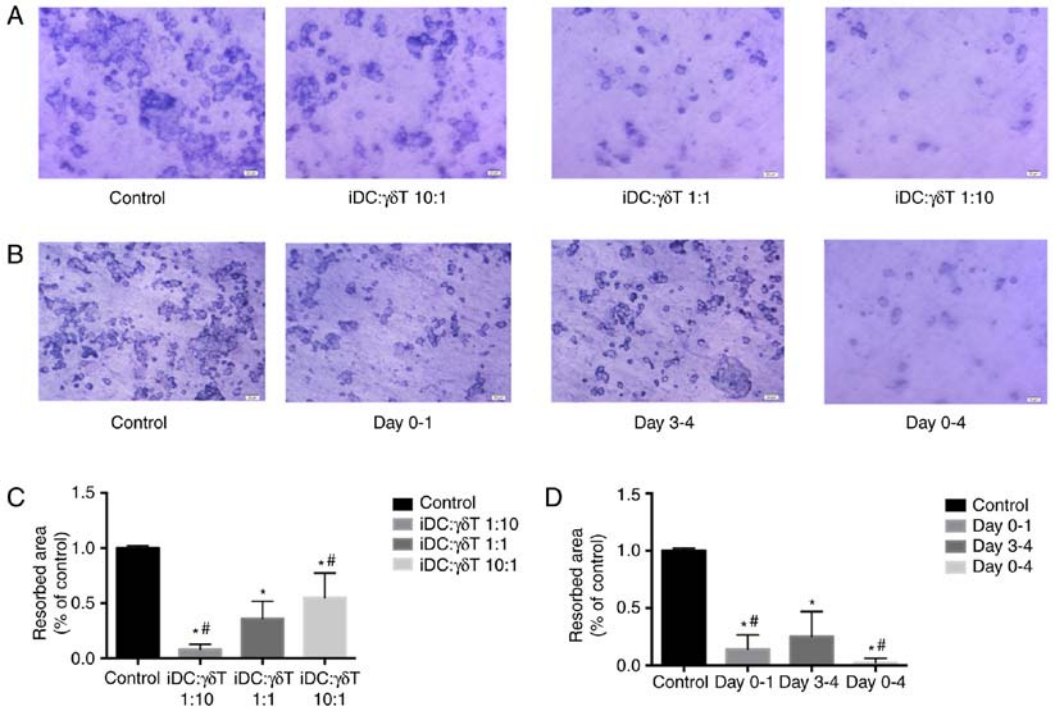

Figure 3. Resorption capacity of OCs generated from iDCs following co-culture with γδ T cells. iDCs were collected following co-culture, maintained in α-MEM supplemented with rh-RANKL and rh-M-CSF for 14 days. Dentine was stained with 1% toluidine. (A) γδ T cells were co-cultured with iDCs in the Transwelll insert at the ratio of 10:1, 1:1 and 1:10 for 24 h. (B) γδ T cells were co-cultured with iDCs in the Transwell insert at the ratio of 1:1 and iDCs were collected at different time-points of culture (duration of treatment, days 0-1, 3-4 or 0-4). (C and D) The ratio of resorbed area of control in (A and B), respectively. Representative images (magnification, x400) and quantification of the resorbed are is shown. Data are presented as the mean ± standard deviation from five experiments. *P<0.001 compared with the control; #P<0.001 compared with the 1:1 group. OCs, osteoclasts; iDCs, immature dendritic cells; rh, recombinant human; RANKL, receptor activator of nuclear factor-κB ligand; M-CSF, macrophage-colony stimulating factor.

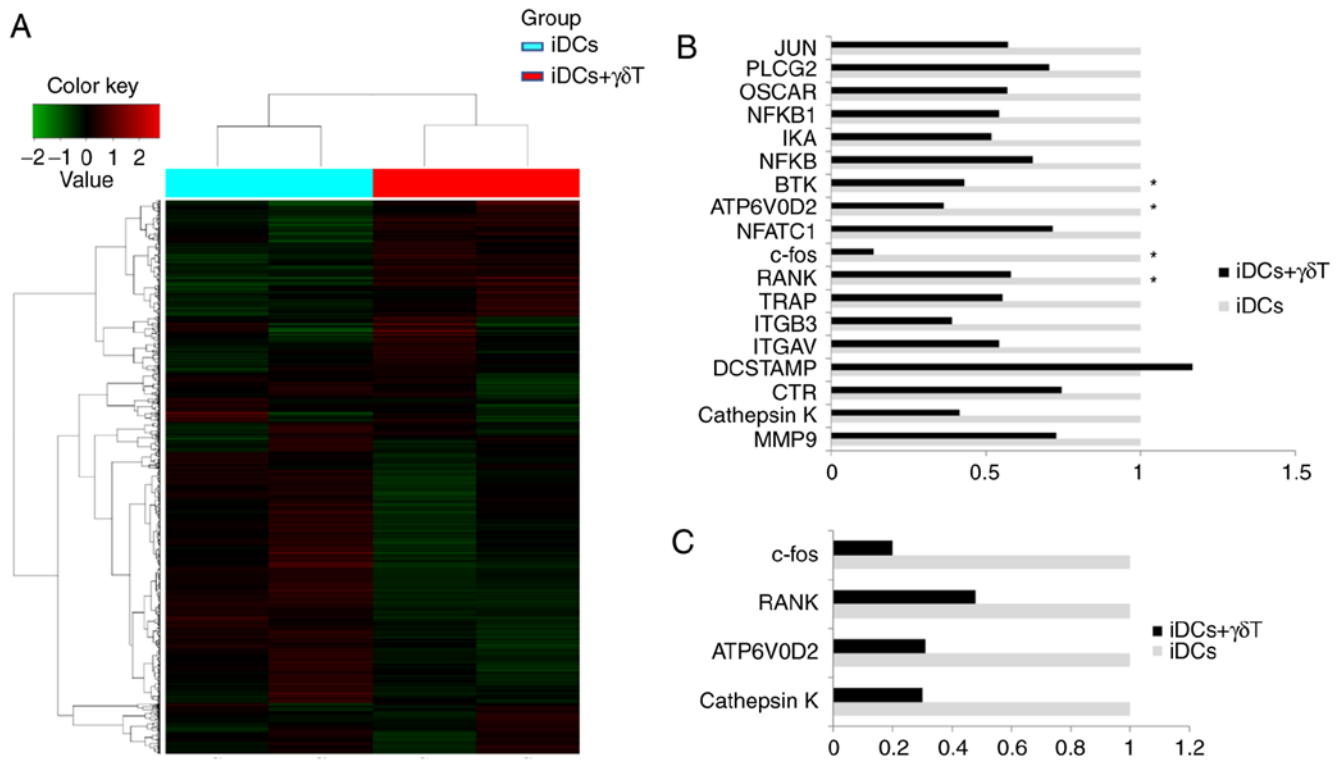

Figure 4. Differential mRNA expression of healthy human OCs transdifferentiated from iDCs. (A) Microarray data uploaded to the Gene Expression Omnibus database (accession no. GSE83464). The heat map illustrates the significant changes in mRNA expression of iDCs with and without γδ T cells co-culture. Red indicates upregulation and green indicates downregulation. (B) Based on the microarray data and KEGG enrichment analysis, the genes that were associated with OC differentiation were selected, and their mRNA fold change was listed in the graph. (C) Microarray results for DEGs were confirmed with reverse transcription-quantitative polymerase chain reaction analysis. Data are presented as the fold change of mRNA expression relative to control. *P<0.05 compared with the control group. OC, osteoclasts; iDCs, immature dendritic cells.

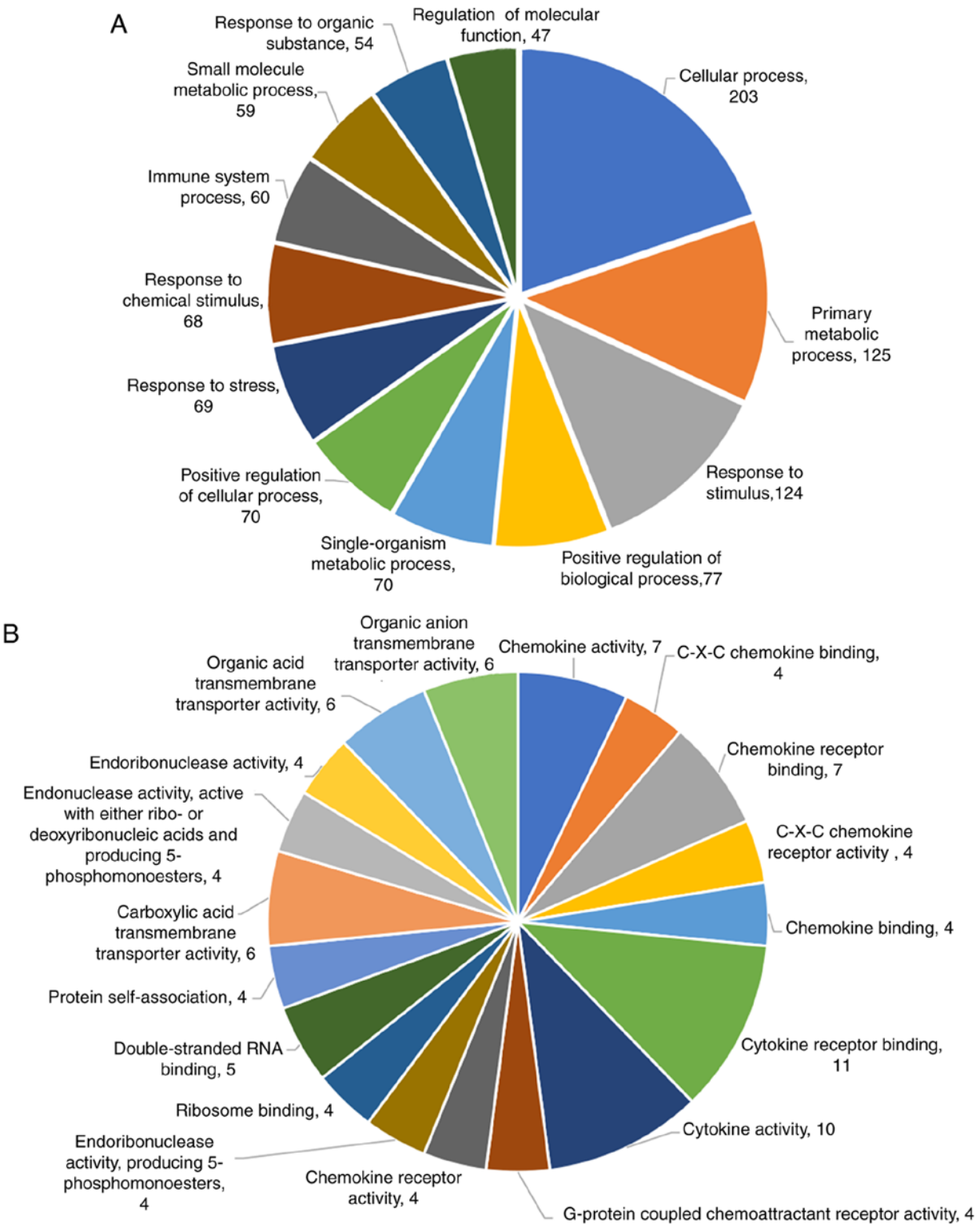

Figure 5. GO analysis of different DEGs were selected from microarray data. (A) Molecular function analysis of the genetic interaction network associated with iDCs -γδ T group compared with control group. (B) Biological process analysis of the genetic interaction network associated with iDCs -γδ T group compared with control group. GO, gene ontology; DEGs, differentially expressed genes; iDCs, immature dendritic cells.

analysis revealed that the DEGs in biological processes were mainly related to cellular process, primary metabolic process and response to stimulus positive regulation of biological process (Fig. 5A). The DEGs in molecular function were involved in chemokine activity, chemokine receptor binding, C-X-C chemokine binding and cytokine receptor binding activity (Fig. 5B). The results from KEGG functional enrichment analysis are listed in Table II. Western blot analysis also confirmed that the protein expression levels of RANK, c-Fos, ATP6V0D2 and cathepsin K in the co-culture groups (γδ T cells: iDCs co-cultures ratios, 1:1 and 10:1) were significantly decreased compared with the control group (iDC alone; Fig. 6).

Table II. KEGG enrichment analysis.

KEGG pathway	Genes involved
[ko04060] Cytokine-cytokine receptor interaction	CCL5; TNFSF10; CXCR5; CXCL3; CCL17; CXCR4; CCL18; VEGFA; IL8; VEGFA; CXCR4; TNFSF15
[ko04620] Toll-like receptor signaling pathway	CCL5; CEP170; IL8; FTSJD1; TLR4
[ko05323] Rheumatoid arthritis	CCL5; ATP6V0D2; CCL5; MMP1; VEGFA; IL8; VEGFA; TRBC1; TLR4
[ko04623] Cytosolic DNA-sensing pathway	CCL5; GOLGA7; FTSJD1; DDX58; FCGR3A; FCGR3B;
[ko04380] Osteoclast differentiation	BTK; FOSL2; ATP6V0D2; BTUK; CTSK
[ko04621] NOD-like receptor signaling pathway	CCL5; IL8; PLD1
[ko04666] Fc Gamma R-mediated phagocytosis	ARPC1B; CEP170; ARPC3; PPAP2B; PPAP2B; PLD1

KEGG, Kyoto Encyclopedia of Genes Genomes.

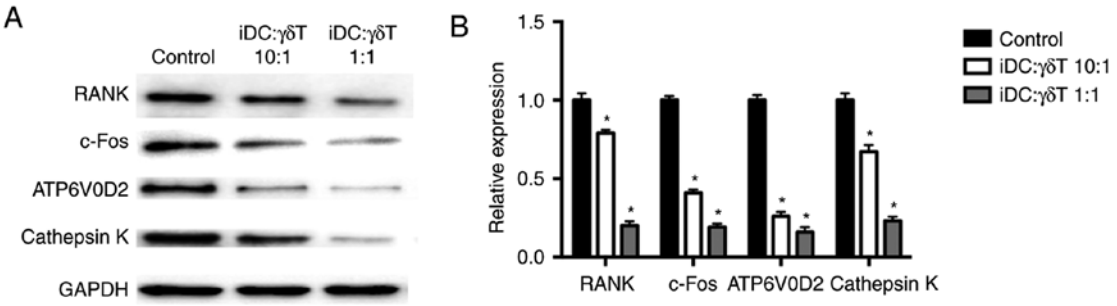

Figure 6. (A and B) Effect of γδ T cell co-culture on protein expression of RANK, c-Fos, ATP6V0D2 and cathepsin K in iDCs. iDCs were seeded at a density of $1.0x10^6$ cells/well and cultured with or without γδ T cells in the upper Transwell inserts for 24 h. The ratios of iDCs and γδ T cells were 10:1 and 1:1. After maintaining the cultures in RANKL- and M-CSF-containing medium for 9 days, the protein expression levels of RANK, c-Fos, ATP6V0D2 and cathepsin K of iDCs were detected by western blot analysis. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05 compared with the control group. RANK, receptor activator of nuclear factor-κB; c-Fos, Fos proto-oncogene AP-1 transcription factor subunit; ATP6V0D2, ATPase H^+ transporting V0 subunit d2; iDCs, immature dendritic cells; RANKL, receptor activator of nuclear factor-κB ligand; M-CSF, macrophage-colony stimulating factor.

Discussion

Previous studies have demonstrated that activated T lymphocytes suppress osteoclastogenesis *in vitro* and *in vivo* (7,19). Amongst the best understood unconventional T cells, human γδ T cells that can readily be expanded *in vitro*, contribute to anti-infection and tumor immune responses and regulate local immune surveillance (20,21). Previous studies have demonstrated that γδ T cells inhibit OC transdifferentiation from monocytes. iDCs are involved in the OC differentiation process and can also give rise to OCs in inflammatory conditions (22). However, the effects and the mechanisms of γδ T cells on iDC osteoclastogenesis have not been clearly elucidated.

In the present study, it was confirmed that γδ T cells inhibited RANKL-mediated OC differentiation from human iDCs and their resorptive function *in vitro*. Previous studies have focused on effects of the T cells on osteoclastogenesis from monocytes (23), while the effects on the transdifferentiation from the iDCs into OCs have seldom been discussed, particularly in humans. In the present study, a co-culture Transwelll system of γδ T cells and iDCs was established and the results demonstrated that γδ T cells, amplified with Zol and rhIL-2 stimulation, suppressed iDC transdifferentiation into OCs. The inhibitory effect of the γδ T cells depended on the radio of γδ T: iDCs in the cultures, which was consistent with previous studies (24,25). The current study also investigated the inhibitory effect of γδ T cells on osteoclastogenesis at different iDC differentiation stages. Notably, the results revealed that there were significantly fewer $TRAP^+$ OCs in the day 0-1 group compared with the day 3-4 group and the days 0-4 group. These results suggest that the early stage of transdifferentiation from iDCs into OCs was more vulnerable to γδ T cell inhibition.

A microarray analysis was performed for further insight into the inhibitory roles of the γδ T cells on osteoclastogenesis. Two different random/unrelated healthy donors were used for sample collection, in order to eliminate genetic variation in the samples. GO and KEGG functional analysis demonstrated that the γδ T cell inhibition on osteoclastogenesis might be associated with the RANK/RANKL signaling pathway, toll-like receptors and the cytokine-cytokine receptor interaction, among other pathway (Table II). In the context of aminobisphosphonate drugs, such as ZOL, the γδ T cells can produce interferon (IFN)-γ, TNF-α and GM-CSF, which mediate monocyte differentiation to iDCs and induce iDCs to further mature (26,27). Pappalardo and Thompson (24) demonstrated that activated human γδ T cells were capable of inhibiting OC formation and the resorption capacity of mature OCs, via the production of GM-CSF and IFN-γ. In the present study, the GO enrichment analysis of the data also revealed that chemokine and chemokine receptor activity, which was inhibited by γδ T cells, may have important roles in the process of osteoclas-

togenesis. The current findings enrich our understanding of T cell function in the regulation of OC differentiation.

Earlier studies have indicated that T lymphocytes suppress osteoclastogenesis by diverting early monocyte/macrophage progenitor lineage commitment towards dendritic cell differentiation (19), the mechanism for which so far remained undiscovered. In the present experiments, an Affymetrix mRNA microarray was used to screen out the differential mRNA expression and then RT-qPCR was used to confirm the changes in mRNA expression of RANK, cathepsin K, c-Fos and ATP6V0D2 that are associated with osteoclastogensis (Fig. 4). The results demonstrated that γδ T cells decreased RANK, c-Fos and ATP6V0D2 expression in iDCs, indicating that γδ T cells suppressed the RANK/RANKL pathway, through downregulating RANK expression, which is essential for OC differentiation and activation.

Previous studies have demonstrated that c-Fos is the switch differentiation mechanism between OC and DC lineages (19,28). c-Fos is a downstream molecule of the NF-κB signaling pathway (29), and c-Fos regulates several transcription factors that are essential for OC formation (30). c-Fos gene knockout mice exhibit severe osteopetrosis because the OC differentiation process is completely inhibited (31). Furthermore, the gene expression of ATP6V0D2 was demonstrated to be suppressed when iDCs were co-cultured with γδ T cells. As ATP6V0D2 is involved in the fusion of osteoclastic precursors (32) and ATP6V0D2 deficiency results in OC precursor cell fusion dysfunction (33), inhibition of ATP6V0D2 might be involved in the process that the γδ T cells use to suppress OC differentiation and decrease their bone resorption activity.

In conclusion, the present results demonstrated that γδ T cells were capable of inhibiting OC formation from iDCs and their resorption capacity. The potential mechanism of the action of γδ T cells might be by suppressing the gene expression of RANK, c-Fos and ATP6V0D2 and the RANK/RANKL pathway.

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Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

XZ performed the experiments and was the major contributor in writing the manuscript. ZZ contributed to the conception and design of the experiments. DQ participated in the study design and performed the statistical analysis. JC analyzed and interpreted the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The ethics committee of the First Affiliated Hospital of Fujian Medical University approved the protocols included in the present study [reference number, 2016 (016)].

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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