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Regulation of ITAM adaptor molecules and their receptors by inhibition of calcineurin-NFAT signalling during late stage osteoclast differentiation

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ABSTRACT

Osteoclasts are specialised bone resorptive cells responsible for both physiological and pathological bone loss. Osteoclast differentiation and activity is dependent upon receptor activator NF-kappa-B ligand (RANKL) interacting with its receptor RANK to induce the transcription factor, nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1 (NFATc1). The immunoreceptor tyrosine-based activation motif (ITAM)-dependent pathway has been identified as a co-stimulatory pathway in osteoclasts. Osteoclast-associated receptor (OSCAR) and triggering receptor expressed in myeloid cells (TREM2) are essential receptors that pair with adaptor molecules Fc receptor common gamma chain (FcRγ) and DNAX-activating protein 12 kDa (DAP12) respectively to induce calcium signalling. Treatment with calcineurin-NFAT inhibitors, Tacrolimus (FK506) and the 11R-VIVIT (VIVIT) peptide, reduces NFATc1 expression consistent with a reduction in osteoclast differentiation and activity. This study aimed to investigate the effects of inhibiting calcineurin-NFAT signalling on the expression of ITAM factors and late stage osteoclast genes including cathepsin K (CathK), Beta 3 integrin (β3) and Annexin VIII (AnnVIII). Human peripheral blood mononuclear cells (PBMCs) were differentiated with RANKL and macrophage-colony stimulating factor (M-CSF) over 10 days in the presence or absence of FK506 or VIVIT. Osteoclast formation (as assessed by tartrate resistant acid phosphatase (TRAP)) and activity (assessed by dentine pit resorption) were significantly reduced with treatment. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis demonstrated that FK506 treatment significantly ($p < 0.05$) reduced the expression of NFATc1, CathK, OSCAR, FcRγ, TREM2 and DAP12 during the terminal stage of osteoclast formation. VIVIT treatment significantly ($p < 0.05$) decreased CathK, OSCAR, FcRγ, and AnnVIII, gene expression. This data suggest FK506 and VIVIT act differently in targeting the calcineurin-NFAT signalling cascade to suppress key mediators of the ITAM pathway during late stage osteoclast differentiation and this is associated with a reduction in both osteoclast differentiation and activity.

1. Introduction

1.1. Background

High numbers of bone resorbing osteoclasts cells have been identified at the sites of bone loss in a number of diseases, such as near arthritic bone erosions in RA [1] and peri-implant osteolysis [2]. Osteoclast differentiation requires receptor activator of NF-κβ ligand (RANKL) and macrophage-colony stimulating factor (M-CSF). RANKL expression by bone stromal cells such as osteoblasts, synovial fibroblasts and T cell is increased adjacent to sites of pathological bone loss [3–5]. RANKL binds to its receptor RANK, expressed on pre-osteoclasts to induce osteoclast differentiation and activation via elevated expression of the transcription factor, nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1 (NFATc1) [6]. Activation of NFATc1 is mediated by the calcium ion dependent protein phosphatase, calcineurin, which dephosphorylates NFATc1 enabling its translocation to the nucleus [7]. Absence of NFATc1 retards osteoclastogenesis [8] and over-expression [9] or ectopic expression of constitutively active NFATc1 [6] stimulates osteoclastogenesis in the absence of RANKL. NFATc1 directly induces osteoclast-specific genes, including cathepsin K (CathK), tartrate resistant acid phosphatase (TRAP), calcitonin receptor (CTR), osteoclast-associated receptor (OSCAR) and β3 integrin [8,10–15], as well as auto-amplifies its own expression [9]. Additionally, the late stage substrate regulated osteoclast factor Annexin VIII (AnnVIII) [16] is also likely to be regulated by NFATc1 (unpublished data). Taken together, the calcineurin-NFATc1 pathway is pivotal to osteoclastogenesis.

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Recently other co-stimulatory pathways involving NFATc1 have been identified in osteoclasts. The immunoreceptor tyrosine-based activation motif (ITAM)-dependent pathway stimulates calcium signals that activate the calcineurin pathway and thus induce NFATc1 [14]. ITAM-containing adaptor proteins, DNAX-activating protein of molecular mass 12 kDa (DAP12) and Fc receptor common gamma chain (FcRγ) bind with their co-receptors; triggering receptor expressed in myeloid cells (TREM2) and osteoclast associated receptor (OSCAR) respectively to induce ITAM signalling [17,18]. We have demonstrated increased expression of these factors adjacent to sites of bone loss in peri-implant loosening [19].

The importance of the ITAM pathway is particularly evident in Nasu-Hakola disease in humans, where a mutation of TREM2 or DAP12 leads to the development of bone cysts [20]. Importantly, mice lacking DAP12 or FcRγ have suppressed NFATc1 expression resulting and defective osteoclast formation [21]. Additionally, DAP12$^{-/-}$ FcRγ$^{-/-}$ mice develop osteopetrosis [22]. Osteoclast differentiation is impeded and bone resorption is remarkably reduced by mutations in DAP12 and TREM2 that result in their loss of function [20]. These studies demonstrate that the ITAM molecules, FcRγ, TREM2 and DAP12 play an important role in osteoclast development and activity.

OSCAR is induced via NFATc1 and OSCAR signalling via the FcRγ adaptor molecule further enhances ITAM signalling on ligand interaction [10,14]. Genetic variations in OSCAR identify it as a potential underlying factor in postmenopausal osteoporosis [23]. Further to this, levels of cell bound and soluble OSCAR are modulated in diseases such as RA [24,25].

The convergence of RANKL-RANK and the ITAM pathways at calcineurin/NFATc1 signalling identifies this interaction as a key therapeutic target for the treatment of osteoclast mediated bone loss [21]. Tacrolimus (FK506) has been identified as an effective treatment of DMARD-resistant or intolerant RA patients [26]. FK506 inhibits the phosphatase activity of calcineurin thus inhibiting translocation and the nuclear localisation of NFATc1 [6]. It has been shown to prevent RANKL-induced osteoclastogenesis *in vitro* [6,27,28] as well as having anabolic effects on the osteoblast *in vivo* and *in vitro* [29].

Side effects associated with treatment with FK506 led to the development of 11R-VIVIT peptide (MAGPHPVIVITGPHEE) (VIVIT) [30]. VIVIT is a more selective and potent inhibitor of the calcineurin-NFAT pathway as VIVIT interferes selectively with alcineurin-NFAT interaction without affecting calcineurin phosphatase activity [31]. We have reported that VIVIT affects osteoclast morphology and suppression of β3 integrin gene expression in murine bone marrow derived osteoclasts [13]. VIVIT has been shown to inhibit titanium particle induced bone resorption and expression of NFATc1 in bone marrow macrophages (BMMs)-derived osteoclasts [28,32].

ITAM-related molecules appear to play a pivotal role in promoting osteoclast differentiation by co-stimulating the calcineurin-NFAT pathway. Therefore, we used FK506 and VIVIT to inhibit calcineurin-NFATc1 signalling assessed their effects on expression of ITAM associated molecules, osteoclastogenic genes and bone resorptive activity during osteoclast differentiation *in vitro*.

2. Materials and methods

2.1. Osteoclast cell culture

Human peripheral blood mononuclear cells (PBMCs) were isolated from whole blood buffy coats obtained from healthy normal donors (Australian Red Cross Blood Service (Adelaide, South Australia)) based on published methods [33]. Monocytes were resuspended in complete medium (α-MEM (Minimum Essential Medium Alpha Medium – Invitrogen, Life Technologies, CA, USA)) with 10% foetal calf serum (FCS) (Invitrogen, CA, USA), 1% 5 μg/mL penicillin–50 U/mL streptomycin (Gibco) and 1% 2 mM L-glutamine (Invitrogen, CA, USA). Cells were seeded at 2×10^6 cells/mL into 6 mm 16-well chamber slides (In Vitro Technologies, Melbourne, Australia) for TRAP staining, or into 12 mm 48-well trays for mRNA expression analysis and onto 8 mm whale tooth dentine in 96-well tray for pit resorption analysis. After incubation for 24 h at 37 °C, 5% CO_2 in a humidified atmosphere, non-adherent cells were removed and replaced with fresh complete medium also containing 25 ng/mL recombinant human M-CSF (Chemicon Australia, Boronia, Australia), medium was replenished 2–3 days.

After 7 days pre-culture, cells were differentiated by the addition of human recombinant RANKL (50 ng/mL) and M-CSF (25 ng/mL) (Chemicon, CA, USA) (denoted as Day 0). Treatments included 0.01 μM, 0.1 μM or 0.5 μM Tacrolimus (FK506) (*sc-24649A*, Santa Cruz Biotechnology, Inc., US) in 0.01% DMSO, or 1.0 μM, 2.0 μM or 5.0 μM 11R-VIVIT peptide (VIVIT) (Calbiochem, Darmstadt, Germany) in 0.1% DMSO. The medium containing factors and treatments was replenished after 3 and 6 days.

Doses were based on previous publications [11,27,28,32] and results from WST-1 proliferation assays (Roche, Mannheim, Germany) for viability of PBMCs from 3 donors after 48 h treatment with inhibitors (data not shown). Control wells were treated with 0.01% DMSO for FK506 and 0.1% DMSO for VIVIT.

2.2. Tartrate resistant acid phosphatase (TRAP) stain

On day 7 pre-osteoclast cells were identified by TRAP staining, according to manufacturer's instructions (Sigma–Aldrich Corporation, St Louis, MO, USA). Slides were viewed under a Nikon FXA Research light Microscope and images at 100× magnification were analysed (Adobe Photoshop Elements 7, 2008). TRAP positive (TRAP^{+ve}) cells with three or more nuclei were counted as pre-osteoclasts.

2.3. Dentine resorption pit formation analysis

On day 10 adherent cells were detached with 0.1% v/v Trypsin in PBS, mounted onto stubs and coated with carbon gold. Three images from three different areas per dentine piece were taken at 200× magnification using the Scanning Electron Microscope (SEM) (Phillips XL20 SEM, Adelaide Microscopy). The images were then traced and areas of resorption filled in Adobe Photoshop Elements 7 (2008). The area of bone resorption was calculated as a percentage of the total area using ImageJ analysis software (Version 1.36b, National Institutes of Health, USA) [19].

2.4. Quantitative real-time polymerase chain reaction (qRT-PCR)

On days 3, 7 and 10, 300 μL Trizol® was added per well and total cellular RNA was isolated from duplicate wells as per manufacturer's instructions (Invitrogen Corporation, Carlsbad, CA, USA) and as previously described [33]. Samples were DNase treated (Turbo DNA-free, Ambion Inc.).

Complementary DNA (cDNA) was prepared from 1 μg of total RNA using random hexamers (Geneworks Pty. Ltd. Adelaide, Australia) and 200 U Superscript™ III (Invitrogen) with Rotor-Gene™ 3000, software version 6.0.38 (Corbett Life Science, Mortlake, NSW, Australia), as per manufacturer's instructions.

The expression of the genes of interest was analysed by qRT-PCR. Amplification was performed using 1 μL of the pre-diluted (1/5) cDNA, 300 nM of each forward and reverse primer, Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen) and DEPC H_2O. Primer pair sequences included; human acidic ribosomal protein (hARP) (reference control gene) [34], CathK, NFATc1, OSCAR, FcRγ,

TREM2 and DAP12 (designed using Primer3Plus) [19], AnnVIII 3′-AAAGGTGCCCCCGAGGTGA-5′ and 3′-GCCGCTGCGTGTTGCTTCT-5′ and β3 integrin 3′-GTGACCTGAAGGAGAATCTGC-5′ and 5′-TTCTTCGAATCATCTGGCC-3′ (Geneworks, Adelaide, SA, Australia). Each Donor sample was prepared in triplicate, including a no RNA RT control. Samples were placed in the Rotor-Gene™ 3000 and analysed using Rotor-Gene™ Series 1.7 software.

2.5. Analysis of mRNA gene expression

Triplicate cycle thresholds (C_t) were averaged for each sample and data obtained from the genes investigated were normalized to the endogenous reference gene hARP [34]. The ΔC_t for each sample was then calculated according to the formula C_t target gene – C_t hARP. The relative quantification was calculated using comparative C_t method, $2^{-\Delta\Delta C_t}$ [35].

2.6. Statistical analysis

TRAP and resorption data were calculated as mean ± standard error (S.E) from 3 donors. For the dose response data analysis one-way ANOVA with Tukey's Post Hoc test was used to compare between two or more groups (GraphPad Prism 5 for Windows, Version 5.03, 2009). Student's *t*-test was used to investigate the difference in mRNA expression between two groups of each individual gene. Statistical significance was accepted when $p < 0.05$.

3. Results

3.1. Effects of FK506 treatment on osteoclast formation and activity

FK506 inhibited the RANKL-induced formation of TRAP expressing multinucleated cells at all concentrations tested (Fig. 1A–D, I). Consistent with the TRAP staining results, FK506 treatment significantly abrogated resorptive activity in a dose-dependent manner (Fig. 1E–H, J). Inhibition of osteoclast activity with 0.5 μM FK506 was significantly greater than 0.1 μM ($p = 0.0026$) and 0.01 μM ($p = 0.0016$) (Fig. 1J), thus effects of 0.5 μM FK506 on gene expression were assessed.

3.2. Effects of VIVIT treatment on osteoclast formation and activity

VIVIT also inhibited formation of TRAP expressing multinucleated cells at 1–5 μM. Resorption was inhibited in a concentration dependent manner (Fig 2). As the effects of VIVIT on resorption were consistently higher at 5.0 μM than 2.0 μM ($p = 0.0002$) and 1.0 μM ($p = 0.0003$) (Fig 2J) thus effects of 5.0 μM VIVIT on gene expression were assessed.

3.3. Effect of FK506 on ITAM gene expression

The effect of 0.5 μM FK506 on osteoclast gene expression over time was assessed. Results are shown for 10 day FK506 treatment, at which time the mRNA expression of each of the ITAM related molecules (OSCAR, FcRγ, TREM2 and DAP12) as well as NFATc1 and CathK (Fig 3) were significantly ($p < 0.05$) inhibited. While expression of integrin β3 and AnnVIII was also reduced, this was not significant statistically. No significant differences in expression of these factors were observed at earlier time points of days 3 and 7 (data not shown).

3.4. Effect of VIVIT on ITAM gene expression

Similar to FK506, the affect of treatment with 5 μM VIVIT for 10 days is presented. VIVIT significantly ($p < 0.05$) reduced the mRNA expression of the ITAM related molecules OSCAR and FcRγ but not TREM2 or DAP12 at day 10. Of note, expression of TREM2 was increased on Day 10 although this was not statistically

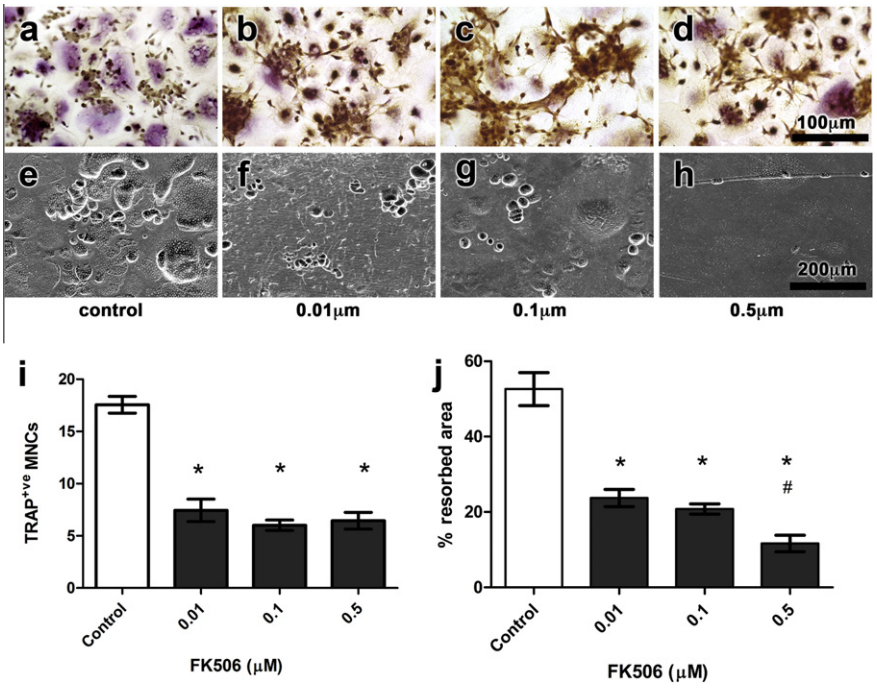

Fig. 1. The effects of Tacrolimus (FK506) on formation and activity of human PBMC-derived osteoclasts. PBMCs were cultured 10 days with RANKL (50 ng/mL) and M-CSF (25 ng/mL) in the absence or presence of various doses FK506. (A–D) Images are representative of $TRAP^{+ve}$ cells (purple) (day 7 with RANKL) at 100× mag, and (E–H) resorption pit formation on dentine (day 10 with RANKL) captured using SEM at 200× mag. (I) $TRAP^{+ve}$ cells with more than three nuclei (in 3 areas of 100× mag per patient) and (J) the percentage pit resorption area are presented as mean ± S.E. Significance was considered as $p > 0.05$ (∗#^); compared to control (∗), 0.5 μM compared to 0.1 μM (#) and 0.5 μM compared to 0.01 μM (^).

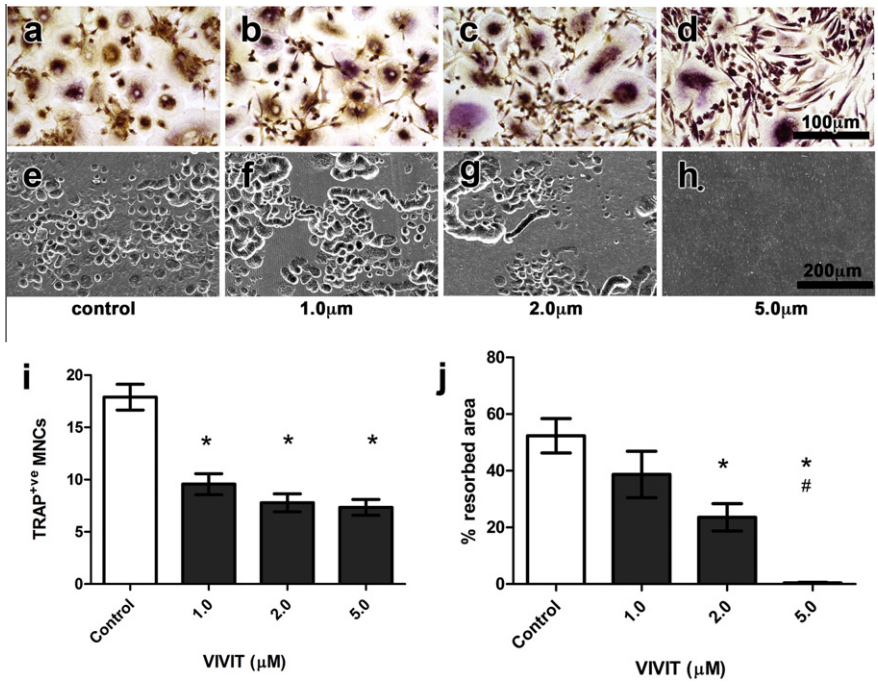

Fig. 2. The effects of 11R-VIVIT peptide (VIVIT) on formation and activity of human PBMC-derived osteoclasts. PBMCs were cultured 10 days with RANKL (50 ng/ml) and M-CSF (25 ng/ml) in the absence or presence of various doses of VIVIT. (A–D) Images are representative of $TRAP^{+ve}$ cells (day 7 with RANKL) at 100× mag, and (E–H) resorption pit formation on dentine (day 10 with RANKL) captured using SEM at 200× mag. (I) $TRAP^{+ve}$ cells with more than three nuclei (in 3 areas of 100× mag per patient) and (J) the percentage pit resorption area with VIVIT treatment are presented as mean ± S.E. Significance was considered as $p > 0.05$ (∗#^); compared to control (∗), 5.0 μM compared to 2.0 μM (#) and 5.0 μM compared to 1.0 μM (^).

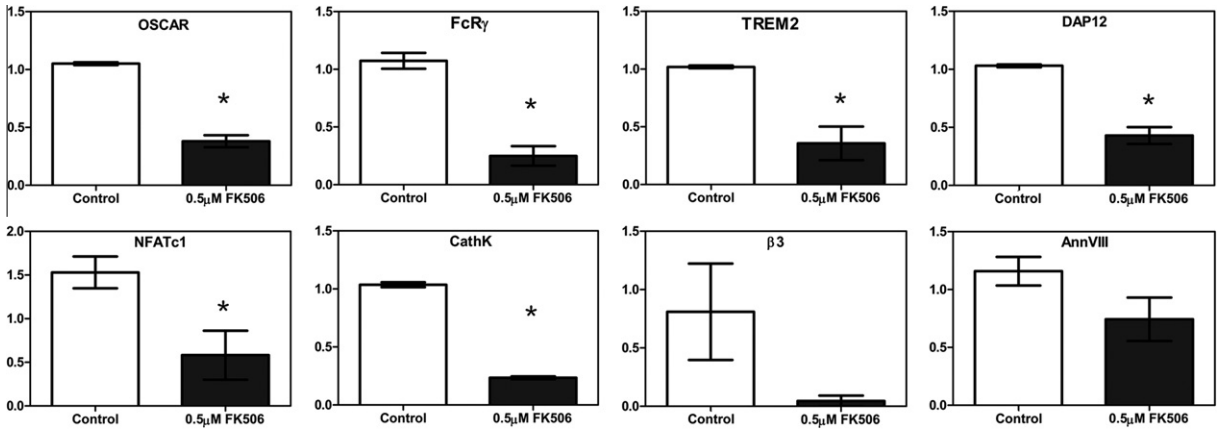

Fig. 3. ITAM molecules and osteoclast gene expression in the presence of 0.5 μM FK506 treatment. Gene expression was assessed by qRT-PCR. The $2^{-\Delta\Delta C_t}$ values (y axis) of the expression of genes of interest relative to hARP as the reference gene are graphed and represented by mean ± S.E obtained form 3 donors. Significance ($^*p < 0.05$) compared to control (0.01% DMSO in media).

significant (Fig 4C). Expression of CathK and AnnVIII was also significantly reduced. Although integrin β3 and NFATc1 were reduced, this was not statistically significant. No significant differences in expression of these factors were seen at earlier time points of days 3 and 7 (data not shown).

4. Discussion

Inhibitors of calcineurin-NFAT signalling have been identified as potentially beneficial in the treatment of pathological bone loss. For example, FK506 not only inhibits T-cell proliferation [36] but also inhibits osteoclastogenesis [6,10]. VIVIT, a more selective inhibitor of calcineurin-NFAT interaction [31] has similar effects. In the present study, we have confirmed that FK506 and VIVIT inhibit RANKL-induced osteoclast differentiation and activity [27]. Consistent with previous publications, FK506 treatment reduced the number of multinucleated cells expressing TRAP as well as resorption at concentrations ranging from 0.01–0.5 μM. VIVIT similarly inhibited the formation of multinucleated TRAP expressing cells and resorption but at higher concentrations (1.0–5.0 μM) consistent with previous findings [32].

We found ITAM related molecules are strongly expressed during the later stages of osteoclastogenesis [10,27]. In a study using murine BMMs derived osteoclasts OSCAR but not TREM2 expression was inhibited by FK506 [10]. Importantly, our study demonstrates using human cells showed that both TREM and OSCAR

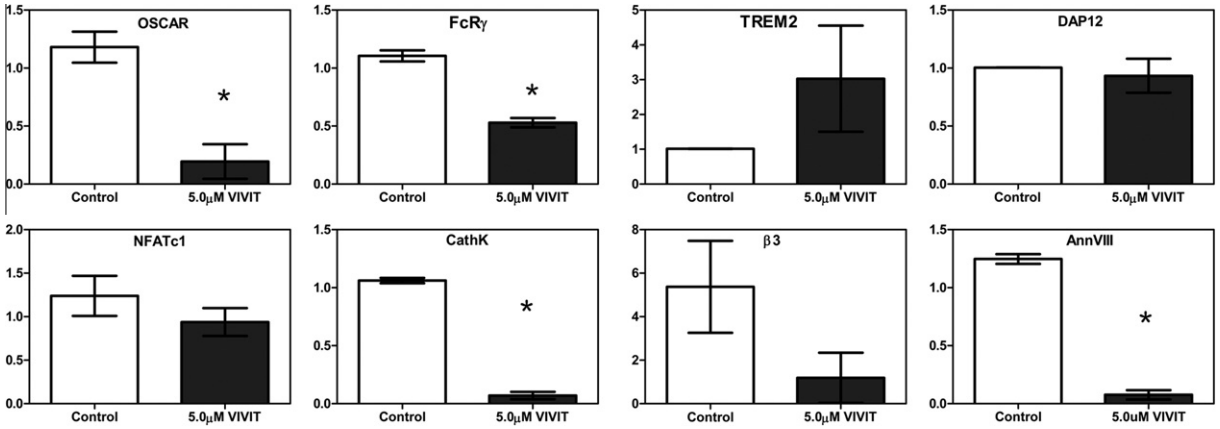

Fig. 4. ITAM molecules and osteoclast gene expression in the presence of 5.0 μM VIVIT. Gene expression was assessed by qRT-PCR. The $2^{-\Delta\Delta C_t}$ values of the expression of genes of interest relative to hARP as the reference gene are graphed and represented by mean ± S.E obtained from 3 donors. Significance ($^*p < 0.05$) compared to control (0.1% DMSO in media).

were inhibited by FK506 in the late stage of osteoclast formation. Interestingly we noted that VIVIT inhibited OSCAR but not TREM2 expression. This may be because VIVIT peptide inhibits calcineurin-mediated dephosphorylation of NFATs without affecting calcineurin phosphatase signalling and non-NFAT mediated signalling. In contrast, FK506 acts further upstream, more broadly affecting both calcineurin phosphatase signalling and non-NFAT mediated signalling [30,31]. This independent regulation of OSCAR and TREM2 is consistent with promoter studies demonstrating direct induction of OSCAR, but not TREM2, by NFATc1 [10].

The ITAM adaptor molecules, FcRγ and DAP12 may also be regulated by different mechanisms. The expression of both FcRγ and DAP12 was significantly reduced by FK506 treatment whereas VIVIT significantly reduced FcRγ but not DAP12. To our knowledge, NFATc1 has not been shown to directly induce DAP12 and this is consistent with the lack of inhibition with the more specifically acting inhibitor VIVIT.

There are few reports demonstrating the effect of FK506 or VIVIT on genes associated with late stages of osteoclast formation [10,27]. However, it has been reported that CTR, CathK and NFATc1 expression is inhibited by VIVIT in a murine model of prosthetic particle-induced osteoclast formation [32]. We found 5.0 μM VIVIT treatment significantly reduced CathK but not NFATc1 expression in late stages of human osteoclast development. In contrast to VIVIT, FK506 significantly reduced both CathK and NFATc1 expression. This is consistent with the work of Miyazaki et al. that demonstrated a reduction in NFATc1 when FK506 treatment was added from seven days when compared to treatment only in the first 3 or 7 days in RANKL-differentiated PBMCs derived from RA patients [27].

β3 integrin is a late stage osteoclast gene essential for osteoclast motility and function that signals through Syk tyrosine kinase, which is regulated by Dap12 and FcRγ [37]. The pairings of ITAM specific receptors and their adaptor molecules phosphorylate tyrosine and activate the Syk-PLCγ pathway [21,22,37,38]. This activation increases the intracellular calcium concentration and activates the calcineurin to dephosphorylate NFATc1 [18,21] which then translocates to the nucleus resulting in further stimulation of osteoclast genes [8,10–15] and NFATc1 expression [9]. We have previously reported that VIVIT affects murine derived osteoclast morphology and the expression of the integrin β3 [13]. In the present study, a reduction in β3 was also noted with VIVIT, but this was not statistically significant. This may be due to low patient numbers or the use of human cells in the present study.

AnnVIII is a calcium regulated gene recently identified by us as upregulated during late stage osteoclast formation on interaction with mineralised substrate [16]. Our *in silico* analysis and co-transfection studies of AnnVIII promoter constructs with NFATc1 expression vectors indicate it is also specifically induced by NFATc1 (unpublished data). VIVIT treatment but not FK506 markedly inhibited AnnVIII expression. Further studies are needed to determine the mechanism by which AnnVIII is affected by calcineurin-NFAT signalling.

The effects of disrupting calcineurin-NFATc1 signalling and the role of OSCAR-FcRγ and DAP12-TREM2 in this process are only just becoming understood. Our findings demonstrate a complex but important interaction of ITAM related molecules, NFATc1 and osteoclastogenesis. Furthermore, modulating ITAM related molecules directly or through calcineurin-NFAT signalling may offer novel ways in inhibiting elevated osteoclast activity seen in many bone loss pathologies.

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