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Suppression of T cell-induced osteoclast formation

Sahar Karieb, Simon W. Fox *

School of Biomedical and Biological Sciences, Plymouth University, Drake Circus, Plymouth, Devon PL4 8AA, UK

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ABSTRACT

Inhibition of T cell derived cytokine production could help suppress osteoclast differentiation in inflammatory skeletal disorders. Bisphosphonates are typically prescribed to prevent inflammatory bone loss but are not tolerated by all patients and are associated with an increased risk of osteonecrosis of the jaw. In light of this other anti-resorptives such as phytoestrogens are being considered. However the effect of phytoestrogens on T cell-induced osteoclast formation is unclear. The effect of genistein and coumestrol on activated T cell-induced osteoclastogenesis and cytokine production was therefore examined. Concentrations of genistein and coumestrol (10^{-7} M) previously shown to directly inhibit osteoclast formation also suppressed the formation of TRAP positive osteoclast induced by con A activated T cells, which was dependent on inhibition of T cell derived TNF-α. While both reduced osteoclast formation their mechanism of action differed. The anti-osteoclastic effect of coumestrol was associated with a dual effect on con A induced T cell proliferation and activation; 10^{-7} M coumestrol significantly reducing T cell number (0.36) and TNF-α (0.47), IL-1β (0.23) and IL-6 (0.35) expression, whereas genistein (10^{-7} M) had no effect on T cell number but a more pronounced effect on T cell differentiation reducing expression of TNF-α (0.49), IL-1β (0.52), IL-6 (0.71) and RANKL (0.71). Phytoestrogens therefore prevent the pro-osteoclastic action of T cells suggesting they may have a role in the control of inflammatory bone loss.

1. Introduction

Excessive osteoclast resorption is a defining characteristic of many inflammatory disorders including rheumatoid arthritis and osteomyelitis [1]. The immune system also has a central role in post-menopausal osteoporosis [2]. Falls in circulating oestrogen are associated with a rapid expansion of the T cell population and T cell deficient mice are resistant to ovariectomy-induced bone loss [3–6]. Oestrogen deficiency enlarges the T cell population by modifying IL-7, TGF-β and IFN-γ production, which promote MHCII expression and antigen presentation to naïve T cell leading to clonal expansion [3,7,8]. T cell derived cytokines in particular TNFα are central to the subsequent osteolytic response. TNF receptor expression positively correlates with hip fracture in older women [9]and TNFα expression is increased in peripheral blood cells from post-menopausal women with oestrogen replacement reducing this [10–12]. TNF-α promotes osteoclast differentiation through several actions it augments receptor activator of NFκB ligand (RANKL) induced osteoclastogenesis, directly stimulates osteoclast formation from human or mouse monocytes [13,14] and has been suggested to activate osteoclast formation independent of RANKL signalling [15].

Bisphosphonates are typically prescribed for post-menopausal bone loss; however they are associated with complications such as induction of the acute phase response and osteonecrosis of the jaw [16]. As a consequence phytoestrogens, a diverse group of plant derived compounds with a structure and function similar to oestradiol, have been examined as alternative therapeutic agents. Diets with high phytoestrogen content are associated with a more robust skeleton [17–19] and women with the highest dietary soy levels have the lowest resorption rates [19,20]. Phytoestrogen supplements also have beneficial effects on spinal [21,22], trochanter [23] and Ward's triangle bone mineral density [24].

The anti-resorptive effect of phytoestrogens is mediated through an action on multiple cell types. However the effect of phytoestrogens on T cell derived cytokines is unknown. An additional effect on T cell could further protect against bone loss, or alternatively phytoestrogens could augment inflammatory cytokine expression thereby antagonizing their direct inhibitory action on osteoclast formation. We therefore investigated the effect of phytoestrogens on T cell-induced bone resorption and pro-inflammatory cytokine and RANKL expression.

2. Materials and methods

2.1. Media and reagents

Jurkat E6.1 T cells a human leukemic T cell line were obtained from ECACC, Porton Down, UK (ECACC cat. num. 88042803) and

* Corresponding author. Address: Room 404 Davy Building, Plymouth University, Plymouth, Devon PL4 8AA, UK.
E-mail address: Simon.fox@plymouth.ac.uk (S.W. Fox).

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cultured in phenol red free RPMI medium supplemented with 10% charcoal stripped foetal calf serum (Autogen Bioclear, UK) 2 mmol/l glutamine, 100 IU/ml benzylpenicillin and 100 mg/ml streptomycin. RAW264.7 monocytes (ATCC, UK, cat. num. TIB-71) were incubated in phenol red free Dulbecco's minimum essential medium supplemented with 10% charcoal stripped foetal calf serum, 2 mmol/l glutamine, 100 IU/ml benzylpenicillin and 100 mg/ml streptomycin. All incubations were performed at 37 °C in 5% CO_2 in a humidified incubator. Cultures were fed every 2–3 days by replacing half of the culture volume with fresh medium. Anti-human TNFα antibodies were purchased from Insight Biotechnology, Wembley, UK. Concanavalin A (con A) and all other reagents were obtained from Sigma (Poole, Dorset, UK) unless stated.

2.2. Real time quantitative PCR analysis of inflammatory cytokine expression

T cells were cultured for four days with genistein (10^{-6}–10^{-9} M) or coumestrol (10^{-6}–10^{-9} M) in the presence of con A (10 μg/ml). Total RNA was extracted and reversed transcribed with M-MLV reverse transcriptase, and real time-PCR performed on a Step One PCR system (Applied Biosystems, UK) using the DNA-binding dye SYBR green as a fluorophore. A total of 2 μl of external plasmid standard or cDNA was added to a final reaction volume of 25 μl containing 0.05 U/μlTaq, SYBR green and specific primers (0.2 μM). Primers used were as follows human β-Actin forward GCG CGG CTA CAG CTT CAC CA and reverse TGG CCG TCA GGC AGCTCG TA; human TNF-α GCT CCA GTG GCT GAA CCG CCand reverse AGC ACA TGG GTG GAG GGG CA; human IL-6 forward TCA ATG AGG AGA CTT GCC TGG TGA and reverse TCT GCA GGA ACT GGA TCA GGA CTT; human IL-1β forward ACG CTC CGG GAC TCA CAG CA and reverse TGA GGC CCA AGG CCA CAG GT; human RANKL ACA GGC CTT TCA AGG AGC TGT GC and reverse ACC AGA TGG GAT GTCGGT GGC. For generation of standard curves, the corresponding cDNA was cloned into pGEM-T Easy (Promega). The concentration of DNA plasmid stock was determined by OD at 260 nm. Copy number for each plasmid was calculated from these measurements. The linear range of the assay was determined by the amplification of log serial dilutions of external plasmid standard from 500 to 5×10^6 copies. The progress of the PCR amplification was monitored by real-time fluorescence emitted from SYBR green during the extension time. Reaction conditions were 94 °C for two minutes, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. At the end of each PCR run a melt curve analysis was performed to show the absence of non-specific bands. For each sample mRNA level was expressed as an absolute copy number normalized against β-actin. The mRNA copy number was calculated for each sample from the standard curves by the instrument's software. Samples were analysed in triplicate.

2.3. Cell proliferation assay

Jurkat E6.1 T cells were cultured in 96 well plates at a density of 1×10^4 cells per well and treated with con A (10 μg/ml) in the presence of coumestrol (10^{-6}–10^{-9} M) or genistein (10^{-6}–10^{-9} M) for four days. Proliferation was then assessed using a commercial AQueous one solution cell proliferation assay (Promega, UK) according to manufacturer's instructions.

2.4. The effect of phytoestrogens on the pro-osteoclastic action of T cells

T cells (1×10^5 cells) were cultured in RPMI media and treated with genistein (10^{-7} M) or coumestrol (10^{-7} M) in the presence of con A (10 μg/ml) for four days. Cells were then centrifuged, washed in medium three times and resuspended in RPMI to remove con A and PEs. T cells were then added to 96 well plates containing RAW264.7 cells (2×10^4 cells/well) and cultured for a further five days. To determine if the pro-osteoclastic effect of T cells was mediated through TNF-α production neutralising mouse anti-human TNF-α antibody (5 μg/ml) was added to replicate wells. Osteoclast formation was evaluated by staining for the specific osteoclastic marker tartrate resistant acid phosphatase (TRAP) using a modification of the method of Burstone [25] using naphthol AS-BI phosphate as a substrate. The number of TRAP-positive cells was counted using an eyepiece graticule at a magnification of ×100 and the results expressed as the number of cells per cm^2. All experiments were performed in triplicate.

2.5. Statistical analysis

Differences between groups were assessed using Fisher's analysis of variance (Statview; Abacus concepts, USA). A difference of $P < 0.05$ was considered significant.

3. Results

3.1. Phytoestrogens blunt the augmentative effect of activated T-cells on osteoclast formation

To examine the effect of T cells on osteoclast differentiation RAW264.7 cells were co-cultured with Jurkat E6.1 cells, which had or had not been treated with concentrations of genistein or coumestrol previously shown to directly inhibit osteoclast formation. No TRAP + osteoclasts were seen in cultures of T cells alone whether they had been activated with con A or not (Fig. 1). Similarly TRAP positive osteoclasts were rarely seen in RAW264.7 cultures in the absence of T cells. Osteoclast number significantly increased following addition of non-activated T cells and was further augmented in co-cultures of con A activated T cells. The stimulatory effect of T cell activation on osteoclast formation was reversed by pre-incubating T cells with genistein (10^{-7} M) or coumestrol (10^{-7} M) (Fig. 1). Osteoclast number in these cultures was reduced to that seen in cultures containing un-stimulated T cells.

To examine the role of TNF-α in T cell-induced osteoclast formation co-cultures were incubated in the presence of anti-human TNF-α antibody. Neutralization of TNF-α had no effect on osteoclast formation induced by un-stimulated T cells. However there was a significant reduction in TRAP + cell number with anti TNF treatment in cultures containing con A activated T cells. Osteoclast formation in these cultures was reduced to that induced by unstimulated T cells. Addition of TNF-α antibodies to genistein or coumestrol treated cultures did not further suppress osteoclast formation (Fig. 1).

3.2. Effect of phytoestrogens on T cell number and pro-osteoclastic cytokine expression

An MTS assay was used to examine the effect of PEs on con A-induced T cell proliferation. Coumestrol at all concentrations significantly decreased T cell number while genistein had no significant effect on cell number at any concentration (Fig. 2).

T cells express a range of inflammatory cytokines that induce bone loss in post-menopausal women. In keeping with this con A stimulated T-cells exhibited a 2.63-fold increase in TNF-α mRNA expression and this was prevented in the presence of PEs. Genistein (10^{-6}–10^{-9} M) and coumestrol (10^{-6}–10^{-9} M) all significantly suppressed con A-induced TNF-α expression (Figs. 3 and 4). Similar effects were seen on IL-1β expression. T cell activation induced a significant2.12-fold increase in IL-1β mRNA which was

suppressed in the presence of genistein (10^{-6}–10^{-9} M) or coumestrol (10^{-6}–10^{-9} M) (Figs. 3 and 4). IL-6 expression was enhanced in activated T cells and this expression was significantly blunted by PE treatment in a dose-dependent fashion. Genistein (10^{-6}, 10^{-8} and 10^{-9} M) and coumestrol (10^{-7} M) decreased IL-6 expression. Activated T cells also express RANKL the key physiological regulator of osteoclast formation. Interestingly RANKL expression was significantly reduced in the presence of genistein (10^{-6}–10^{-9} M) and coumestrol (10^{-8} M).

4. Discussion

The immune system has a major impact on bone cell activity and many common skeletal disorders arise as a consequence of immune dysfunction [2]. For instance increased T cell number and TNFα are thought to at least in part mediate the excessive rate of bone resorption associated with post-menopausal osteoporosis [5,26]. We have previously shown that genistein and coumestrol directly inhibit TNF-induced osteoclast formation [27]. However this direct suppressive effect could be overridden if phytoestrogens promote T cell proliferation or augment pro-osteoclastic cytokine production. Therefore we examined the effect of genistein and coumestrol on T cell-induced osteoclast formation. Co-culture of T lymphocytes with osteoclast precursors enhanced osteoclast formation and con A stimulated T cells further augmented this effect similar to previous reports [28,29]. This augmentative action was significantly blunted by pre-incubation of T cells with genistein (10^{-7} M) or coumestrol (10^{-7} M). This suggests that phytoestrogens possess both direct and indirect actions that reduce osteoclast formation and this dual action may be more beneficial than other anti-resorptives.

To examine the role of TNF-α in T cell-induced osteoclast formation co-cultures were incubated in the presence of anti-human TNF-α antibody. Neutralization of TNF-α had no effect on osteoclast formation induced by un-stimulated T cells. This basal level of osteoclastogenesis may result from IL-6 and RANKL expression,

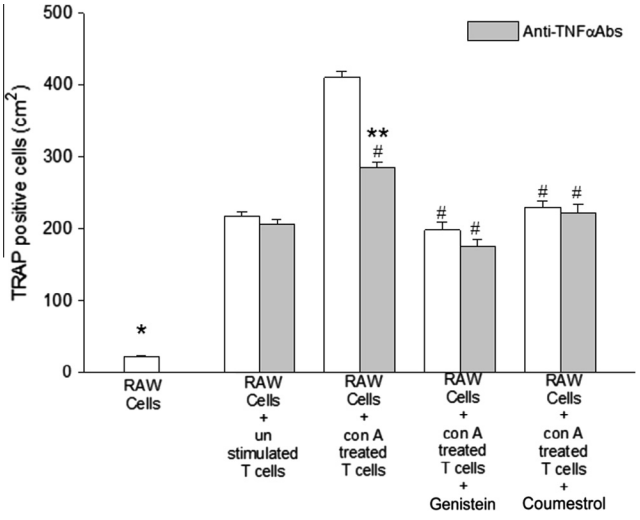

Fig. 1. Genistein and coumestrol blunt activated T cell-induced osteoclast formation. Jurkat E6.1 T cells were cultured in the presence or absence of con A, genistein (10^{-7} M) and coumestrol (10^{-7} M) for 4 days. Jurkat cells (1×10^5 cells) were then collected, repeatedly washed and co-cultured with RAW264.7 cells (2×10^4 cells per well) for 5 days. Some cultures were treated with anti-human TNF alpha antibodies (5 μg/ml) to determine the role of TNF in T cell induced osteoclast formation. Values are expressed as mean ± SEM of three separate experiments. *Significantly different versus all other groups $P < 0.05$. $^{\#}P < 0.05$ versus RAW264.7 + con A treated T cell group without anti TNF alpha antibodies $^{**}P < 0.05$ versus corresponding group without anti-TNF antibody.

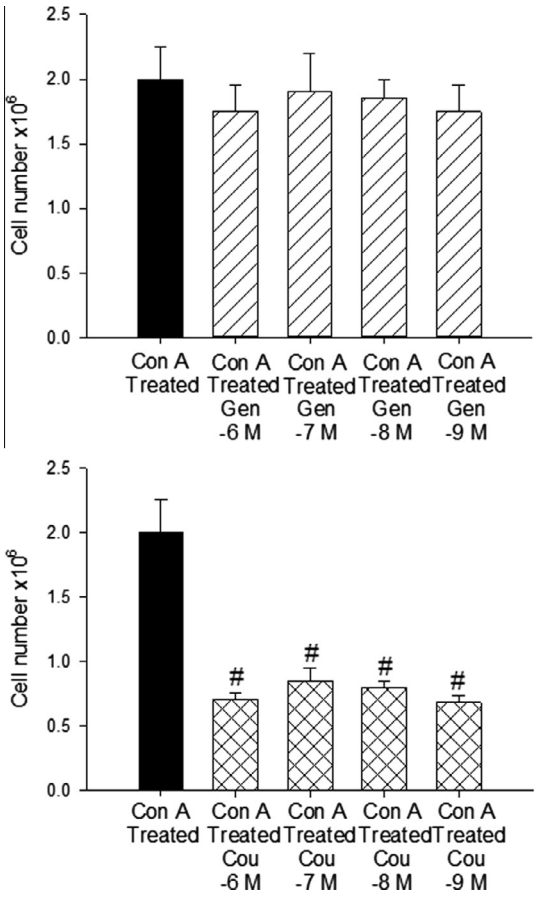

Fig. 2. Coumestrol decreased T cell number. Jurkat E6.1 T cells were incubated in combination of con A (10 μg/ml), genistein (10^{-5}–10^{-9} M) or coumestrol (10^{-5}–10^{-9} M) for 4 days. Cell number was then assessed using an MTS assay. Values are expressed as the mean ± SEM from three separate experiments. $^{\#}P < 0.05$ versus con A treated group.

which are known to augment osteoclast formation. In addition T cells may induce pro-osteoclastic cytokine expression by the osteoclast precursors themselves as monocytic IL-6 and IL-1β secretion is elevated by T cells in rheumatoid arthritis [30]. This is dependent on a reciprocal interaction in which monocytes induce T cell CD40L expression which then turn promotes monocytic IL-6 and TNF [31]. This may be the case but is not possible to assess in the current co-culture experiments. Alternatively T cell derived RANKL may augment its effect by enhancing osteoclast precursor RANK expression as low levels of RANKL have previously been shown to stimulate RAW cell M-CSF production leading to elevated RANK expression and augmentation of osteoclast formation [32].

In contrast TNF-α appears to mediate the augmentative effect of con A as neutralizing antibodies significantly reduced the stimulus provided by activated T cells. Con A also enhanced the expression of a range of other pro-osteoclastic inflammatory cytokines although their importance in the pro-osteoclastic action is secondary to that of TNF, as neutralizing antibodies restored osteoclast number to near un-stimulated levels. This change in cytokine profile is consistent with the results of Kawai et al. [29], which showed that activated T lymphocytes expressed significantly greater levels of TNF-α, IL-1β and IL-6 followed by increases in the ability of T cells to induce osteoclast formation. Surprisingly Con A did not increase RANKL expression as T cell RANKL expression has been suggested to be one mechanism promoting osteoclastogenesis in inflammatory disorders [33]. The reason for this is unclear but may represent a limitation of con A activation in contrast to other

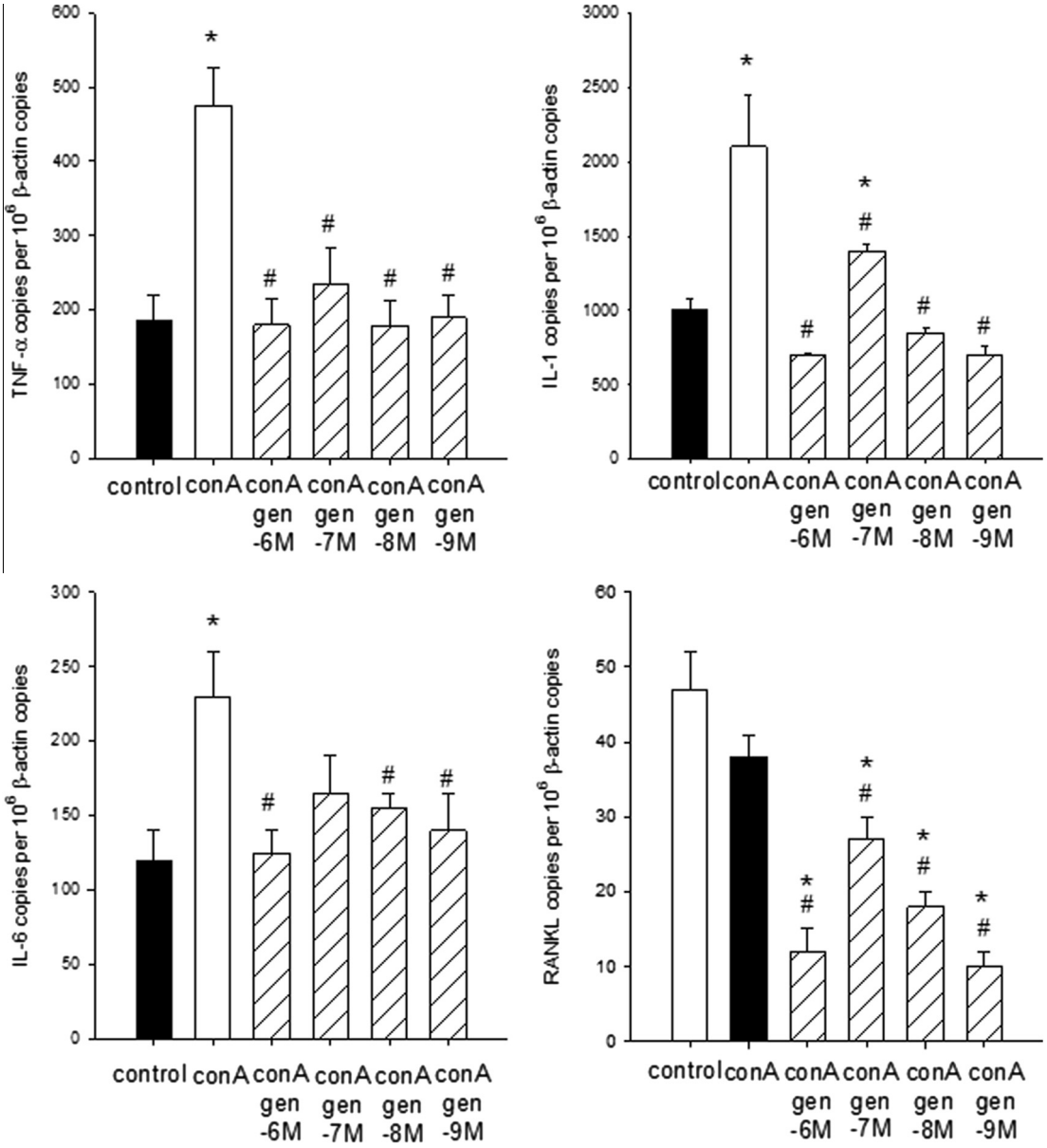

Fig. 3. Genistein suppresses inflammatory cytokine and RANKL expression in activated T cells. Jurkat E6.1 T cells were incubated in combinations of con A (10 μg/ml) and genistein (10^{-5}–10^{-9} M) for 4 days. TNF-α, IL-1β, Il-6 and RANKL mRNA expression was then assessed by quantitative real time PCR and data normalized to 10^6 copies of β-actin. Data is expressed as the mean ± SEM for three separate experiments. $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus con A treated group.

stimuli such as bacterial exotoxins that have been shown to increase Jurkat RANKL expression [34].

The suppressive effect of genistein and coumestrol on T cell-induced osteoclast formation is mediated through different cellular actions. Genistein had no effect on T cell viability whereas it reduced con A stimulated TNF-α, IL-1 and RANKL expression. Thus, its anti-osteoclastic action was mediated through suppression of differentiation rather than clonal expansion. While a reduction in T cell number would further enhance the anti-osteoclastic effect the concentration required (10^{-5} M) to cause mitochondrial dysfunction and reduce T cell viability [35,36] is in the range of those reported to directly stimulate TNF-α-induced osteoclast formation [27]. These concentrations would therefore be unlikely to have a greater effect on osteoclast formation and in addition would be difficult to generate from dietary sources or supplements. In light of the significant effect of anti-TNF-α antibodies on osteoclastogenesis it is likely that the ability of genistein to reduce TNF-α expression represents the most important aspect of its action, although this does not preclude a further contribution from decreased IL-6 or RANKL expression, as genistein treated cultures had fewer osteoclast than co-cultures of activated T cells administered TNF-α antibodies. On the other hand, the anti-osteoclastic action of coumestrol arises from a combination of a smaller pool of viable T cells and reduced TNF-α, IL-6 and IL-1 expression although no greater effect on osteoclast formation was noted in comparison to genistein. The mechanism thorough which genistein and coumestrol reduce cytokine expression is not known and could potentially be meditated through several effects. It may be oestrogen receptor dependent as seen for the direct effect on osteoclast formation [27] or alternatively it could be caused by the antioxidant action of phytoestrogens which could modify the redox status and inhibit TNF-α secretion. The effect of genistein could also be mediated through its ability to suppress tyrosine kinase signaling which plays an important role in inflammation [37].

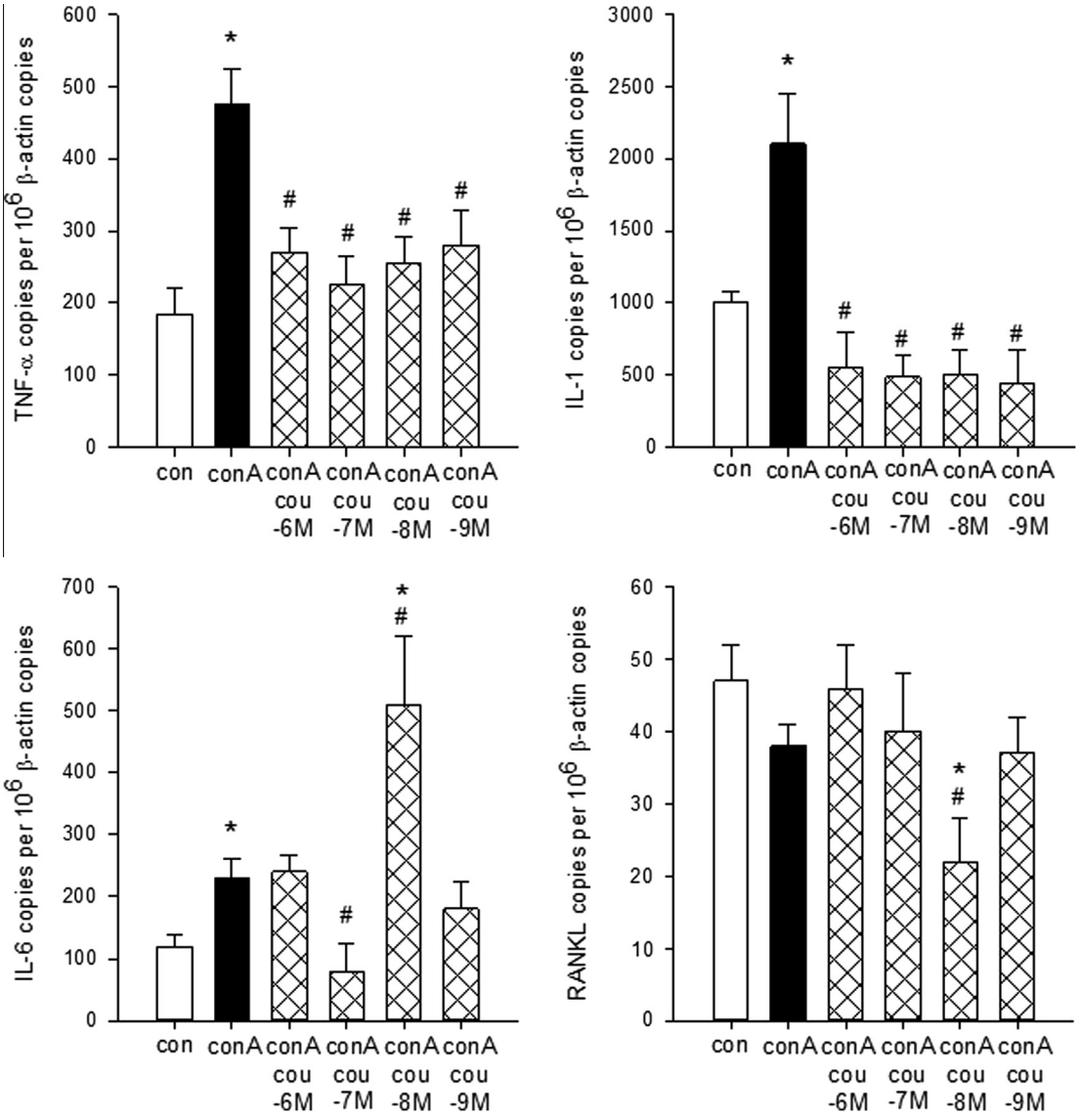

Fig. 4. Coumestrol suppresses inflammatory cytokine expression in activated T cells. Jurkat E6.1 T cells were incubated in combinations of con A (10 μg/ml) and coumestrol (10^{-5}–10^{-9} M) for 4 days. TNF-α, IL-1β, Il-6 and RANKL mRNA expression was then assessed by quantitative real time PCR and data normalized to 10^6 copies of β-actin. Data is expressed as the mean ± SEM for three separate experiments. $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus con A treated group.

In conclusion the data shows an inhibitory effect of genistein and coumestrol on activated T cell-induced osteoclast formation. This effect is observed at concentrations previously shown to directly inhibit osteoclast formation and promote bone matrix formation. This provides evidence that a further mechanism through which PEs may inhibit bone loss in vivo is via a reduction in T lymphocyte driven remodelling. PEs could therefore prove useful in the attenuation of bone destruction in not only post-menopausal osteoporosis but also in other inflammatory disorders such as rheumatoid arthritis.

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