ICAM-1-mediated osteoblast-T lymphocyte direct interaction increases mineralization through TGF-β1 suppression

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Running title: Osteoblast-T cell contact and mineralization

Abstract

Modulation of osteoblast functions by T lymphocytes is important in inflammation-associated mineralized tissue diseases. The study aimed to determine whether direct interaction between these two cell types affects osteoblast functions and mineralization. The results showed that direct contact between the two cell types was evident by SEM and TEM. Under osteogenic induction, higher hydroxyapatite precipitation was observed in co-cultures with direct contact with T lymphocytes compared with that by osteoblasts cultured alone. Co-cultures without direct cell contact caused a decrease in mineralization. Direct cell contact also up-regulated intercellular adhesion molecule (ICAM)-1 and simultaneously down-regulated transforming growth factor (TGF)-β1 in osteoblasts. However, down-regulation of TGF-β1 was reversed by ICAM-1 blocking. Exogenously added TGF-β1 in co-cultures with direct cell contact suppressed mineralization. In conclusion, studies are consistent with ICAM-1-mediated direct contact between osteoblasts and T lymphocytes increasing mineralization via down-regulation of TGF-β1 in osteoblasts *in vitro*. This suggests a possible unexpected, but crucial, role of T lymphocytes in enhancing matrix mineralization during the repair process *in vivo*. The study identifies ICAM-1/TGF-β1 as possible novel therapeutic targets for the treatment and prevention of inflammation-associated mineralized tissue diseases.

Keywords: ICAM-1; Direct contact; Osteoblasts; T lymphocytes; Mineralization; TGF-β1

Introduction

T lymphocytes play an important part in the regulation of bone cell function and chronic inflammatory conditions of bone such as osteoarthritis, loss of alveolar bone in periodontal diseases and osteomyelitis of jaw bones [1-7]. These pathologies generally involve the secretion of soluble inflammatory cytokines by activated T lymphocytes, acting mainly as negative regulators of osteoblast differentiation and eliciting bone breakdown [2, 5-12]. However, T lymphocyte accumulation has also been reported in the area of ectopic bone formation in many diseases such as cardiac valve calcification [13], fibrodysplasia ossificans progressiva (FOP) [14], intraabdominal myositis ossificans [15] and myositis ossificans traumatica [16]. Bone morphogenetic proteins, potent osteogenic morphogens, were also expressed by myofibroblasts and preosteoblasts in areas adjacent to T lymphocyte infiltration in cardiac valves where heterotopic ossification was evident [13]. Moreover, the very early stage of FOP involving extensive perivascular lymphocytic accumulation in apparently normal skeletal muscle suggests the possibility that lymphocytes may play a part in the pathogenesis of heterotopic ossification in this disease [14]. Thus, apart from mediating tissue breakdown via soluble mediators, T lymphocytes might also have an important role in mineralization during the repair process, possibly through cell adhesion-mediated signaling initiated via the direct contact between local mesenchymal cells and T lymphocytes in the area of lymphocytic infiltration. Such cell-cell interactions have previously been shown to play a fundamental part in regulating the functional activity of many types of cells, including osteoblasts [17-21]. Moreover, a direct link between T cells and bone formation during the healing after bone fracture has previously been reported *in vivo* [22].

It has been suggested that direct cell-to-cell contact between osteoblasts and other cell types controls their functions *in vitro* and *in vivo* [23]. Osteoblasts express particular adhesion molecules, such as ICAM-1 and VCAM-1, that participate in the adhesion (to cells expressing the receptors to these adhesion molecules) and activity of osteoblasts [18]. Cellular adhesion of osteoblasts through ICAM-1 as well as VCAM-1 possessed a capability to transduce intracellular activation signals. For example, cross-linking of these two adhesion molecules on osteoblasts regulated the production of multiple soluble factors by osteoblasts, such as IL-1β, TNF-α and IL-6, which have an important role in bone repair during inflammation [18, 24, 25]. It is thus possible that these adhesion molecules may regulate the production of soluble factors by osteoblasts, in particular osteogenic growth factors TGF-β1, BMP-2 and IGF-1 [26]. However, little is known about the direct interaction between osteoblasts and T lymphocytes and whether this process of cell-cell contact can also modulate osteogenic differentiation and bone formation. The present study was therefore carried out to examine the direct interaction between osteoblasts and T lymphocytes and determine the functional consequences, particularly the formation of mineralized nodules by primary human osteoblasts *in vitro*.

Materials and Methods

Isolation and culture of primary human osteoblasts

Primary human osteoblasts were grown from the cortico-lamellar bone of the maxilla, as previously reported [27, 28], after obtaining informed consent according to the protocol approved by the Joint Research and Ethics Committee of the Eastman Dental Institute and Hospital and the Ethics Review Sub-Committee for Research Involving Human Research Subjects of Thammasat University No. 3 (COA No. 068/2564). Cells were cultured at 37°C in a

humidified atmosphere of 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco Life Technologies Ltd, Paisley, UK) containing 10% heat-inactivated fetal calf serum (FCS) (PAA Laboratories, Yeovil, UK) (10% FCS-DMEM) supplemented with 100 U/ml of penicillin (Gibco), 100 μg/ml of streptomycin (Gibco) and 2.5 μg/ml of amphotericin B (Gibco). Cells were used between passages 3 and 5.

Isolation and culture of primary human T lymphocytes

Human T lymphocytes were isolated and cultured as previously reported, and more than 85% of these cells were T lymphocytes, as assessed by flow cytometry (FCM) analysis of the expression of the T cell marker CD3 [29]. Buffy coat fractions were obtained from the National Blood Service, Colindale, London, UK and the National Blood Center, Thai Red Cross Society, Thailand. The buffy coat was diluted 1:1 with phosphate-buffered saline (PBS), layered on Ficoll-Paque PLUS (GE Healthcare Life Sciences, Little Chalfont, UK) and centrifuged at 800 g for 40 min at 18˚C. The lymphocyte/monocyte interface layer was collected, and after 2 h at 37˚C to allow the monocytes to adhere, the non-adherent T lymphocytes were collected and maintained in 10% FCS-DMEM supplemented with 100 U/ml of penicillin and 100 μg/ml of streptomycin at $1x10^6$ cells/ml.

Osteoblast-T lymphocyte co-cultures

T lymphocytes were resuspended in standard culture media at a concentration of $1x10^6$ cells/ml and added to culture dishes containing osteoblast monolayers that had reached confluence with a ratio of 20 T lymphocytes per one osteoblast. In some experiments, the two cell types were separated by a semi-permeable porous membrane (0.4 μm) using a cell culture

insert (Nunc, VWR Ltd, Lutterworth, UK). A schematic diagram of the co-culture is shown in Fig. S1.

Treatments of cells

To block the activity of ICAM-1, an ICAM-1 neutralizing antibody (5-20 μg/ml, mAb IgG1 Clone # BBIG-I1 (11C81), R&D systems) was used to pretreat cell cultures for 30 min before further treatments/assays. A corresponding isotype control was also used (R&D Systems). In some experiments, osteoblasts were pretreated with SB203580 (10 μM), SP600125 (10 μM) and UO126 (1 μM) (all from Sigma), which are potent inhibitors specific to p38 mitogen-activated protein kinases (p38 MAPK), Jun N-terminal kinases (JNK) 1/2/3 and extracellular regulated kinases (ERK) 1/2, respectively, for 30 min before being co-cultured with T lymphocytes to explore the involvement of these signaling pathway(s) in T lymphocyte-mediated suppression of TGF-β1 in osteoblasts. Moreover, a range of TGF-β1 (0.1-10 ng/ml; R&D Systems) was used to confirm the involvement of TGF-β1 reduction by osteoblast-T lymphocyte direct contact in biomineralization.

Scanning electron microscopy (SEM) and SEM energy dispersive X-ray microanalysis (SEM-EDX)

The samples were fixed in 3% glutaraldehyde in 0.14 M sodium cacodylate buffer (pH 7.3) at 4˚C overnight, then dehydrated in a graded series of alcohols (50%, 70%, 90% and two changes of 100%), washed with hexamethyldisilazane (TAAB Laboratories; Berkshire, UK) for 5 min, air-dried, and then sputter-coated with gold/palladium using a Polaron E5100 coating device. Cell morphology and direct cell contact were observed using a JEOL JSM 5410LV SEM

(JEOL UK, Welwyn Garden City, UK). In some experiments, elemental analysis was also carried out to examine the calcium phosphate content of the apparent mineralized deposits in the samples using SEM combined with INCA 300 energy dispersive X-ray microanalysis system (Oxford Instruments, High Wycombe, UK).

Transmission electron microscopy (TEM)

Co-cultures were prepared on sterile plastic coverslips (Thermanox®), as described above. The samples were then fixed with 0.5% glutaraldehyde at 4°C for 30 min, dehydrated in cold ethanol and embedded at -20°C in LR White resin (Agar Scientific, Stansted, UK). Ultra-thin sections (approximately 0.1 μm) were collected on specimen grids (Agar Scientific), rehydrated with distilled water and then stained with uranyl acetate (1 min) and lead citrate (1 min). The samples were visualized and photographed using a JEOL 100 CX II Transmission Electron Microscope (JEOL UK).

Immunocytochemistry (ICC)

Immunocytochemical analysis was used to examine the expression of CD3, a surface antigen expressed by all T lymphocytes, and CD25, a surface antigen expressed by activated T lymphocytes. Monolayers of osteoblasts ($6x10^4$ cells) were prepared on sterile plastic coverslips, and T lymphocytes ($1.2x10^6$ cells) were seeded onto the osteoblasts, as described above. The co-cultures were incubated in a standard medium for 72 h. After washing extensively with PBS to remove non-adherent T lymphocytes, ICC was carried out as previously reported [27] using primary mouse anti-human CD3 or primary mouse anti-human CD25 antibodies (DAKO) diluted 1:100 in PBS containing 2% FCS-PBS for 1 h at RT. Non-specific mouse IgG was used as the

negative control. The samples were incubated with the HRP-conjugated goat anti-mouse IgG antibody diluted 1:500 in FCS-PBS for 1 h at RT and then developed using 3,3'-diaminobenzidine tetrachloride (DAB) (Sigma) for 10 min. Counter-staining was performed using Mayer's hematoxylin (Merck). The stained cells were examined under an Olympus BX 50 light microscope (Olympus UK, Southall, UK) and photographed with a Nikon Coolpix 4500 digital camera (Best Scientific, Swindon, UK).

Cell-enzyme-linked immunosorbent assay (C-ELISA).

The expression of the T cell CD3 surface antigen, corresponding to the number of T lymphocytes, was carried out using C-ELISA [30]. Briefly, confluent osteoblast monolayers (approximately $2x10^4$ cells/well) were obtained after culturing the cells in flat-bottom 96-well culture plates at a density of $1x10^4$ cells/well in 10% FCS-DMEM for 48 h. After removal of the non-adherent T lymphocytes by extensively washing, C-ELIZA was carried out on the adherent cells (osteoblasts and adherent T lymphocytes) using primary mouse anti-human CD3 antibody (DAKO) (1:100) for 3 h at RT. Non-specific mouse immunoglobulin G (IgG) (DAKO) was used as the negative control. The samples were then incubated with HRP-conjugated goat anti-mouse IgG antibody (DAKO) (1:500) in FCS-PBS for 1 h at RT, and then the substrate 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma) added for 30 min at RT. The reaction was stopped by adding 0.5 M H_2SO_4, and the absorbance at 450 nm was measured using an ELISA plate reader (Titertek Multiskan® Plus MKII; Labsystems, Helsinki, Finland).

Matrix mineralization assay

Calcium-containing deposits and mineralized matrices were examined as previously reported [31]. Briefly, osteoblasts were plated at a density of $2x10^4$ cells/cm^2 in 24-well plates in a standard culture medium. After 48 h in standard culture media, the T lymphocytes were added, and the co-cultures were incubated in an osteogenic medium (OM), which consisted of a standard culture medium supplemented with 0.1 mM L-ascorbic acid 2-phosphate and 10 mM β-glycerophosphate. After 14-28 days indicated in each experiment, the cell monolayers were stained with 1% alizarin red S (Sigma), then rinsed twice with methanol, air dried and photographed. Incorporated alizarin red S was extracted from the samples with 100 mM cetylpyridinium chloride (Sigma) for 20 min at room temperature, and the absorbance (A570 nm) was measured.

Raman spectroscopic analysis

Raman analysis was carried out using a LabRam spectrometer (Horiba Jobin Yvon, Middlesex, UK) to determine whether the bone-like mineralized nodules obtained in the cultures contain key inorganic and organic compartments of bone (*i.e.,* hydroxyapatite and collagen, respectively). Maps of an area of (100×100 μm) of the surface of the mineralized deposits formed by osteoblasts cultured alone and in T lymphocyte-osteoblast co-cultures on sterile plastic coverslips, as described above, were obtained at a step size of 5 μm, using a 633 nm laser, 50× objective and 1800 grating. The mean Raman spectra of the corresponding Raman maps were obtained following background subtraction and normalization using LabRam software. Standard Raman spectra of the hydroxyapatite, plastic coverslip Thermanox® and collagen were also recorded.

Flow cytometry (FCM)

FCM was conducted to examine the expression of TGF-β1, BMP-2, IGF-1, ICAM1 and VCAM-1 proteins in osteoblasts. The samples were then prepared for FCM as described previously [32]. In brief, the cells were detached using a scraper and 20 mM EDTA without trypsin and then fixed with 1% paraformaldehyde for 30 min. After each step described below, they were washed with PBS containing 2% FCS (PBS-FCS). Following centrifugation, approximately 50,000 osteoblastic cells were treated for 30 min at room temperature with antibodies specific to human TGF-β1, BMP-2, IGF-1, ICAM1, VCAM-1 (all from R&D Systems, Abingdon, UK) and phosphorylated forms of p-38, ERK1/2, JNK MAPKs (all from Cell Signaling Technology, Leiden, Netherlands), diluted in 0.1% saponin containing PBS-FCS. Corresponding isotype control antibodies (R&D Systems) were used as the negative control. The samples were incubated with appropriate fluorescence-conjugated secondary antibodies, and the stained cells were analyzed by flow cytometry (FACScan; Becton-Dickinson, Cowley, UK). Analysis of data was performed using the CELLQuest software program (Becton-Dickinson). The geometric means of the fluorescence values of the cell population are shown as the average fluorescence intensity (AFI) of 5,000 individual cells. In some experiments, the cultured samples were treated for 4 h before cell harvesting with 1 μM monensin (Sigma) to prevent the secretion of TGF-β1, BMP-2, IGF-1.

Statistical analysis

The data are presented as the mean ± SD from triplicates, with similar results obtained from three independent experiments. Statistical differences were analyzed using SPSS software

(SPSS, Inc., Chicago, IL) using a one-way ANOVA followed by the post hoc Dunnett's test with *a p* value < 0.05 considered statistically significant.

Results

Direct cell contact between osteoblasts and T lymphocytes and T lymphocyte activation

Fig. 1a shows that after 72 h in co-cultures, some T lymphocytes adhered to the osteoblast surface and were not dissociated by extensive washing, as described in the Materials and Methods. This interaction between these two cells was confirmed by SEM, as shown in Fig 1b, in which approximately 10-15 T lymphocytes (arrows) were estimated to be in contact with an underlying osteoblast. A higher magnification SEM is shown in Fig 1c, which also demonstrates the presence of an apparently activated T lymphocyte with characteristic extended cytoplasmic processes (arrows) very closely associated with the osteoblast. Further evidence suggestive of direct osteoblast-T lymphocyte cell-cell contact is shown in Fig. 1d-1e, in which the intimate nature of the apparently direct cell-cell interaction was visualized at the ultrastructural level. Fig. 1d shows a light microscopic photograph of a co-culture section stained with crystal violet, which demonstrates extended cytoplasmic processes (arrows) of an apparently activated T lymphocyte directly interacting with the osteoblast. TEM results also revealed the presence of some activated T lymphocytes (arrows) directly adhering to the osteoblast (Fig. 1e). The cell-cell apposition established by membrane-to-membrane interaction at the site of the cell-cell contact, often reached up to more than 5 μm in length as shown in Fig. 1e. No lymphocytes were observed in areas that did not contain adherent osteoblasts (Fig. 1a and 1b). The greatest number of T lymphocytes adherent to the osteoblasts was obtained when a ratio

of 20 T lymphocytes per one osteoblast was added to the osteoblast cultures for 72 h of incubation (Fig. S2); this cell ratio was therefore used in subsequent experiments.

The expression of CD3 and CD25 in co-cultures was used to examine whether the mononuclear cells in co-cultures are T lymphocytes and, more specifically, activated T lymphocytes, respectively. The representative immunocytochemical micrograph in Fig. 1f demonstrated that all the mononuclear cells that had remained adherent to the osteoblasts after 72 h of co-culture expressed CD3 and would therefore be considered T lymphocytes. Moreover, most of these adherent cells were also observed to be positive for CD25 (as shown in Fig. 1f), indicating that they had presumably become activated by cell membrane-dependent signal transduction processes following the addition of their non-activated T cell precursors to the osteoblasts.

Effect of direct contact with T lymphocytes on the formation of mineralized nodules by osteoblasts

To examine whether the presence of T lymphocytes affects osteogenic mineralization, the osteoblasts were cultured with and without T lymphocytes in osteogenic media. The results showed that by day 14 of incubation, mineralized deposits were observed only in T lymphocyte co-cultures (Fig. 2a (iii)) but not in osteoblast cultures alone (Fig.2a (i)). Moreover, a greater amount of nodule formation was also observed on day 28 in the presence of T lymphocytes compared with the culture of osteoblasts alone (Fig. 2a (iv) vs. Fig. 2a (ii)). To further investigate whether cell-cell contact plays a part in the increased formation of mineralization in T lymphocyte-osteoblast co-cultures, a porous membrane was used to block direct cell interaction physically. The results showed that under osteogenic conditions, the amount of alizarin red-

positive nodules was greater in co-cultures, without membrane separation, than that in the osteoblasts alone (Fig. 2b). In marked contrast, prevention of direct cell contact of these two cell types by a porous membrane dramatically reduced the level of alizarin red-positive deposit to the level even lower than that in the control group (osteoblasts alone) (Fig. 2b). A summary of the mineralization analysis in Fig. 2c shows that co-cultures possessed an over 3-fold increase in biomineralization. In contrast, those with membrane separation resulted in an over 8-fold decrease when compared with the control osteoblasts. In some experiments, pre-activated T lymphocytes were used in the co-cultures, and similar matrix mineralization results were observed (Fig. S3).

Characterization of mineralized deposits in co-cultures of osteoblasts and T lymphocytes was further investigated by SEM-EDX and Raman spectroscopy. The results in Fig. 2d and 2e show the elemental analysis of the mineralized deposits observed using SEM-EDX. When cultured under osteogenic conditions for 28 days, the presence of Ca and P, key elements of mineralized bone, were detected in the calcified matrix both in osteoblasts cultured alone (2d and spectrum 2) and in T lymphocyte-osteoblast co-cultures (2e and spectrum 4). The Ca/P ratio of the calcification was found to be 1.50 and 1.57 in osteoblast cultures and in T lymphocyte-osteoblast co-cultures, respectively. No Ca and P were detected in the area of the non-mineralized matrix (spectrum 1 in 2d and spectrum 3 in 2e). Fig. 2e also demonstrates that calcification is readily visible around and near the (smaller and more spherical) lymphocyte cells attached to the osteoblasts. Moreover, Raman microscope images were consistent with the SEM images in showing that with osteoblasts alone, 5 μm diameter hydroxyapatite crystals aggregated together in large clumps of the order of 40 μm (*i.e.,* of the size of a spread osteoblast) (Fig. 2f). The Raman spectra of these crystals (green areas in Figs. 2h and 2i) showed a dominant peak at

960 cm^{-1} (Fig. 2j), consistent with a broadened hydroxyapatite peak (Fig. 2m). Regions in between the hydroxyapatite clumps (blue areas in Fig. 2h) had Raman spectra that were dominated by Thermanox® peaks (Fig. 2m). In the co-cultures, the Raman image and mapping again indicated the presence of approximately 5 μm hydroxyapatite crystals. However, these were more dispersed across the whole sample (Figs. 2g and 2i). Further, in these co-cultures, areas with spectra consistent with pure Thermanox® were difficult to detect. Average Raman spectra of osteoblasts and co-cultures were comparable, indicating a mixture of hydroxyapatite, Thermanox® and other organic peaks consistent with the presence of protein similar to collagen (Figs. 2k and 2l, respectively). Raman spectra of collagen, Thermanox® and hydroxyapatite (HA) are also shown in Fig. 2m as standards for comparison.

Direct cell contact up-regulated ICAM-1 and simultaneously down-regulated TGF-β1 in osteoblasts

In co-cultures with the presence of direct cell contact, the expression levels of TGF-β1 and IGF-1 decreased by 52% and 47%, respectively, in osteoblasts, while ICAM-1 increased by about 27 folds compared with those in osteoblast mono-cultures (Fig. 3). No changes in the levels of BMP-2 and VCAM-1in osteoblasts were detected in co-cultures compared with the osteoblast alone. Prevention of direct cell contact by a transwell membrane reversed the effect of T lymphocytes on TGF-β1 and ICAM-1, but not IGF-1, in osteoblasts. In some experiments, pre-activated T lymphocytes were used in the co-cultures, and similar results were observed (Fig. S4). The results indicated that direct cell contact between either non-activated or pre-activated T lymphocytes and osteoblasts might play an important role in the increased ICAM-1 level and the decreased TGF-β1 level in osteoblasts.

ICAM-1 signaling-mediated direct cell contact was involved in the osteoblastic expression of TGF-β1 in co-cultures.

To examine whether ICAM-1 signaling-mediated direct cell contact play a role in the osteoblastic expression of TGF-β1 in co-cultures, the ability of ICAM-1 blocking antibody to prevent T lymphocyte-osteoblast direct contact was first determined. The results in Fig. 4a indicate that the blocking antibody at 5-10 μg/ml significantly blocked the adhesion of T lymphocytes to osteoblasts by 60-65% for up to 72 h. Treatment of the co-cultures with the ICAM-1 blocking antibody at 5 μg/ml also rescued TGF-β1 expression to the level of approximately 70% of that in the control osteoblast mono-culture (Fig. 4b). This suggests that direct adhesion mediated by ICAM-1 in the co-culture at least partly suppressed the expression of TGF-β1 in osteoblasts when co-cultured with T lymphocytes. The involvement of MAPK signalings in the regulation of osteoblastic TGF-β1 expression by ICAM-1-mediated direct adhesion was further investigated, and the results are shown in Figs. 4c and 4d. The levels of phosphorylated MAPKs, *i.e.,* p-p38, p-ERK1/2 and p-JNK1/2/3, in osteoblast mono-cultures were undetectable in the present study (data not shown). Treatment of co-cultures with an ICAM-1 blocking antibody resulted in significant reductions in all MAPKs (Fig. 4c). However, in co-cultures, only a p38 inhibitor SB203580 reversed osteoblastic TGF-β1 suppression to the level of approximately 87% of the control osteoblast mono-cultures (Fig. 4d). This indicates that p-38-dependent ICAM-1 signaling plays a role in osteoblastic TGF-β1 suppression when co-cultures with T lymphocytes.

Exogenously added TGF-β1 in co-cultures, in the presence of direct cell contact, suppressed mineralization

The involvement of TGF-β1 in matrix mineralization in co-cultures in the presence of direct T lymphocyte-osteoblast contact was assessed, and the results are shown in Fig. 5. While exogenously added TGF-β1 dose-dependently inhibited matrix mineralization in osteoblast mono-cultures, it also suppressed matrix mineralization in co-cultures. High doses at 5 and 10 ng/ml of TGF-β1 could completely suppress the increased mineralization induced by T lymphocyte direct contact in co-cultures. The results indicated that in the presence of direct contact between these two cell types, exogenously added TGF-β1 in co-cultures with direct cell contact strongly inhibited matrix mineralization by osteoblasts. This further supports the involvement of TGF-β1 suppression in enhanced matrix mineralization in co-cultures.

Discussion

Although several lines of evidence support the role of T lymphocytes in chronic inflammation-associated bone diseases [1, 4, 12, 21, 33], little is known about the effects of direct cell-to-cell interaction on bone remodeling in chronic inflammation of the bone. This process involves interactions between T lymphocytes, the major inflammatory cell observed to be present, and osteoblasts. The results of the present study have shown, for the first time, that naturally non-adherent human T lymphocytes could become activated and adherent when co-cultured with human osteoblasts, at least in tissue culture. The interaction between these two types of the cell was evident by both SEM and TEM, which suggested that an intimate relationship was established via numerous lymphocyte cytoplasmic processes and bone cell microvilli-like structures, leading to extensive membrane-to-membrane contact (as per Fig. 1). It has previously been shown that resting lymphocytes established only limited direct contact with dermal fibroblasts. In contrast, activated lymphocytes formed extensive sites of direct interaction

along the fibroblast cell surface without evidence of cell fusion at the contact zones or the formation of specialized junctions between the two types of cells [34].

Interactions between surface adhesion molecules of osteoblasts and T cell receptors could play an important part in the activation of T lymphocytes, as distinct intracellular signaling has been reported to be initiated at adhesion molecule / T cell receptor-enriched regions [35]. In the present study, T lymphocytes appear to have become activated at some point during the establishment of direct cell-to-cell contact with the osteoblasts, as evident by the distinct hand mirror-like morphological features and the expression of CD25. Similar observations are likely to occur during the inflammatory response, where circulating lymphocytes become activated and firmly adherent to endothelial cells, concomitantly changing their morphology from small rounded cells to a far more polarized shape [36]. Furthermore, the adhesion molecule LFA-1, a T cell receptor for ICAM-1 and highly expressed in osteoblasts [18], has a pivotal role in T lymphocyte-mediated cell contact and T lymphocyte activation [37]. These observations provide evidence for the importance of adhesion molecule / T cell receptor signaling in the activation of T lymphocytes. However, there have also been some reports of an inhibitory role of cell-cell contact on T lymphocyte activation, most probably mediated via soluble cytokines rather than cell-to-cell contact signals [38].

In the present study, we also showed a potentially important functional consequence of direct cell contact between T lymphocytes and osteoblasts - an increase in bone matrix mineralization, possibly resulting from signal transduction via cell adhesion. Previous studies have also suggested that integrin adhesion and signaling could facilitate the initiation of matrix mineralization by osteoblasts and stem cells [39, 40] via induction of the expression of a key osteogenic transcription factor Runx2 [41]. The present results also showed that ablation of the

direct cell-to-cell contact between T lymphocytes and osteoblasts by a semi-permeable membrane resulted in a profound decrease in mineralized nodule formation by osteoblasts, in contrast to the suppression of matrix mineralization by osteoblasts mediated by soluble cytokines from T lymphocytes, *e.g.,* IL-1β and TNF-α, which have previously been shown to inhibit osteoblast differentiation and thus bone mineralization [9, 10, 12, 42]. It is noteworthy that when pre-activated T lymphocytes were used in co-cultures, their effects on matrix mineralization (Fig. S3) and osteoblastic expression of the growth factors and adhesion molecules (Fig. S4) were comparable to those using T lymphocytes (without pre-activation). It is possible that following the addition of non-activated T lymphocytes to osteoblasts, cell membrane-dependent signal transduction may induce T cell activation pathway(s) similar to those occurred in pre-activated T lymphocytes. Molecular mechanisms of T cell activation by direct adhesion to osteoblasts warrant further investigation.

In the co-cultures studied here, mineralization was observed around and near the lymphocytic cells attached to the osteoblasts, suggesting that these cells may help increase the number of crystal nucleation sites ensuring that a large number of smaller size calcified crystals, presumably hydroxyapatite, are formed. The Raman spectra of these crystals showed a dominant peak at 960 cm^{-1} consistent with a broadened hydroxyapatite peak (Fig. 2), possibly as a consequence of calcium deficiency of the hydroxyapatite and/or partial substitution of other ions into the hydroxyapatite lattice structure. The Ca/P ratio of the calcification deposited in T lymphocyte-osteoblast co-cultures was 1.57, indicating the presence of calcium-deficient hydroxyapatite in bone (Ca/P ratio of stoichiometric hydroxyapatite = 1.67). Calcium-deficient hydroxyapatite rather than stoichiometric hydroxyapatite is of special interest since the Ca/P

ratio in bone is lower than 1.67, as it has been suggested that calcium-deficient hydroxyapatite may play an important part in bone remodeling and bone formation [43].

Several previous studies have reported the biological importance of the direct cell-to-cell contact between T lymphocytes and other connective tissue cells [17-19]. For example, Olsen et al. showed direct transfer of α-D-mannosidase from T lymphocytes that directly adhered to fibroblasts [17], while direct contact with human gingival fibroblasts resulted in the up-regulation of CD13 in the T cells [19]. In addition, direct contact of T cells with human osteoblasts stimulated the secretion of IL-1β and IL-6 via a process that involved the adhesion molecules ICAM-1 and VCAM-1[18]. The present study showed that regardless of the T cell pre-activation, direct cell-to-cell contact between T lymphocytes and osteoblasts regulated the expression of TGF-β1, but not BMP-2 and IGF-1, and ICAM-1, but not VCAM-1, in osteoblasts (Fig. 3 and Fig. S4). A reduction in osteoblastic TGF-β1 expression appeared to be induced by ICAM-1-mediated direct cell-to-cell contact. The decrease in TGF-β1 in turn enhanced mineralization, as evident by reversal experiments that exogenously added TGF-β1was used (Fig. 5). As previously reported, following binding to its ligands on T lymphocytes, ICAM-1 initiates several intracellular signaling pathways, such as MAPKs and PI3K, to control the expression of several genes associated with inflammation [44, 45], and TGF-β1 inhibits matrix mineralization by suppressing BMP-4-induced osteoblast maturation, stimulating the expression of inhibitory Smads and suppressing SMURF1-C/EBPβ-DKK1 axis [46-48]. The present results indicated that osteoblastic ICAM-1 signaling activated all three principal MAPKs, as previously reported [49-51], while only the signal transduction via p38, but not the other two pathways, controlled osteoblastic TGF-β1 expression. Certain transcription factors activated by the p38 signaling cascade may regulate the TGF-β1 promoter, as previously reported for the signal transduction initiated by IL-6- mediated activation of p-38 [52]. Precise

signaling pathways and transcriptional regulation involved in the regulation of matrix mineralization by ICAM-1-mediated direct cell contact warrants further *in vitro* and *in vivo* studies.

As shown in Fig. 5, the effect of exogenously added TGF-β1 on mineralization by osteoblasts appeared to be dose-dependent and reached a plateau at 5 ng/ml regardless of the presence of T lymphocytes, suggesting that TGF-β1 may be a key regulator for bone repair in bone diseases associated with chronic inflammation. However, at low concentrations of TGF-β1 treatment of the co-cultures, other direct adhesion-mediated signals may overcome this TGF-β1 effect. Increased IL-1β, IL-6 and PGE-2 levels in osteoblasts directly in contact with T lymphocytes via ICAM-1 have been reported previously [18, 53]. The marked increase in osteoblastic ICAM-1 following co-cultured with T lymphocytes may suggest positive feedback from T lymphocytes [49]. Furthermore, it has previously been reported that the expression of ICAM-1 in non-immune cells can also regulate the secretion of soluble inflammatory cytokines by activated T cells [54]. It is thus possible that ICAM-1-mediated direct contact between osteoblasts and T cells may control matrix mineralization via regulation of cytokine secretion by T cells. Future studies to explore these hypotheses are required.

Conclusions

The present study has shown, for the first time, that there is direct cell-to-cell contact between T lymphocytes and osteoblasts, and studies are consistent with ICAM-1-mediated direct contact between osteoblasts and T lymphocytes increasing mineralization via down-regulation of TGF-β1 in osteoblasts *in vitro*. This suggests a possible unexpected, but crucial, role of T lymphocytes in biomineralization during the repair process *in vivo*, thus identifying ICAM-

1/TGF-β1 as possible novel therapeutic targets for the treatment and prevention of inflammation-associated mineralized tissue diseases.

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Conflict of Interest statements

No competing conflict of interests exist.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics Approval Statement

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Joint Research and Ethics Committee of the Eastman Dental Institute and Hospital, the Ethics Review Sub-Committee for Research Involving Human

Research Subjects of Thammasat University No. 3 (COA No. 068/2564) and the Institutional Biosafety Committee Thammasat University (057/2564).

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Figure legends

Fig. 1. Direct contact between osteoblasts (OB) and T lymphocytes (T). In (a), the image shows microscopic visualization of T lymphocyte-bone cell interaction on day 3 of co-culture. The SEM image in (b) demonstrates that approximately10-15 T lymphocytes (arrows) were in direct contact with one bone cell. Image (c) shows a higher magnification SEM of the direct cell-to-cell contact between both cell types, which reveals an activated T lymphocyte with characteristic extended cytoplasmic processes (arrows) directly attached to the bone cell. Image (d) shows a representative section stained with crystal violet and observed under the light microscope before TEM analysis (e). In (d), arrows indicate extended cytoplasmic processes of an activated T lymphocyte directly interacting with an osteoblast. Under TEM in (e), T lymphocytes were observed to be in direct contact with an osteoblast, and at higher magnification, the extensive area of close membrane-membrane contact is visible, as indicated by the arrows. In (f), ICC analysis of the expression of CD3 and CD25, cell surface antigens characteristic of T lymphocytes and activated T lymphocytes, respectively, in co-cultures. These markers are expressed as intense brown staining, whereas unstained cells are observed in the negative control.

Fig. 2. Effect of T cells on the formation of mineralized nodules in osteoblast-T lymphocyte co-cultures under osteogenic induction. In (a), mineralized deposits were not present in osteoblasts cultured alone (OB) for 14 days but were observed by 28 days (i and ii, respectively) and were also observed in co-cultures (OB+T) both at 14 and 28 days (iii and iv) and at higher magnification in v and vi, respectively). Alizarin red-S staining confirmed the presence of calcium-containing deposits (b), which was far more intense in co-cultures in direct cell-cell contact than when the two types of cells were separated by a membrane (M). Control osteoblasts

cultured in a non-osteogenic medium showed no staining. The levels of extracted alizarin red dye were also shown (c). The results are presented as the mean percent ± SD obtained from triplicate samples. * $p < 0.05$. In (d)-(e), elemental analysis of mineralized deposits formed in osteoblast cultures (d) and in co-cultures (e) by SEM-EDX. Note that the spectra of Ca and P are shown in the figure, while the spectra of other main ions, *e.g.,* Au and Pd (as coating materials) and oxygen, have been omitted. The results are presented as the mean Ca/P ratio ± SD obtained from 10 different mineralized deposits in a sample. Similar results were found in three separate experiments. Raman spectroscopy of biomineralization in osteoblast-T lymphocyte co-cultures is shown in (f)-(m). Raman images, mappings and spectra of representative mineralized deposits formed in osteoblast cultures (f, h and k, respectively) and co-cultures (g, i and l, respectively). In Raman mappings, areas in green have a strong peak at 960 cm^{-1} consistent with hydroxyapatite-like inorganic deposits, while areas in blue have characteristic spectra consistent with an organic phase (j). Raman spectra of collagen, Thermanox® and hydroxyapatite (HA) are also shown in (m) as standards for comparison. Similar results were found in three separate experiments.

Fig. 3. Flow cytometric analysis of the expression of growth factors and adhesion molecules in osteoblasts. Osteoblasts were cultured alone (OB), cultured with T lymphocytes (OB+T) and cultured with T lymphocytes in the presence of a porous membrane (OB+T+M) to prevent direct contact between these two cell types for 72 h. The expression levels of TGF-β1, BMP-2, IGF-1, ICAM-1 and VCAM-1 were then assessed by FCM (a). The results are presented as the mean percent ± SD obtained from triplicate samples, and the expression levels in the OB group were defined as 100%. Representative histograms demonstrating the expression levels of TGF-β1 (b)

and ICAM-1 (c) are also shown. Similar results were found in three separate experiments. * $p < 0.05$.

Fig. 4. Role of ICAM-1 signaling-mediated direct cell contact in the osteoblastic expression of TGF-β1 in co-cultures. In (a), osteoblasts were pre-incubated with an ICAM-1 blocking antibody at various concentrations (0-20 μg/ml). Cells were then co-cultured with T lymphocytes for 24-72 h, and the CD3 levels, proportional to the numbers of T lymphocytes, were determined by C-ELIZA. The results are presented as mean absorbance ± SD from 6 replicates. In (b and c), osteoblasts were pre-incubated with an ICAM-1 blocking antibody (10 μg/ml) before co-cultured with T lymphocytes (OB+T+ICAM-1 blocking), and in (d), pre-treatments of cultures with MAPK inhibitors were performed. The expression of TGF-β1 (b and d) and phosphorylated MAPKs (c) in osteoblasts was examined by FCM. The results are presented as mean percent ± SD from triplicates, defined as 100% in the control osteoblast alone (OB). * $p < 0.05$.

Fig. 5. Effect of exogenously added TGF-β1 on matrix mineralization in osteoblast mono-cultures and osteoblast-T lymphocyte co-cultures under osteogenic conditions. Osteoblasts were cultured with and without T lymphocytes in the presence of varying concentrations of TGF-β1 (0-10 ng/ml) and osteogenic supplements for 28 days. Matrix mineralization was assessed using the alizarin red S staining assay, and the levels of extracted alizarin red dye were determined. The results are presented as the mean percent ± SD obtained from triplicate samples. Similar results were found in three separate experiments. * vs OB, 0 ng/ml TGF-β1, # vs OB+T, 0 ng/ml TGF-β1

Fig. 1

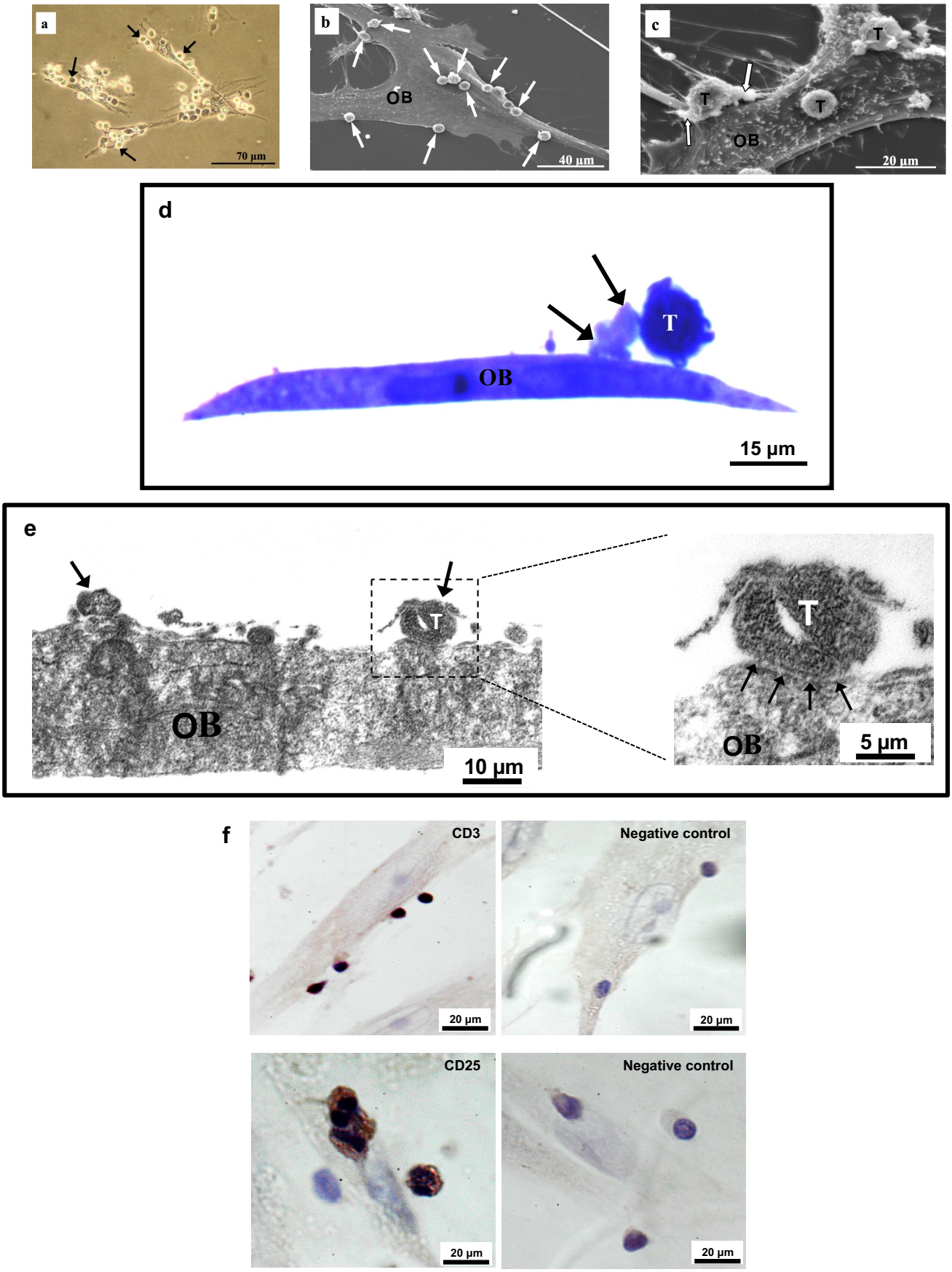

a
70 µm
b
OB
40 µm
c
T
T
T
OB
20 µm
d
T
OB
15 µm
e
OB
T
10 µm
T
OB
5 µm
f
CD3
20 µm
Negative control
20 µm
CD25
20 µm
Negative control
20 µm

Fig. 2

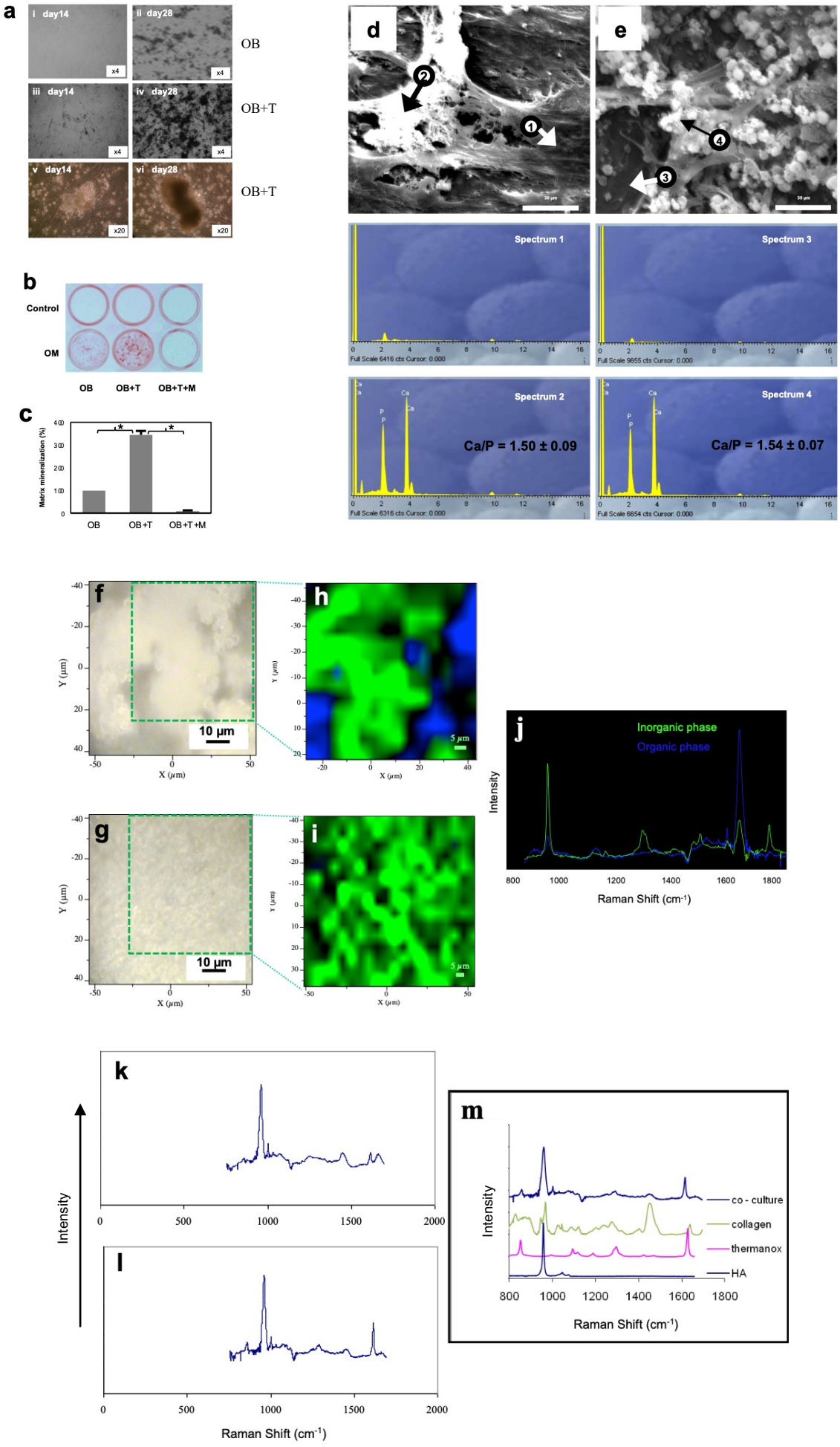

a
i day14
ii day28
iii day14
iv day28
v day14
vi day28
x4
x20
OB
OB+T
OB+T
b
Control
OM
OB
OB+T
OB+T+M
c
Matrix mineralization (%)
OB
OB+T
OB+T+M
d
e
Spectrum 1
Spectrum 3
Spectrum 2
Spectrum 4
Ca/P = 1.50 ± 0.09
Ca/P = 1.54 ± 0.07
f
g
h
i
10 µm
5 µm
X (µm)
Y (µm)
j
Inorganic phase
Organic phase
Intensity
Raman Shift (cm-1)
k
l
m
co - culture
collagen
thermanox
HA

Fig. 3

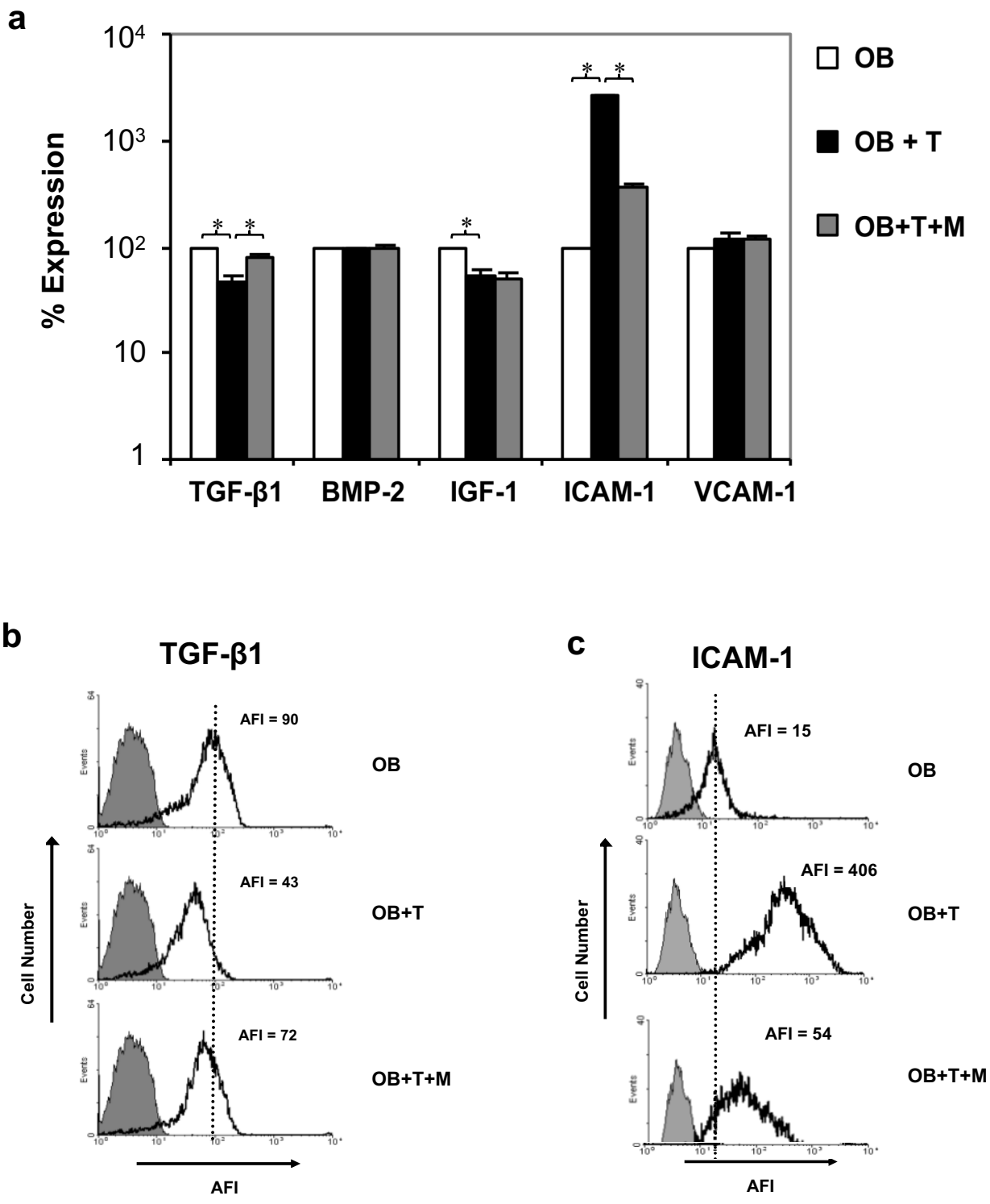

a
% Expression
10⁴
10³
10²
10
1
OB
OB + T
OB+T+M
TGF-β1
BMP-2
IGF-1
ICAM-1
VCAM-1
b
TGF-β1
AFI = 90
AFI = 43
AFI = 72
OB
OB+T
OB+T+M
Cell Number
AFI
c
ICAM-1
AFI = 15
AFI = 406
AFI = 54
OB
OB+T
OB+T+M
Cell Number
AFI

Fig. 4

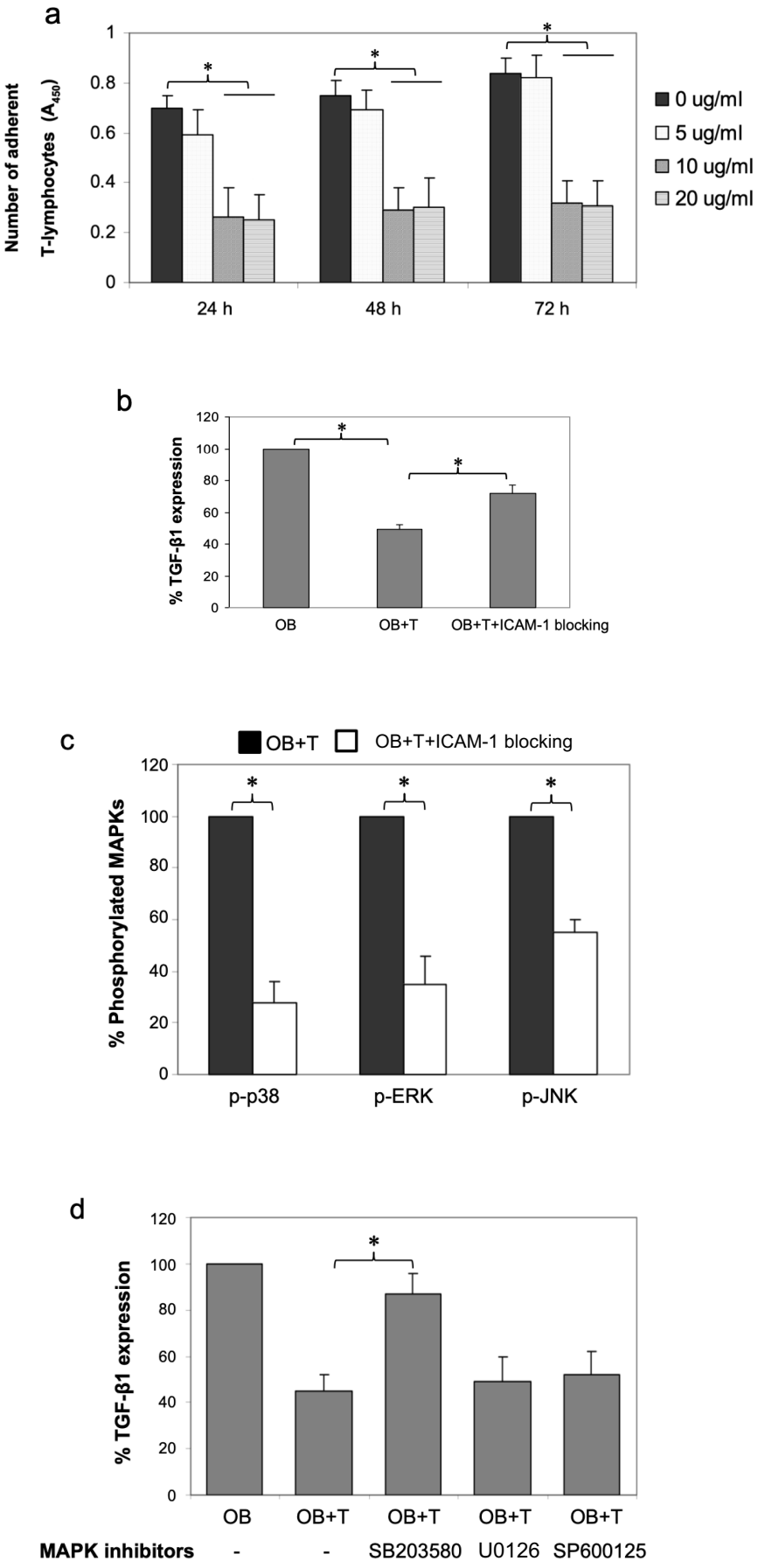
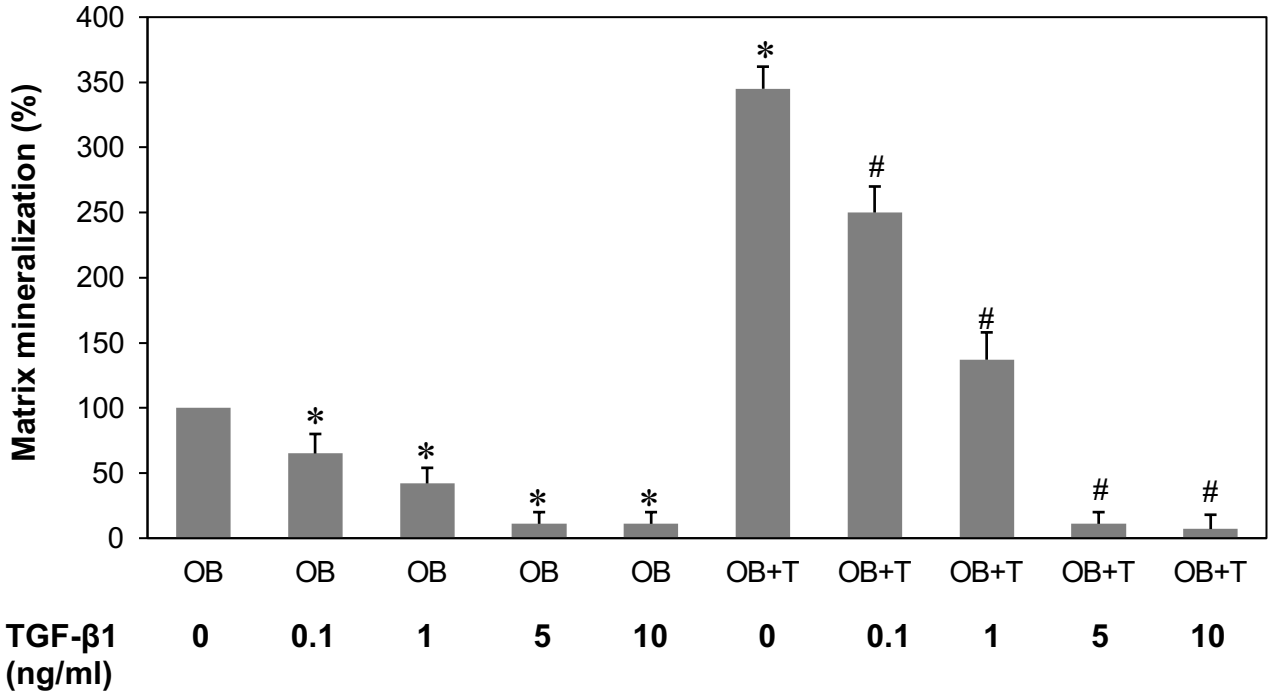

a
Number of adherent T-lymphocytes (A_{450})
1
0.8
0.6
0.4
0.2
0
*
*
*
24 h
48 h
72 h
0 ug/ml
5 ug/ml
10 ug/ml
20 ug/ml
b
% TGF-β1 expression
120
100
80
60
40
20
0
*
*
OB
OB+T
OB+T+ICAM-1 blocking
c
OB+T
OB+T+ICAM-1 blocking
% Phosphorylated MAPKs
120
100
80
60
40
20
0
*
*
*
p-p38
p-ERK
p-JNK
d
% TGF-β1 expression
120
100
80
60
40
20
0
*
OB
OB+T
OB+T
OB+T
OB+T
MAPK inhibitors
-
-
SB203580
U0126
SP600125

Fig. 5


Matrix mineralization (%)
400
350
300
250
200
150
100
50
0
*
*
*
*
*
#
#
#
#
OB
OB
OB
OB
OB
OB+T
OB+T
OB+T
OB+T
OB+T
TGF-β1 (ng/ml)
0
0.1
1
5
10
0
0.1
1
5
10

Supporting information

ICAM-1-mediated osteoblast-T lymphocyte direct interaction increases mineralization through TGF-β1 suppression

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Supporting Information: Materials and Methods

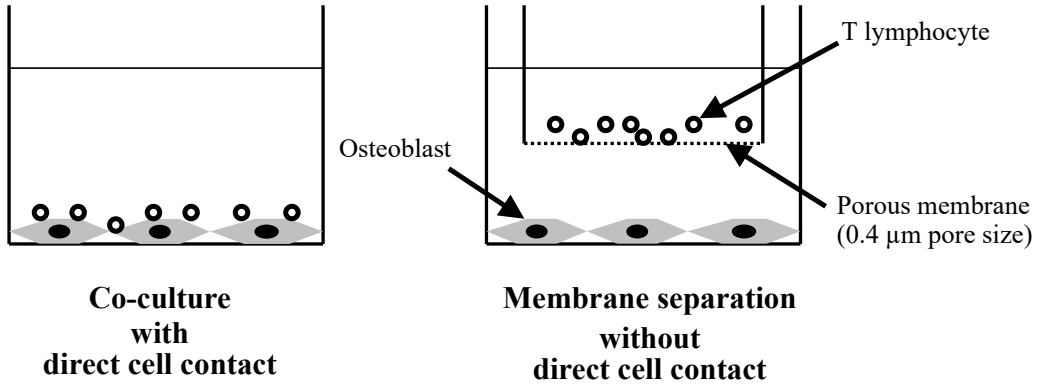

Fig. S1. A diagram schematically shows the T lymphocyte-osteoblast co-cultures in which the suspensions of T lymphocytes are physically separated from adherent osteoblast monolayers by a porous membrane.

Supporting Information: Results

Effect of increasing numbers of T lymphocytes on cell-to-cell contact with osteoblasts

In Fig. S2, C-ELISA analysis showed that increasing the initial number of T lymphocytes added to the osteoblast monolayers progressively increased the total number of T lymphocytes that remained adherent to the osteoblasts up to a co-culture period of 72 h. The greatest number of T lymphocytes adherent to the osteoblasts was obtained when a ratio of 20 T lymphocytes per one osteoblast was added to the osteoblast cultures for 72 h of incubation; this cell ratio was therefore used in subsequent experiments. Notably, at higher initial seeding densities (30-40 T cells/osteoblast), the number of adherent T cells significantly decreased as time progressed in the co-cultures (Fig. S2).

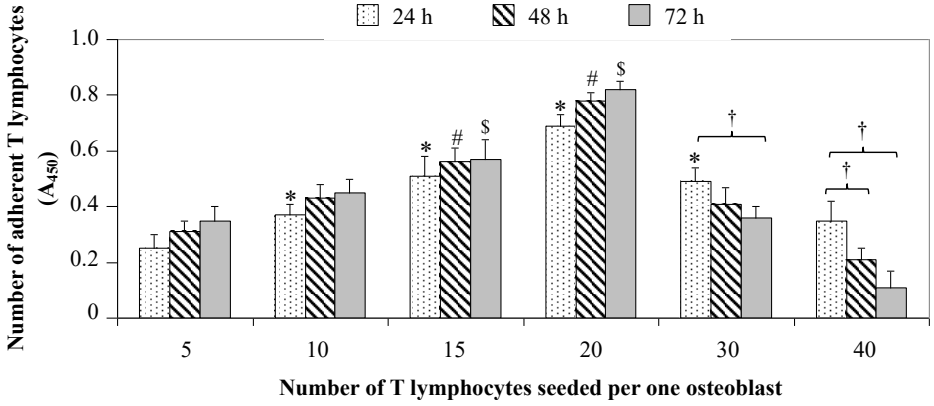

Fig. S2. C-ELISA analysis of the number of T lymphocytes attached to osteoblast monolayers in co-cultures. Osteoblasts were cultured in a 96-well plate until they reached confluence and non-activated T lymphocytes then added at 5, 10, 15, 20, 30 and 40 cells per one osteoblast for 24, 48 and 72 h, as described in the Materials and Methods The co-cultures were extensively washed with PBS to remove the non-adherent T lymphocytes and the adherent T lymphocyte-osteoblast monolayers were fixed and analyzed for CD3 expression using C-ELISA. The absorbance measured is proportional to the number of T lymphocytes remaining (adherent) in the co-culture. The results are presented as the mean A450 ± SD of triplicate wells. Note that, up to 72 h in co-cultures, the highest number of T lymphocytes that had adhered to the osteoblasts was obtained when 20 T lymphocytes per one osteoblast were co-cultured.
* $p < 0.05$ vs 5 T cells/osteoblast at 24 h, $^{\#}$ $p < 0.05$ vs 5 T cells/osteoblast at 48 h, $^{\$}$ $p < 0.05$ vs 5 T cells/osteoblast at 72 h, † $p < 0.05$.

Effect of pre-activated T lymphocytes on matrix mineralization by osteoblasts

To examine whether the presence of pre-activated T lymphocytes affects matrix mineralization, T lymphocytes were pre-activated with 5 μg/ml concanavalin A (ConA; Sigma) for 72 h before co-cultured with osteoblasts under osteogenic induction. In addition, a porous membrane was used to block direct cell interaction. The results showed that under osteogenic conditions, the amount of alizarin red-positive deposits was greater in co-cultures, without membrane separation, than that in the osteoblasts alone (Fig. S3a). In marked contrast, prevention of physical direct cell contact of these two cell types markedly decreased alizarin red-positive deposits to the level much lower than that in the control group (osteoblasts alone) (Fig. S3a). A summary of the mineralization analysis in Fig. S3b shows that co-cultures between osteoblasts and pre-activated T lymphocytes possessed an approximately 3-fold increase in matrix mineralization. In contrast, those with membrane separation resulted in an over 9-fold decrease when compared with the control osteoblasts.

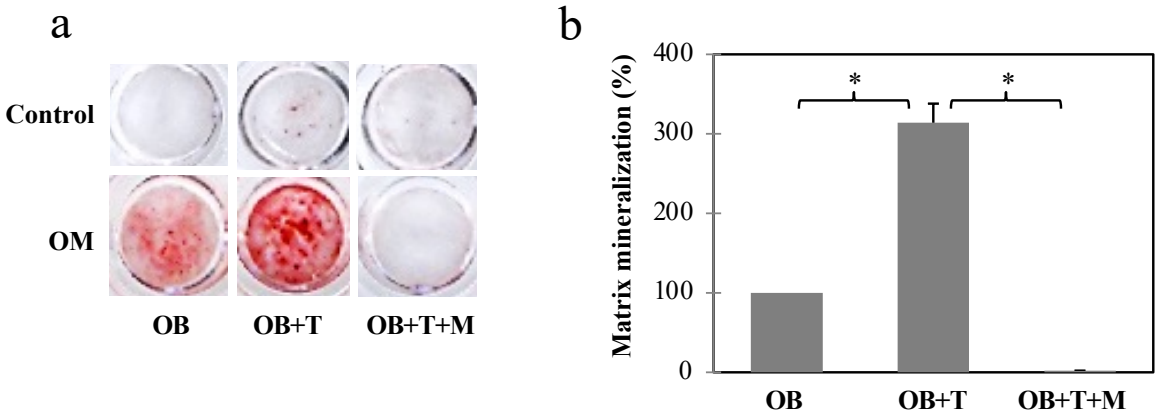

Fig. S3. Effect of pre-activated T lymphocytes on the formation of mineralized nodules in osteoblast-pre-activated T lymphocyte co-cultures under osteogenic induction. In (a), osteoblasts (OB) were cultured alone or with pre-activated T lymphocytes (T) with and without osteogenic medium (OM), and mineralized deposits stained with alizarin red-S are shown as blight red deposits. A porous membrane (M) was also used to physically prevent direct cell contact between these two cell types. A summary of matrix mineralization is shown in (b). The results are presented as the mean percent ± SD obtained from triplicate samples. Similar results were obtained from three separate experiments. * $p < 0.05$.

Direct cell contact to pre-activated T lymphocytes up-regulated ICAM-1 and simultaneously down-regulated TGF-β1 in osteoblasts

In co-cultures between pre-activated T lymphocytes and osteoblasts with the presence of direct cell contact, the expression level of TGF-β1 decreased by 42%, in osteoblasts, while ICAM-1 increased by about 29 folds compared with those in osteoblast mono-cultures (Fig. S4). No changes in the levels of BMP-2, IGF-1and VCAM-1in osteoblasts were detected in co-cultures compared with the osteoblast alone. Prevention of direct cell contact by a transwell membrane reversed the effect of T lymphocytes on TGF-β1 and ICAM-1 in osteoblasts. The results indicated that direct cell contact of pre-activated T lymphocytes to osteoblasts might play an important role in the increased ICAM-1 level and the decreased TGF-β1 level in osteoblasts.

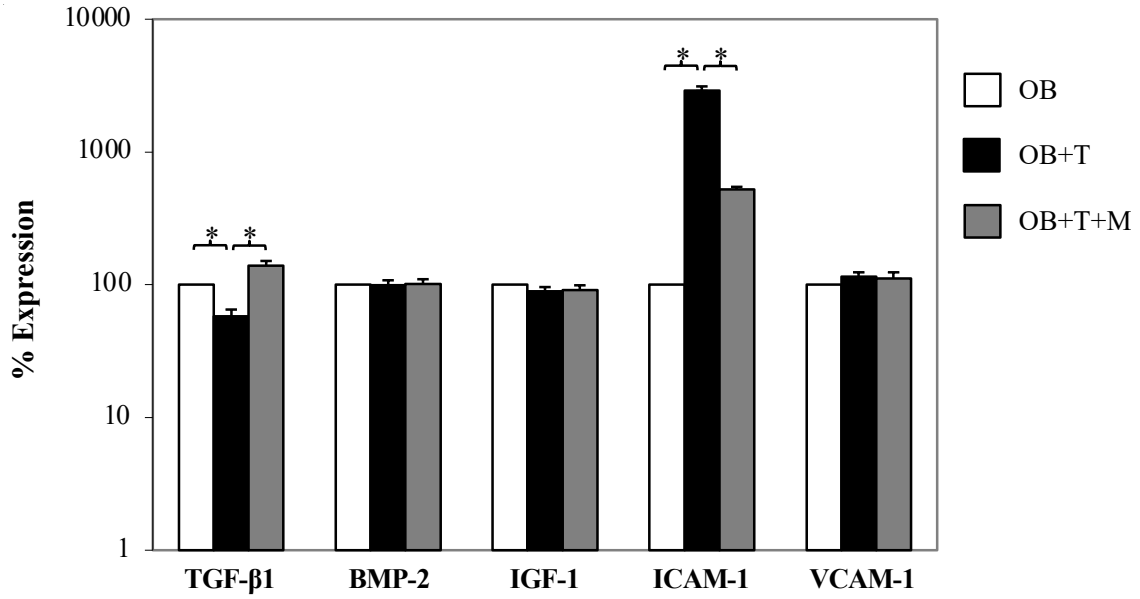

Fig. S4. Flow cytometric analysis of the expression of growth factors and adhesion molecules in osteoblasts. Osteoblasts were cultured alone (OB), cultured with ConA pre-activated T lymphocytes (OB+T) and cultured with ConA pre-activated T lymphocytes in the presence of a porous membrane (OB+T+M) for 72 h. The expression levels of TGF-β1, BMP-2, IGF-1, ICAM-1 and VCAM-1 were then assessed by FCM. The results are presented as the mean percent ± SD obtained from triplicate samples, and the expression levels in the OB group were defined as 100%. Similar results were obtained from three separate experiments. * $p < 0.05$