

$\alpha 1\beta 1$ integrin and interleukin-7 receptor up-regulate the expression of RANKL in human T cells and enhance their osteoclastogenic function

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Introduction

The homeostasis of the skeletal system depends on a balance between bone-forming osteoblasts and bone-resorbing osteoclasts. The ability of the immune system, through tissue-persisting activated/effector CD4⁺ T cells, to regulate this balance towards osteoclasts contributes to pathological bone resorption seen in chronic inflamma-

Summary

Activated T cells, through the production of the receptor activator of NF- κ B ligand (RANKL) cytokine, have been implicated in the osteoclast development and bone loss that are associated with autoimmune diseases such as rheumatoid arthritis. However, the cellular pathways that regulate the expression of RANKL and the induction of osteoclasts are still unclear. In this study, we show that, in human effector CD4⁺ T cells, activation of $\alpha 1\beta 1$ integrin and interleukin (IL)-7 receptor (IL-7R) up-regulates the expression and production of RANKL but has no effect on the production of interferon- γ , an inhibitor of T-cell-mediated osteoclastogenesis. Thus, both $\alpha 1\beta 1$ integrin and IL-7R enhance the ability of these cells to induce the formation of osteoclasts from human monocytes. Furthermore, we found that simultaneous activation of effector CD4⁺ T cells via $\alpha 1\beta 1$ integrin and IL-7R synergistically increases the production of RANKL and enhances their osteoclastogenic function. We also show that, although $\alpha 1\beta 1$ integrin does not protect human effector CD4⁺ T cells from IL-2-withdrawal-induced apoptosis, it does enhance the pro-survival effect of IL-7, further emphasizing the importance of the $\alpha 1\beta 1$ /IL-7R synergistic effect. Together our results identify a new function of $\alpha 1\beta 1$ integrin in T cells and suggest that activation of effector CD4⁺ T cells through $\alpha 1\beta 1$ integrin and IL-7R is an important regulatory pathway in T-cell-dependent osteoclastogenesis. Further understanding of the mechanisms by which IL-7R and $\alpha 1\beta 1$ integrin promote T-cell-mediated osteoclastogenesis will lead to new insights into the regulatory pathways of T-cell-dependent bone resorption associated with autoimmune diseases.

Keywords: effector T cells; integrins; osteoclastogenesis; RANKL

tory diseases such as rheumatoid arthritis (RA), periodontitis and inflammatory bowel disease.^{1–4} This is in part attributable to the ability of activated T cells to produce the osteoclastogenic cytokine receptor activator of NF- κ B ligand (RANKL), which following its binding to its receptor RANK expressed on osteoclast precursors initiates the differentiation programme that leads to the formation of mature osteoclasts.^{1–5} Activated T cells *in vitro* or isolated

Abbreviations: 7-AAD, 7-aminoactinomycin; Coll I, collagen type I; Coll IV, collagen type IV; ECM, extracellular matrix; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; IFN- γ , interferon- γ ; IL-7, interleukin-7; IL-7R, interleukin-7 receptor; mAb, monoclonal antibody; M-CSF, macrophage-colony-stimulating factor; MFI, mean fluorescence intensity; OPG, osteoprotegerin; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PE, phycoerythrin; PLL, poly-L-lysine; RA, rheumatoid arthritis; RANKL, receptor activator of NF- κ B ligand; RT-PCR, reverse transcriptase-polymerase chain reaction; TCR, T-cell receptor; TNF, tumour necrosis factor; TRAP, tartrate-resistant acid phosphatase.

T cells from patients with chronic inflammatory diseases such as RA produce RANKL and directly induce the development of osteoclasts from autologous monocytes used as osteoclast precursors.^{6,7}

The development of osteoclasts is also dependent on additional cytokines such as osteoprotegerin (OPG), which can be produced by stromal cells⁸ OPG is a decoy receptor of RANKL and interferes with RANKL-induced differentiation of osteoclasts.² Interferon (IFN)- γ , which can be produced by activated T cells, also blocks the RANKL/RANK signalling pathway and the subsequent formation of osteoclasts.^{9,10} In this context, activated T cells that produce large amounts of IFN- γ , such as in response to T-cell receptor (TCR) stimulation, are unable to induce the formation of osteoclasts.^{9–11} In contrast, interleukin (IL)-7, which is an important cytokine in T-cell development and survival,¹² has been shown to induce the development of osteoclasts through T-cell production of RANKL,^{13,14} suggesting that the ability of activated T cells to induce the formation of osteoclasts is probably dependent on how they are activated. It is noteworthy that most of the above studies were conducted with freshly isolated resting T cells, which are unlikely to be representative of the effector T cells found at the sites of tissue injury and which are associated with the inflammatory response. It is therefore still unclear how effector T cells are activated to potently induce the formation of osteoclasts.

Integrins are α/β heterodimeric membrane proteins that mediate cell–cell interactions and cell adhesion to the surrounding extracellular matrix (ECM). In addition, integrins can elicit a wide variety of intracellular signals that modulate cell growth, proliferation and apoptosis.^{15,16} T cells express several members of the $\beta 1$ integrin family, which following cellular activation mediate their attachment to the ECM.^{17,18} In addition, T-cell interactions with the ECM in injured tissues also regulate their effector functions. Recent studies have focused on the function of collagen-binding integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$, which are expressed on effector/memory T cells infiltrating the tissues.^{18–20} In this regard, several studies, including ours, have demonstrated that these integrins co-stimulate TCR-dependent proliferation and cytokine production and protect effector T cells from apoptosis.^{21–24} In addition, $\alpha 1\beta 1$ integrin has been shown to be involved in the development of RA, a chronic inflammatory disease associated with bone resorption. Indeed, infiltrating T cells from the arthritic synovium of patients express high levels of $\alpha 1\beta 1$ integrin^{25,26} and its blockade with antibodies significantly reduces the inflammatory response and the development of arthritis in animal models.^{27,28} Whether $\alpha 1\beta 1$ integrin has a direct role in T-cell-mediated osteoclastogenesis has not been explored. To explore this possibility, we have examined whether activation of $\alpha 1\beta 1$ integrin

regulates the expression of RANKL and the ability of effector CD4⁺ T cells to induce the formation of osteoclasts. Furthermore, we have also examined whether IL-7 regulates RANKL expression in these cells, and whether $\alpha 1\beta 1$ integrin enhances the osteoclastogenic function of IL-7, as integrins can act as co-stimulatory molecules.

Our results show that ligation of $\alpha 1\beta 1$ integrin with its ligand collagen IV (Coll IV) or activation of IL-7R with IL-7 up-regulates to the same extent the expression of RANKL in human effector CD4⁺ T cells. This is correlated with the ability of Coll IV- and IL-7-activated T cells to induce the formation of osteoclasts from monocytes. Importantly, activation of effector CD4⁺ T cells with both Coll IV and IL-7 led to a synergistic induction of RANKL and development of osteoclasts. Finally, we show that ligation of $\alpha 1\beta 1$ integrin also augments the ability of IL-7 to protect these cells from IL-2-withdrawal-induced apoptosis. Our results indicate that $\alpha 1\beta 1$ integrin and IL-7R may represent two major regulatory pathways that contribute to the osteoclastogenic function of effector CD4⁺ T cells and may thus be important in T-cell-dependent bone loss associated with the development of autoimmune and chronic inflammatory diseases.

Materials and methods

Antibodies and reagents

Anti-CD3 (OKT3), anti-CD28 (CD28-2), anti-CD127 (hIL-7R-M21) and isotype control monoclonal antibodies (mAbs) were purchased from BD Biosciences (San Diego, CA). The anti- $\alpha 1$ integrin (FB12) and poly-L-lysine (PLL) were purchased from Chemicon (Temecula, CA). Fluorescein isothiocyanate (FITC)-conjugated anti-mouse mAbs were purchased from Jackson ImmunoResearch (West Grove, PA), Coll IV and IL-2 from Sigma (St Louis, MO), and IL-7, macrophage–colony-stimulating factor (M-CSF) and anti-human RANKL mAb (70525) from R&D Systems (Minneapolis, MN).

Isolation of primary CD4⁺ T cells and generation of long-term activated lymphoblasts

Peripheral blood mononuclear cells (PBMC) from healthy adult volunteers were isolated on Ficoll gradients. Primary human naïve CD4⁺ T cells were then purified by negative selection using magnetic beads from StemCell Technologies (Vancouver, BC, Canada) according to the manufacturer's instructions. Staining with anti-CD3 and anti-CD4 mAbs and flow cytometry [fluorescence-activated cell sorter (FACS)] analysis indicated that more than 97% of the isolated cells were CD3/CD4 double-positive T cells. Freshly isolated blood T cells do not express the

collagen-binding integrins α 1 β 1 and α 2 β 1 but do so after *in vitro* activation.^{18,19,24} Thus, the cells were activated with immobilized anti-CD3 (2.5 μ g/ml) and soluble anti-CD28 (5 μ g/ml) mAbs for 24 hr in complete RPMI 1640 medium supplemented with 10% of fetal bovine serum (FBS), 2 mM/l glutamine and 100 units/ml penicillin and streptomycin. The cells were then washed, transferred to flasks and cultured for 10 more days in the presence of 20 U/ml of recombinant IL-2, which was added every 2 days. Because IL-2 down-regulates the expression of IL-7R on activated/effector T cells,²⁹ the cells at day 10 were cultured for one more day in fresh medium in the absence of IL-2 before being used in subsequent experiments. These cells, which will be referred to as effector CD4⁺ T cells, are responsive to TCR activation, which leads to IL-2 and IFN- γ production.

Cell surface molecule expression

The expression of cell surface receptors on effector CD4⁺ T cells was determined by immunostaining and flow cytometry analysis. The cells were incubated on ice with 10 μ g/ml of anti- α 1 integrin or anti-CD127 or with control isotypic mAbs for 45 min in PBS containing 1% FBS. The cells were washed and incubated with FITC-conjugated anti-mouse mAb for another 45 min. Cells were washed in PBS and analysed by flow cytometry using a FACScan instrument (BD Biosciences).

Enzyme-linked immunosorbent assays (ELISAs)

The production of IFN- γ and soluble RANKL was measured using specific ELISAs from R&D Systems (Minneapolis, MN) and Biomedica (Vienna, Austria), respectively. After 24 hr of activation, cell supernatants were harvested and loaded on an ELISA plate according to the manufacturer's instructions.

Apoptosis assays

Apoptosis was determined using the Annexin V-PE/7AAD detection kit from BD Biosciences. After stimulation, the cells were washed in cold PBS, and 10⁵ cells were incubated in 1 \times buffer containing 5 μ l of Annexin V-PE and 5 μ l of 7-aminoactinomycin (7-AAD) for 15 min at room temperature, in the dark. The cells were then washed and analysed by flow cytometry using the FACScan instrument (BD Biosciences) and apoptotic cells were identified as cells that were Annexin V-positive.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted with Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad,

CA). First-strand cDNA was prepared from 1 μ g of total RNA using the ThermoScript RT-PCR system from Invitrogen. RANKL and β -actin transcripts were amplified by PCR using specific primers as previously described.³⁰ PCR reactions were performed with 1 U Taq polymerase in a total volume of 50 μ l, and amplifications were carried out in a Peltier Thermal Cycler (MJ Research, Ramsey, MN). The amplification for each gene was in the linear curve, i.e. under non-saturating conditions during the exponential phase. A volume of 10 μ l of the reaction mixture was size-separated on a 2% agarose gel, and specifically amplified products were detected by ethidium bromide staining and UV illumination.

Immunoblot analysis

The cells were resuspended in RPMI 1640 medium and stimulated in six-well plates. At the end of cultures, the cells were harvested and washed in cold PBS, and cell lysates were prepared in radio-immune precipitation assay (RIPA) buffer containing protease and phosphatase inhibitors as previously described.²⁴ Cell lysates were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and analysed by immunoblot using specific anti-RANKL mAb. The blots were stripped and re-probed with control anti-actin mAb (Santa Cruz Biotechnology, Santa Cruz, CA) to ensure equal loading. In all experiments, immunoblots were visualized using a horseradish peroxidase (HRP)-conjugated secondary antibody followed by enhanced chemiluminescence detection (Pierce, Rockford, IL).

Osteoclastogenesis experiments

The ability of RANKL-expressing effector CD4⁺ T cells to induce the development of osteoclasts was tested in a coculture assay with human monocytes used as osteoclast precursors. Human CD16-negative monocytes, which have the ability to differentiate into osteoclasts,³¹ were isolated from the peripheral blood of healthy volunteers by immunomagnetic negative selection (StemCell Technologies, Vancouver, BC, Canada). Monocytes (1 \times 10⁵ cells) were seeded in 48-well plates and were activated with M-CSF (100 ng/ml) in alpha minimum eagle medium (α MEM) supplemented with serum and antibiotics. Shortly thereafter, effector T cells (1 \times 10⁵ cells) that had been activated or not for 12 hr with IL-7, Coll IV, anti-CD3 mAb, IL-7 + Coll IV or anti-CD3 + Coll IV were added to the monocytic cultures. Culture media and activated T cells were replaced every 2 days with fresh media and freshly activated effector T cells, respectively, as above. At the end of the experiment (day 8), osteoclast development was visualized by tartrate-resistant acid phosphatase (TRAP) staining (Sigma) according to the manufacturer's instructions.

After staining, multinucleated (≥ 4 nuclei) TRAP-positive cells were counted as osteoclasts.

Results

Expression of $\alpha 1\beta 1$ integrin and IL-7R on effector CD4⁺ T cells

To determine the involvement of $\alpha 1\beta 1$ in T-cell-mediated osteoclastogenesis, we used effector CD4⁺ T cells generated after *in vitro* activation. These cells express high levels of $\alpha 1$ integrin, as we and others have previously shown.^{19,24,32} After 12 days of activation, almost 78% of the total cell population stained positive for $\alpha 1$ integrin, with an average mean fluorescence intensity (MFI) of 25.71 (Fig. 1a). These cells were also found to express high levels of IL-7R, as 90% of the total cell population stained positive for the α chain of IL-7R (CD127) with an MFI of 50 (Fig. 1b; left panel). The expression of IL-7R detected on human effector CD4⁺ T cells is in agreement with reports showing that effector T cells can maintain significant levels of IL-7R.^{33–35} The cells were also responsive to IL-7 stimulation, as this led to a significant

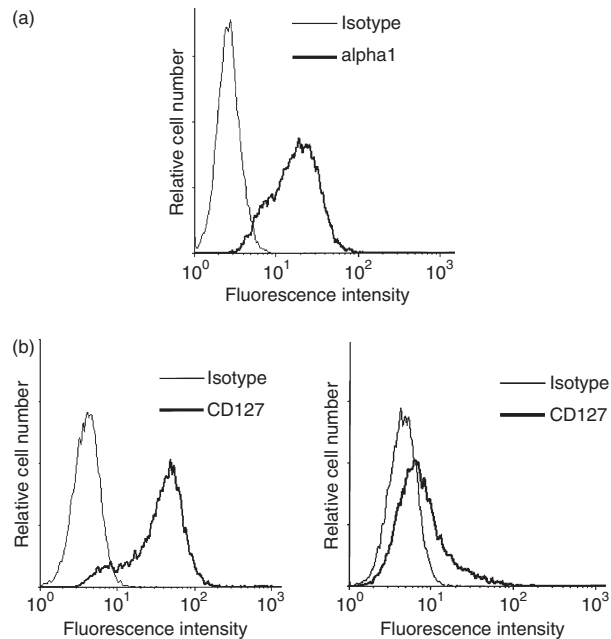


Figure 1. Human effector CD4⁺ T cells express $\alpha 1\beta 1$ integrin and the interleukin-7 receptor (IL-7R). The expression of these cell surface receptors was determined by fluorescence-activated cell sorting (FACS) analysis as described in the *Materials and methods*. (a) Expression of $\alpha 1\beta 1$ integrin. (b) Expression of IL-7R on effector CD4⁺ T cells without stimulation (left panel) and upon stimulation with IL-7 for 8 hr (right panel). The results are representative of three independent experiments performed with T cells from different blood donors.

reduction in the levels of CD127 on the cell surface (Fig. 1b, right panel) as previously observed.³⁶

$\alpha 1\beta 1$ integrin and IL-7R up-regulate the expression and production of RANKL in effector CD4⁺ T cells

To examine whether $\alpha 1\beta 1$ integrin regulates RANKL gene expression, we stimulated effector CD4⁺ T cells with Coll IV, the ligand of $\alpha 1\beta 1$ integrin, IL-7 or IL-7 + Coll IV. As shown in Fig. 2a, effector CD4⁺ T cells expressed low levels of RANKL mRNA, which were significantly increased upon stimulation for 6 hr with IL-7 or with Coll IV. Importantly, the combination of IL-7 and Coll IV led to a stronger up-regulation of RANKL mRNA levels. As a control, PLL, a non-integrin-binding ligand, had no effect on RANKL gene expression and did not affect the IL-7 response. Similar results were also obtained when the cells were activated for 24 hr (Fig. 2a). These results indicate that $\alpha 1\beta 1$ integrin and IL-7R are important regulatory pathways of RANKL gene expression in human effector CD4⁺ T cells. To determine whether the up-regulation of RANKL mRNA could also be observed at the protein level, we measured the levels of RANKL protein by immunoblot analysis. As shown in Fig. 2(b), IL-7 and Coll IV increased the protein levels of RANKL and their combination led to enhanced production of RANKL.

Similar to other tumour necrosis factor (TNF) ligands, RANKL also undergoes molecular shedding, leading to the production of a soluble form that is measured in cell supernatants.³⁷ We therefore measured the levels of soluble RANKL and, as shown in Fig. 2(c), effector CD4⁺ T cells produced detectable levels of soluble RANKL, which were significantly increased by IL-7 and Coll IV. The combination of IL-7 + Coll IV also led to a synergistic production of soluble RANKL. The up-regulatory effect of IL-7, Coll IV and IL-7 + Coll IV on RANKL gene expression and production was not a result of augmented cell proliferation as no changes in cell numbers were detected in any of the tested conditions (data not shown). Together these results indicate that ligation of $\alpha 1\beta 1$ with Coll IV up-regulates the levels of RANKL in human effector CD4⁺ T cells and synergizes with IL-7R in this process.

Activation of effector CD4⁺ T cells through $\alpha 1\beta 1$ and IL-7R does not modulate IFN- γ production

As activated T cells are known to produce IFN- γ , which is an inhibitor of RANKL-induced osteoclastogenesis, we verified the expression of IFN- γ in effector CD4⁺ T cells. As shown in Fig. 3, these cells expressed low levels of IFN- γ , which were not modulated by IL-7, Coll IV or IL-7 + Coll IV. In contrast, and in agreement with our recent report,²³

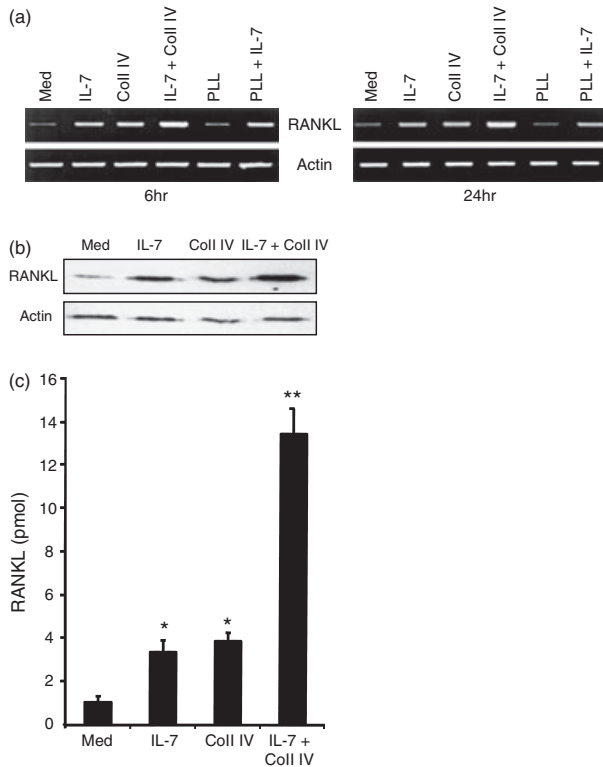


Figure 2. Interleukin (IL)-7 and collagen IV (Coll IV) induce the expression of receptor activator of NF- κ B ligand (RANKL). (a) Effector CD4⁺ T cells were activated with IL-7 (2.5 ng/ml), Coll IV (20 μ g/ml), poly-L-lysine (PLL; 20 μ g/ml), IL-7 + Coll IV, and IL-7 + PLL for 6 or 24 hr. The cells were harvested, total RNA was prepared and the expression of RANKL was determined by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. As a control, the levels of actin mRNA were also determined. The results are representative of three independent experiments performed with T cells from different blood donors. (b) Cells were activated as indicated for 24 hr and cell lysates were prepared. The expression of RANKL was then determined by immunoblot analysis. The blot was stripped and re-probed with anti-actin monoclonal antibody to ensure equal loading. The results are representative of two independent experiments performed with T cells from different blood donors. (c) Cells were activated as indicated for 24 hr, and then cell supernatants were harvested and the production of soluble RANKL was determined by enzyme-linked immunosorbent assay (ELISA). The results are presented as mean values from three independent experiments performed in triplicate with T cells from different blood donors, with standard errors as indicated. Statistical analysis was carried out using Student's *t*-test: **P* < 0.05 between IL-7-treated and untreated samples (med), and between Coll IV-treated and untreated samples (med). ***P* < 0.05 between IL-7 + Coll IV- and IL-7-treated samples, and between IL-7 + Coll IV- and Coll IV-treated samples.

cells activated via TCR (anti-CD3 mAb) produced high levels of IFN- γ , and Coll IV did not modulate these levels (Fig. 3). Furthermore, and in agreement with previous findings,^{11,30,38,39} we found that effector CD4⁺ T cells

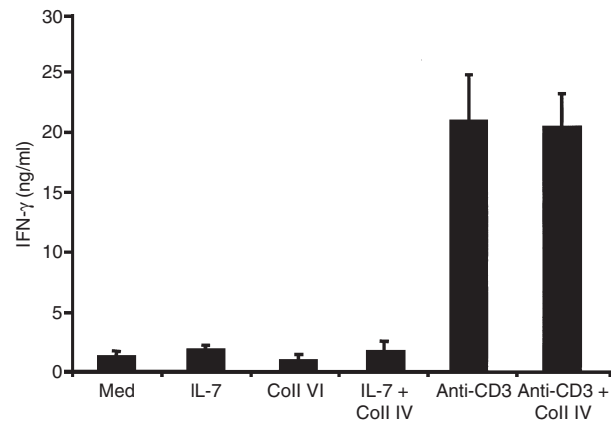


Figure 3. Interleukin (IL)-7 and collagen IV (Coll IV) co-stimulation does not increase the production of interferon (IFN)- γ . Cells were activated as indicated for 24 hr, and then cell supernatants were harvested and the production of IFN- γ was determined by enzyme-linked immunosorbent assay (ELISA). The results are presented as mean values from four independent experiments performed in triplicate with T cells from different blood donors, with standard errors as indicated.

activated or not with IL-7, Coll IV or IL-7 + Coll IV did not express osteoprotegerin (OPG) (data not shown).

$\alpha 1\beta 1$ integrin and IL-7R up-regulate the osteoclastogenic function of effector CD4⁺ T cells

To examine the functional role of IL-7R and $\alpha 1\beta 1$ integrin in T-cell-dependent osteoclastogenesis, we sought to determine the ability of effector CD4⁺ T cells activated with IL-7, Coll IV or both to induce the formation of osteoclasts as measured by the number of TRAP-positive multinucleated cells. As shown in Fig. 4, IL-7-activated effector CD4⁺ T cells significantly increased the formation of osteoclasts from monocytes compared with non-activated cells. Furthermore, Coll IV-activated T cells also increased the formation of osteoclasts (Fig. 4a,b). Importantly, IL-7 + Coll IV-activated T cells were the most potent in inducing the formation of osteoclasts (Fig. 4a,b), indicating that the synergistic effect of IL-7 and Coll IV on RANKL expression also occurs at the functional level. The formation of osteoclasts was attributable to RANKL as it was significantly reduced by recombinant OPG, and monocytes cultured without effector T cells did not form osteoclasts (Fig. 4a,b).

As TCR-activated effector T cells produce large amounts of IFN- γ , we investigated whether these cells have the ability to induce the formation of osteoclasts, and found that this is not the case (Fig. 5). Interestingly, activation of effector CD4⁺ T cells via TCR also induced RANKL expression, which was also up-regulated by Coll IV (Fig. 6). Together, these results indicate that activation of IFN- γ -producing effector T cells has the ability to

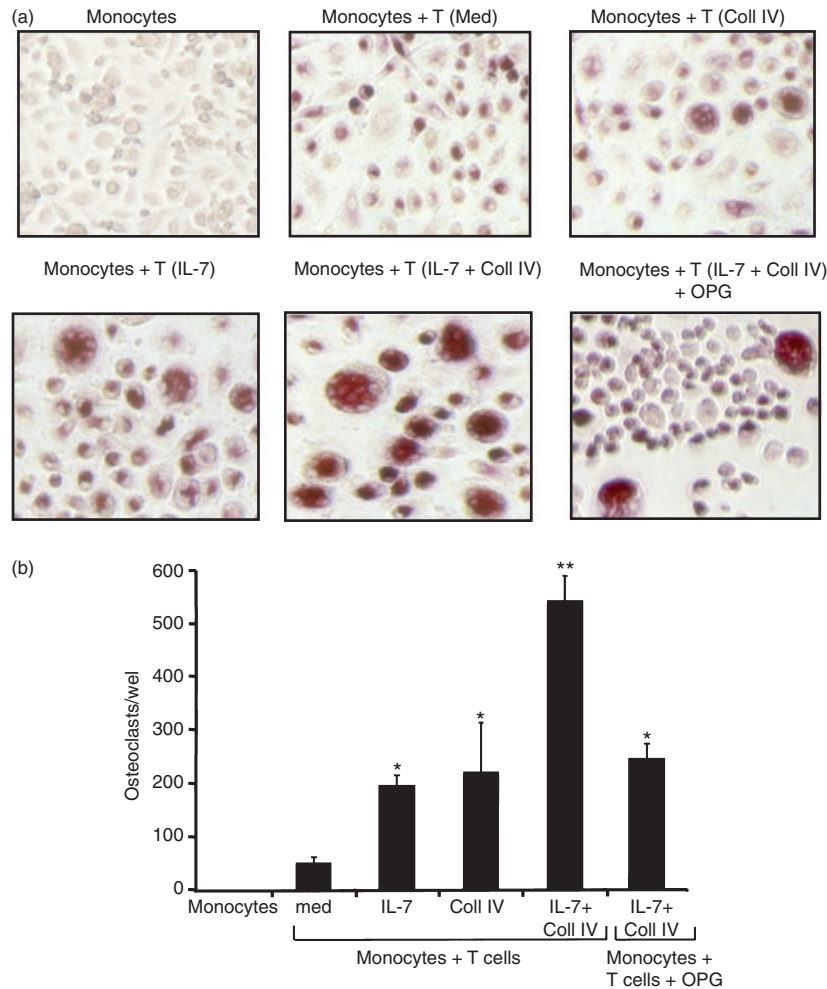


Figure 4. Interleukin (IL)-7- and collagen IV (Coll IV)-activated effector CD4⁺ T cells induce the formation of osteoclasts. Effector CD4⁺ T cells (T) were activated with IL-7, Coll IV or IL-7 + Coll IV or left untreated (med). They were washed and co-cultured with monocytes in the presence of macrophage colony-stimulating factor (M-CSF). Monocytes cultured alone and monocytes cultured with T cells activated with IL-7 + Coll IV in the presence of recombinant osteoprotegerin (OPG) are also shown. After 8 days, the cultures were washed and the adherent cells stained for tartrate-resistant acid phosphatase (TRAP) activity as described in the *Materials and methods*. (a) Representative images showing the formation of TRAP-positive multinucleated osteoclast cells. (b) Quantification of osteoclast formation induced by activated T cells. After TRAP staining, the total numbers of TRAP-positive cells with at least four nuclei were counted. The results are presented as mean values from three independent experiments performed in duplicate with T cells from different blood donors, with standard errors as indicated. Statistical analysis was carried out using Student's *t*-test: **P* < 0.05 between IL-7-treated and untreated samples (med), between Coll IV-treated and untreated samples (med), and between IL-7 + Coll IV + OPG-treated samples and untreated samples. ***P* < 0.05 between IL-7 + Coll IV- and IL-7-treated samples, between IL-7 + Coll IV- and Coll IV-treated samples, and between IL-7 + Coll IV- and IL-7 + Coll IV + OPG-treated samples.

induce the development of osteoclasts when cells are activated through IL-7R and $\alpha 1\beta 1$ integrin.

Coll IV potentiates the survival effect of IL-7

Protection of effector CD4⁺ T cells from IL-2-withdrawal-induced apoptosis can contribute to their accumulation in inflamed tissues^{40,41} and thus to T-cell-mediated tissue injury, and IL-7 is known to protect T cells from this form of apoptosis.^{12,42–44} We therefore investigated whether ligation of $\alpha 1\beta 1$ integrin with Coll IV also regulates T-cell survival or potentiates the anti-apoptotic function of IL-7.

Effector CD4⁺ T cells were activated or not in the absence of IL-2 with IL-7, Coll IV or IL-7 + Coll IV and their apoptosis was determined. As shown in Fig. 7, IL-2-starved effector T cells showed significant cell death after 72 hr, as nearly 60% of the cells were Annexin V-positive. The presence of IL-7 reduced apoptosis by 45%; Coll IV by itself had no effect but potentiated the IL-7 effect. Indeed, IL-7 + Coll IV reduced apoptosis by 73% compared with the 45% inhibition of apoptosis elicited by IL-7 alone. Together these results suggest that Coll IV enhances not only IL-7-induced production of RANKL but also IL-7-induced survival, suggesting that Coll IV + IL-7 may

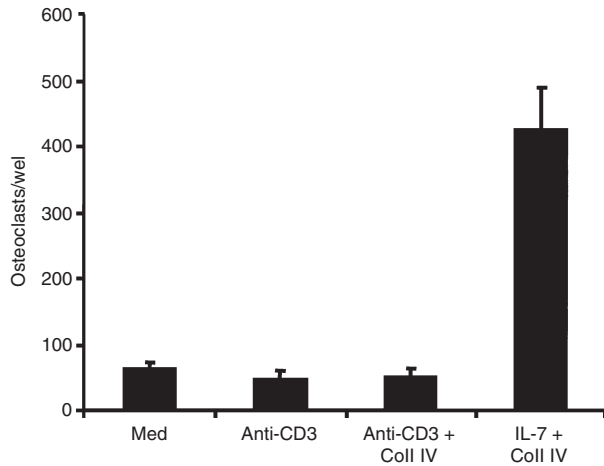


Figure 5. T-cell receptor (TCR)-activated effector $CD4^+$ T cells do not induce the formation of osteoclasts. The cells were activated with anti-CD3 monoclonal antibody in the presence or absence of collagen IV (Coll IV). The cells were washed and co-cultured with monocytes in the presence of macrophage colony-stimulating factor (M-CSF). After 8 days, the cultures were washed and stained for tartrate-resistant acid phosphatase (TRAP) activity as described in the *Materials and methods*. The results are presented as in Fig. 4(b).

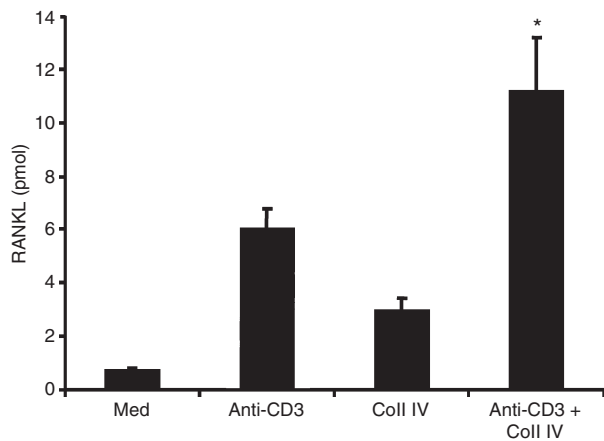


Figure 6. Activation of effector $CD4^+$ T cells via the T-cell receptor (TCR) and $\alpha 1\beta 1$ integrin also augments the production of receptor activator of NF- κ B ligand (RANKL). The cells were activated or not with anti-CD3 monoclonal antibody, collagen IV (Coll IV) or both stimuli. After 24 hr, cell supernatants were harvested and the production of soluble RANKL assessed by enzyme-linked immunosorbent assay (ELISA). The results are presented as in Fig. 2(c). Statistical analysis was carried out using Student's *t*-test: * $P < 0.05$ between anti-CD3 + Coll IV- and anti-CD3-treated samples.

represent an important signalling pathway in the immunopathogenic function of effector $CD4^+$ T cells.

Discussion

The mechanisms by which effector T cells are activated to induce the formation of osteoclasts are not well under-

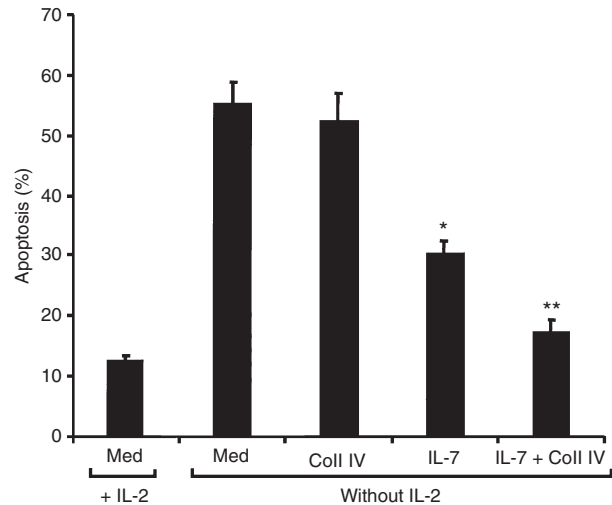


Figure 7. Collagen IV (Coll IV) enhances interleukin (IL)-7-induced cell survival. Effector $CD4^+$ T cells were stimulated or not in the absence of IL-2 with IL-7, Coll IV or IL-7 + Coll IV. As a control, the cells were also cultured in the presence of IL-2. After 72 hr, cell apoptosis was determined by Annexin V/7AAD staining and fluorescence-activated cell sorting (FACS) analysis. The results are presented as mean values from three independent experiments performed in triplicate with T cells from different blood donors, with standard errors as indicated. Statistical analysis was carried out using Student's *t*-test: * $P < 0.05$ between IL-7-treated and untreated (med) samples. ** $P < 0.05$ between IL-7 + Coll IV- and IL-7-treated samples.

stood. In the present study, we have demonstrated that $\alpha 1\beta 1$ integrin, which is expressed on effector $CD4^+$ T cells found in inflamed tissues, induces the expression of RANKL and enhances the formation of osteoclasts, thus identifying a novel role of $\alpha 1\beta 1$ integrin in T-cell functions. In addition, our results extend the previously reported osteoclastogenic function of IL-7 in naïve T cells¹⁴ to human effector $CD4^+$ T cells. More importantly, our results show that IL-7R and $\alpha 1\beta 1$ integrin on these cells synergize to augment their expression of RANKL, their ability to induce the formation of osteoclasts and their survival. Together these results indicate that IL-7R and $\alpha 1\beta 1$ integrin represent major regulatory pathways involved in T-cell-mediated osteoclastogenesis.

Our results show that stimulation of IFN- γ -producing effector $CD4^+$ T cells with Coll IV and IL-7 augments the levels of RANKL but does not affect the expression of IFN- γ ; an inhibitor of osteoclast development. This is correlated with the ability of these activated cells to induce the formation of osteoclasts. Although they express basal levels of IFN- γ , it is likely that these levels are insufficient to prevent the formation of osteoclasts. This has also been reported with naïve T cells stimulated with phytohaemagglutinin,⁴⁵ suggesting that IFN- γ -producing effector $CD4^+$ T cells can induce the formation of osteoclasts when they are activated with stimuli that

limit the production of IFN- γ . This possibility is also supported by our finding that effector CD4⁺ T cells activated through the TCR were unable to induce the formation of osteoclasts, despite the fact that these cells also produce RANKL. These results are in agreement with previous studies showing that activation of T cells via the TCR, which leads to the production of high levels of IFN- γ , interferes with their ability to induce osteoclastogenesis.^{9–11}

We also examined whether activation of effector T cells through the TCR and IL-7R and α 1 β 1 integrin enhances their osteoclastogenic function. We found that simultaneous activation of effector T cells via IL-7R and TCR led to slightly increased production of both IFN- γ and RANKL, and Coll IV/ α 1 β 1 slightly enhanced IL-7R + TCR-mediated production of RANKL but not IFN- γ . However, these cells were still unable to induce the formation of osteoclasts because of the high levels of IFN- γ produced by these T cells (data not shown). These results further indicate the importance of the IL-7R/ α 1 β 1 integrin pathway in T-cell-mediated osteoclastogenesis.

Although Th1 cells have been associated with the pathogenesis of RA, an autoimmune disease associated with bone resorption, it was not possible to measure significant levels of IFN- γ in the synovial tissue.⁴⁶ This suggests that, in the synovial tissue, these T cells can be activated with pro-inflammatory signals that do not lead to the production of high amounts of IFN- γ , such as through α 1 β 1 integrin and IL-7R, as proposed herein. In this context, α 1 β 1 integrin is expressed on effector CD4⁺ T cells infiltrating the synovium and has been implicated in the development of RA.^{25–28} Furthermore, synovial T cells from patients were also shown to express IL-7R.⁴⁷ IL-7 is an important cytokine in T-cell development and survival and is produced by stromal cells found in the extracellular compartment of many tissues.¹² Importantly, IL-7 levels are augmented in chronic inflammatory diseases such as RA, and IL-7 has been implicated in the activation of synovial T cells⁴⁸ and in the induction of bone loss *in vivo*.¹³ Therefore, our study strongly suggests that α 1 β 1 integrin and IL-7R could represent two major signalling pathways implicated in the regulation of T-cell-mediated bone loss associated with RA. Our results further support the notion that activation of effector T cells can occur independently of the TCR,^{49–51} especially in chronic inflammatory diseases such as RA, where cytokines and, as shown herein, ECM proteins can be critical in driving the activation of effector T cells.

In addition to T cells, osteoblasts also produce RANKL and induce the formation of osteoclasts. In this regard, it was recently demonstrated that adhesion of human osteoblasts to collagen type I (Coll I) also up-regulates RANKL and the induction of osteoclasts.⁵² Together with ours, these studies identify cell–ECM interactions as important not only in cell adhesion but also in the regulation of

bone resorption. We previously demonstrated that ligation of α 2 β 1 integrin with Coll I reduces TCR- and doxorubicin-dependent expression of RANKL in Jurkat T cells.³⁰ We have also shown that α 2 β 1 integrin enhances the production of TCR-dependent production of IFN- γ ²³ and, similar to Coll IV, we found that anti-CD3 + Coll I-activated effector T cells also do not induce the formation of osteoclasts (data not shown). However, although Coll I enhanced IL-7-dependent RANKL expression in human effector CD4⁺ T cells, it did not enhance their ability to induce the development of osteoclasts (data not shown). The mechanisms underlying the effect of Coll I in the context of IL-7 co-stimulation are currently unknown but are not due to the up-regulation of IFN- γ . Additional experiments are required to determine how Coll I modulates T-cell-mediated osteoclastogenesis. Although α 2 β 1 integrin is expressed along with α 1 β 1 integrin on effector/memory T cells, its blockade had only a minor effect on the development of arthritis compared with the blockade of α 1 β 1 integrin.²⁷ Thus, these studies suggest that α 1 β 1 integrin may be more important than α 2 β 1 integrin in the regulation of T-cell-dependent bone loss.

Both Coll IV and IL-7 are present in the bone tissue,^{48,53} and IL-7 has previously been shown to increase the adhesion of T cells to ECM proteins including Coll IV.⁵⁴ Thus, during bone resorption, IL-7 can increase the adhesion of effector T cells to Coll IV, which will result in their retention in the bone tissue and to the synergistic production of RANKL. Subsequently, this will lead to the development of osteoclasts either from monocytes recruited from the periphery or from myeloid cells of the bone marrow, which will ultimately lead to bone erosion.

In addition to the up-regulation of RANKL, our results also show that α 1 β 1 integrin enhances the pro-survival function of IL-7. While Coll IV had no effect on IL-2-withdrawal-induced apoptosis of effector CD4⁺ T cells, it did enhance the protective effect of IL-7. This form of apoptosis occurs during the contraction phase of the immune response and protection from it has been suggested to be a critical step in the development of memory T cells as well as in the persistence of activated T cells during chronic inflammation.^{40,41} Our results may help to explain why synovial T cells, which were shown to be resistant to apoptosis, manage to survive in the inflamed synovium, where only minor amounts of IL-2 were detected.^{55,56} The role of IL-7 in T-cell survival and in the development of T-cell memory has been well studied.^{12,43,44} However, recent studies indicate that IL-7R alone is not sufficient for the efficient survival of effector T cells and their differentiation towards memory T cells.^{35,57} In contrast, α 1 β 1 integrin has been implicated in the protection of effector T cells from apoptosis during influenza infection.⁵⁸ The results of the present study suggest that it is highly possible that the functional interaction between these two receptors may be

the key for long-term survival of effector/memory T cells. Thus, our results suggest that, during chronic inflammation associated with bone loss, IL-7R and $\alpha 1\beta 1$ integrin signalling pathways can rescue effector/memory T cells and promote bone resorption through the up-regulation of RANKL expression.

Our findings strongly suggest that $\beta 1$ integrins can also provide signals that are co-stimulatory to cytokine signalling. A recent study showed that $\alpha 1\beta 1$ integrin synergizes with the TNF II receptor in protecting effector CD8⁺ T cells from Fas-induced apoptosis.²¹ Thus, in addition to TCR signalling, integrins can be important modulators of cytokine signalling in T cells.

In summary, we have identified a new signalling pathway composed of $\alpha 1\beta 1$ integrin and IL-7R that contributes to T-cell survival and to T-cell-mediated osteoclastogenesis. Further investigation of the signalling mechanisms underlying the effects of IL-7 + Coll IV is likely to provide new insights into how T cells contribute to the T-cell-mediated bone erosion seen in autoimmune diseases.

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