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Activated T Lymphocytes Support Osteoclast Formation *in Vitro*

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Osteoblastic stromal cells are capable of supporting osteoclast formation from hematopoietic precursors in the presence of osteotropic factors such as $1\alpha,25(OH)_2D_3$, PTH, and IL-11. Osteoblastic stromal cells produce receptor activator of NF-κB ligand (RANKL), a type II membrane protein of the TNF ligand family, in response to these agents. Activated T lymphocytes also produce RANKL; however, the ability of this cell type to support osteoclast formation *in vitro* is unknown. Human PBMC-derived T cells, extracted using αCD3-coated magnetic beads, were cocultured with adherent murine spleen cells in the presence of Con A and a panel of cytokines. In the presence of Con A, *bona fide* osteoclasts were formed *in vitro* with activated T cells: IL-1α and TGFβ further enhanced osteoclast numbers. PBMC-derived lymphocytes showed an increase in the mRNA expression of RANKL within 24 h of treatment with the same agents that were used to induce osteoclast formation. In synovial tissue sections with lymphoid infiltrates from RA patients, the expression of RANKL was demonstrated in $CD3^+$ T cells. The ability of activated T lymphocytes to support osteoclast formation may provide a mechanism for the potentiation of osteoclast formation and bone resorption in disease states such as rheumatoid arthritis.

The maintenance of skeletal integrity requires a strict balance between the anabolic and resorptive actions of osteoblasts and osteoclasts, respectively. Osteoclasts are large multinucleated cells derived from the monocyte/macrophage lineage that are responsible for the resorption of bone. Osteoclast differentiation is supported by osteoblasts that express M-CSF and RANKL. The presence of macrophage-colony stimulating factor (M-CSF) is necessary but not sufficient to support osteoclast formation from hemopoietic precursors alone (1). Recently, a novel membrane-bound member of the TNF-ligand family responsible for osteoclast differentiation has been identified and termed osteoclast differentiation factor (ODF: 2). This factor is also known as receptor activator of NF-κB ligand (RANKL), TNF-related activation-induced cytokine (TRANCE) or osteoprotegerin ligand (OPGL) (2). We will subsequently refer to it as RANKL as it describes the relation of this molecule to its signaling receptor and its post-receptor signaling actions, and does not imply tissue specificity. Both mouse spleen cells and human peripheral blood mononuclear cells were able to differentiate into mature, bone resorbing osteoclasts in the presence of soluble RANKL and M-CSF without osteoblastic stromal cells (1, 2). Furthermore, several bone-resorbing factors were shown to promote the expression of RANKL mRNA by osteoblasts (3). The cognate signaling receptor, receptor activator of NF-κB (RANK), for RANKL has been identified (2) and determined to be the signaling receptor for osteoclastogenesis mediated by RANKL (4); while a decoy receptor, osteoprotegerin (OPG), is secreted by osteoblastic cells and limits the biological activity of RANKL (5).

Cytokine-mediated bone loss is a major feature of rheumatoid arthritis (RA) and consists of three types: generalized osteoporosis, periarticular osteopenia and erosion of the marginal and sub-chondral bone (6). The importance of T cells in the pathogenesis of RA has been controversial. Although T lymphocytes are abundant in the synovium, T lymphocyte-associated cytokines such as IFN-γ are found at much lower levels compared to IL-1, IL-8 and TNFα in arthritic joints (7). Nevertheless there is compelling evidence for the selective accumulation of $CD4^+$ T cells in the joints, and for T cell-dependent production of cytokines and metalloproteinases in the SCID mouse/human synovium chimera (8). Since stimulated $CD4^+$ T cells produce RANKL (2), we investigated the possibility that T cells can directly support osteoclast formation.

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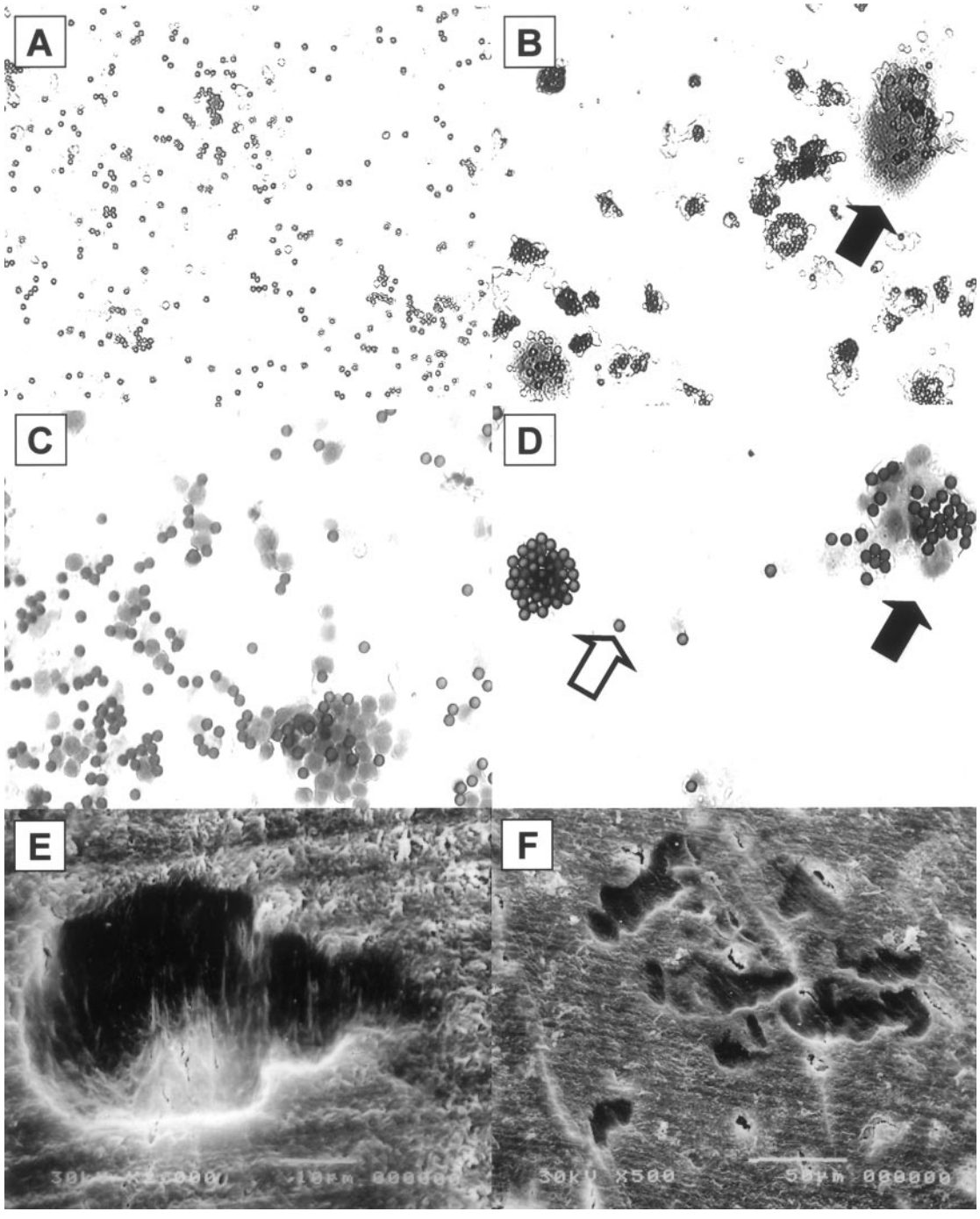

FIG. 1. Identification of osteoclasts in human T cell/murine spleen cell cocultures. (A) Negative control. TRAP staining in the absence of any treatment. (B) $TRAP^+$ staining of multinucleated cells in the presence of Con A (1 μg/ml), IL-1 (1 ng/ml) and TGFβ (10 ng/ml). A $TRAP^+$ MNC is indicated by the black arrow. (C) Negative control. Calcitonin receptor immunohistochemistry in the absence of any treatment. (D) Calcitonin receptor immunohistochemistry of multinucleated cells in the presence of Con A, IL-1, and TGFβ. A calcitonin receptor expressing MNC is indicated by the black arrow, while the open arrow indicates an example of the spherical Dynabeads scattered throughout the culture. (E) Resorption lacunae electronmicroscopy. Bar, 10 μm. (F) Resorption lacunae electronmicroscopy. Bar, 50 μm. Original magnification: (A, B) $\times$125, (C, D) $\times$225.

MATERIALS AND METHODS

Animals, cell lines, and drugs. Newborn (0–1 day old) C57/Bl6J mice were purchased from Monash University Animal Services Centre (Clayton, Australia). Recombinant human macrophage-colony stimulating factor (M-CSF) was kindly provided by Genetics Institute (Cambridge, MA) and concanavalin A (Con A) was purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant human

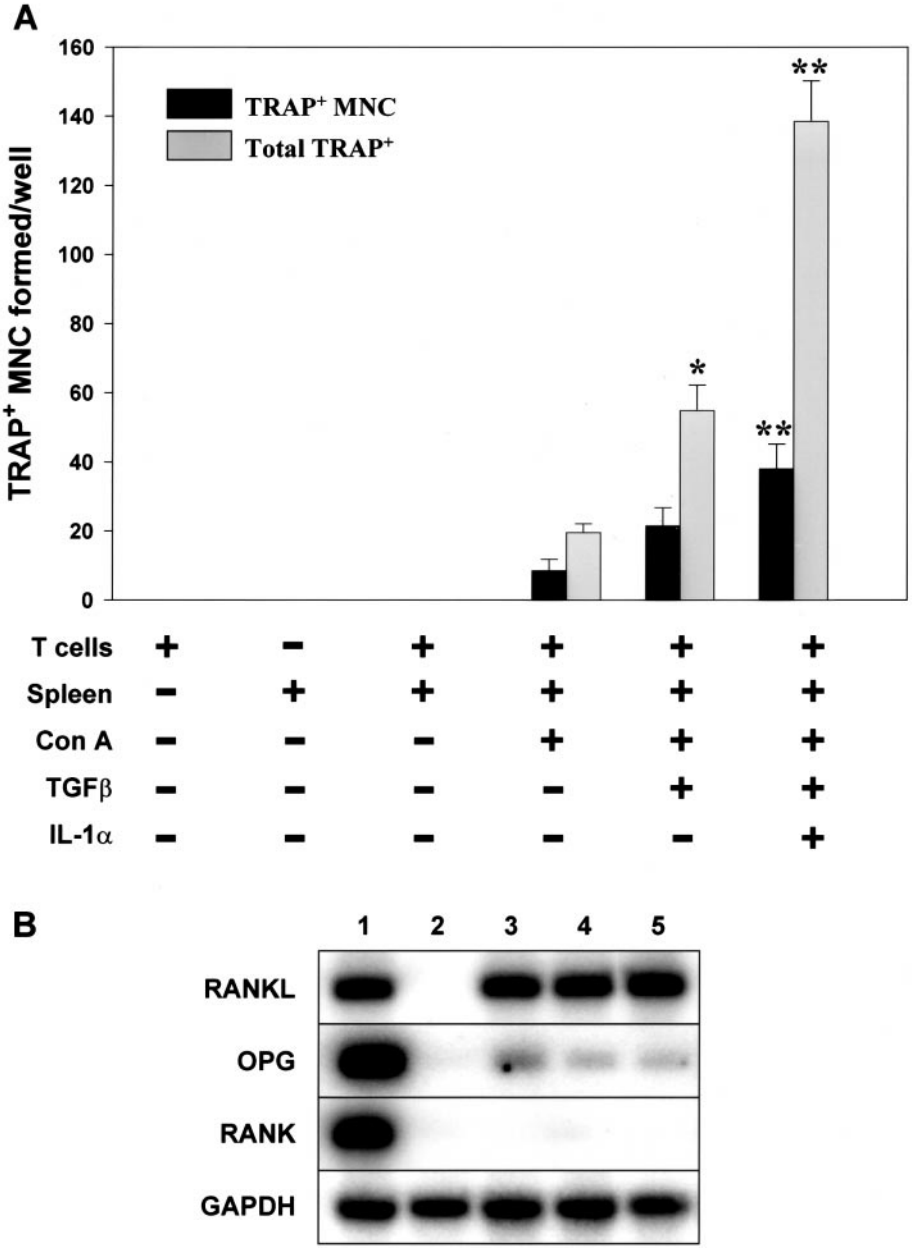

FIG. 2. (A) TRAP$^+$ staining for multinucleated osteoclasts formed in T cell/murine spleen cell cocultures. Negative controls for the cultures were performed in the absence of Con A and cytokines except for 25 ng/ml M-CSF and 1 ng/ml IL-2, with spleen cells or T cells alone, or the two cell types in combination. Treatment of T cells with Con A alone or in combination with TGFβ or IL-1 and TGFβ was able to support TRAP$^+$ cell formation from precursors of splenic origin. These data are representative of 10 separate experiments with very similar results and is the mean $\pm$ SEM of quadruplicate wells. (B) Semiquantitative RT-PCR analysis of RANKL, OPG, RANK, and GAPDH mRNA. A human giant cell tumor of bone RT was used as a positive control for each mRNA species (lane 1). PBMC-derived T cells were treated with IL-2 (lane 2), treated for 24 h with αCD3 Ab, IL-2, and Con A (lane 3), treated for 24 h with αCD3 Ab, IL-2, Con A and TGFβ (lane 4), or treated for 24 h with αCD3 Ab, IL-2, Con A, IL-1, and TGFβ (lane 5). This result is representative of three independent RT-PCR experiments of PBMC-derived T cells isolated from three volunteers.

cytokines were purchased from R & D Systems (Minneapolis, MN) unless otherwise stated.

Patients. Blood (30 ml) was obtained from 17 healthy donors (11 female, 6 male). Synovial tissue was obtained from the knee joints of 15 patients (age range 51–75) with RA, as defined by the American College of Rheumatology revised criteria, during synovectomy or joint replacement surgery. Tissue sections of RA synovium were stained with hematoxylin and eosin, and samples with lymphoid aggregates were used in these studies.

T cell preparation. PBMC were isolated by Ficoll-Paque (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) discontinuous gradient centrifugation essentially as described (1). PBMC were then rinsed in PBS and resuspended in RPMI 1640 containing 10% FBS. T cells were obtained from the PBMC incubation with washed Dyna-

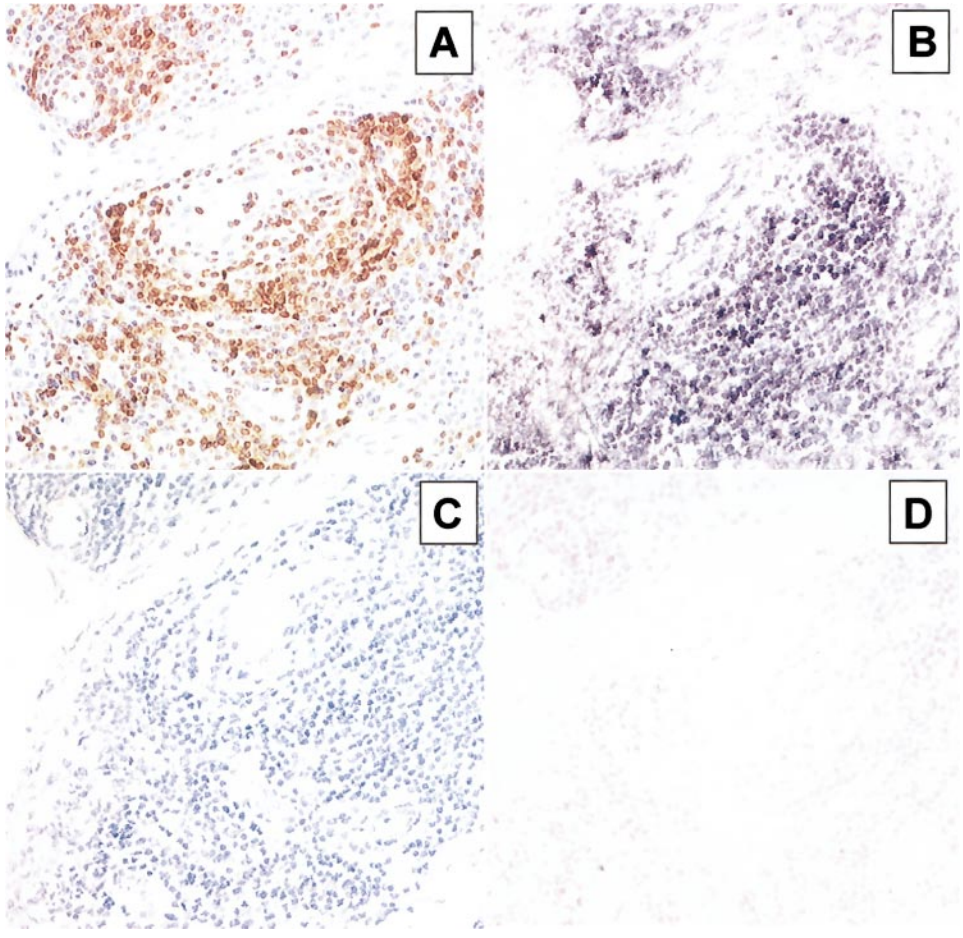

FIG. 3. Localization of RANKL in tissue sections from a synovectomy of the right elbow joint of a patient with rheumatoid arthritis and its relation to the T cell receptor antigen, CD3. (A) Immunohistochemical detection of CD3 in lymphoid aggregates. (B) *In situ* hybridization for RANKL mRNA expression showed strong signal in the lymphoid aggregates. (C) Immunohistochemistry non-immune negative control. (D) *In situ* hybridization negative control using a sense strand probe. Original magnification, ×125.

beads M-450 CD3 (pan T) at a ratio of 5 beads per target cell. The $CD3^+$ T cell fraction was isolated according to the manufacturer's directions (Dynal, Oslo, Norway) resulting in a $CD3^+$ T lymphocyte population of >95% purity.

Functional assay for RANKL. Osteoclast formation was determined as previously reported (1, 9, 10). Spleen cells derived from newborn C57BL/6J mice were disaggregated through a wire sieve and resuspended in α-MEM containing 10% FBS and 25 ng/ml M-CSF and plated at 1×10^6 cells/well in 48 well plates. The spleen cells were settled overnight and washed to remove non-adherent cells before adding T cells at 1×10^5 cells/well. T cells and exogenous treatments [IL-1 (1 ng/ml), TGFβ (10 ng/ml), IL-2 (1 ng/ml), M-CSF (25 ng/ml) and Con A (1 μg/ml)] were replaced at every 3 days resulting in a total culture period of 10 days. Cultures were subjected to histochemical staining for TRAP, calcitonin receptor immunohistochemistry, and electronmicroscopy of bone slices at the conclusion of the coculture period as previously described (1).

Semiquantitative reverse transcription-PCR. The following oligonucleotides were used in RT-PCR experiments: RANKL-15 (5′-TGG ATC ACA GCA CAT CAG AGC AG-3′; 533–555), RANKL-16 (5′-TGG GGC TCA ATC TAT ATC TCG AAC-3′; 1065–1088) and RANKL-6 (5′-TCT AAC CAT GAG CCA TCC-3′; 569–586); OPG-4 (5′-GGG GAC CAC AAT GAA CAA GTT G-3′; 85–106), OPG-5 (5′-AGC TTG CAC CAC TCC AAA TCC-3′; 473–493) and OPG-6 (5′-GCT GTC TGT TGT GTA GTA GTG GTC AG-3′; 291–313); RANK-10 (5′-GGG AAA GCA CTC ACA GCT AAT TTG-3′; 753–776), RANK-11 (5′-GCA CTG GCT TAA ACT GTC ATT CTC C-3′; 1181–1205) and RANK-15 (5′-GCT GAC CAA AGT TTG CCG TG-3′; 854–873). The nucleotide sequence was derived from the following GenBank Accession No.: RANKL (AF019048), OPG (MMU94331) and RANK (AF019046). Glyceraldehyde-3-phosphate dehydrogenase primers were GAPDH-1, GAPDH-2 and GAPDH-4, and internal oligonucleotides (RANKL-6, OPG-6, RANK-15 and GAPDH-1) used for the hybridization studies were as previously described (3).

Tissue preparation. Synovial tissue samples were fixed by immersion in 4% paraformaldehyde in diethyl ester pyrocarbonic acid (DEPC)-treated phosphate buffered solution (PBS) pH 7.4 and maintained overnight at 4°C (11). Tissues were processed and embedded in paraffin under sterile conditions.

Immunohistochemistry. Sections were blocked for endogenous peroxidase followed by blocking with swine serum. Polyclonal CD3 (DAKO Corporation, CA, USA) was applied for 1 h followed by biotinylated secondary antibody and peroxidase conjugated streptavidin. Positive cells were detected with DAB staining and counterstained in Harris hematoxylin.

Riboprobe construction and in situ hybridization. RT-PCR was used to generate 750 bp of murine RANKL derived from mouse

calvarial osteoblasts using the oligonucleotides RANKL-1 (5′-ATC AGA AGA CAG CAC TCA CT-3′, nucleotides 381–400) and RANKL-2 (5′-ATC TAG GAC ATC CAT GCT AAT GTT C-3′, nucleotides 1106–1130). This fragment was purified using PCR Wizard column (Promega, Madison, WI) and cloned into pGEM-T (Promega). The plasmid was linearized with *Nde*I or *Sac*II and transcribed with T7 or SP6 RNA polymerase to generate antisense and sense riboprobes, respectively. These riboprobes were labeled with digoxigenin (DIG) during the transcription procedure using an RNA labeling kit (Boehringer Mannheim, Mannheim GmbH, Germany). *In situ* hybridization was performed as previously described (11).

RESULTS

Osteoclast formation in cocultures of murine spleen and normal human T cells. Since T cells have previously been shown to produce RANKL (2), the ability of T cells to act directly on hematopoietic precursor cells to promote osteoclast formation was investigated using adherent murine spleen cells cocultured with normal human PBMC-derived T cells. All cell combinations were treated with M-CSF (25 ng/ml) and IL-2 (1 ng/ml) to promote the survival of osteoclastic precursors and T cells, respectively. Murine spleen cells were used as a source of osteoclastic precursors in preference to human adherent mononuclear cells, because murine hemopoietic precursor cells differentiate at twice the rate of human monocytes, thereby reducing the duration of the coculture period (1). Further, the generation of osteoclasts in these coculture conditions from murine spleen cells resulted in virtually all $TRAP^+$ cells expressing CTR (1). As crosslinking of the T cell receptor/CD3 complex results in dramatic T cell apoptosis within 72 h through the production of TNFα and FasL (12), T cells were replaced every three days in the coculture. In the absence of any further exogenous treatment, no $TRAP^+$, CTR^+ multinucleated (MNC) cells (3 or more nuclei) were formed (Figs. 1A and 1C). Cytokines, such as IL-1α (1 ng/ml) or TGFβ1 (10 ng/ml), were unable to support $TRAP^+$ MNC formation when added individually to the coculture. However, in the presence of Con A (1 μg/ml) alone, a small number of $TRAP^+$ MNC were formed (8.5 $\pm$ 6.6/well: Fig. 2A). The addition of Con A in combination with IL-1 and TGFβ, resulted in a four fold increase in osteoclast formation (38 $\pm$ 14.4/well: Fig. 2A) as determined by histochemical staining for $TRAP^+$ MNC, the presence of calcitonin receptors and the presence of bone resorption pits (Figs. 1B, 1D, 1E, and 1F). Treatment of adherent spleen cells with Con A, IL-1α and TGFβ in the absence of T cells, or T cells with Con A, IL-1α and TGFβ in the absence of hemopoietic cells, did not result in the formation of any $TRAP^+$ MNC (data not shown). Interestingly, when purified T cells obtained from the synovial fluid of a patient exhibiting severe rheumatoid arthritis associated with marked T cell infiltration were cultured together with adherent mouse spleen cells, substantial $TRAP^+$ MNC were formed (data not shown). These cells did not require activation with αCD3 and were even able to form a limited number of $TRAP^+$ mononuclear cells in the absence of exogenous treatments such as IL-1α and TGFβ.

Stimulated T cells produce RANKL. RT-PCR analysis for RANKL, OPG and RANK was performed on PBMC-derived human T cells following 24 h treatment with agents determined to induce osteoclast formation: αCD3 Ab, IL-2 (1 ng/ml) and Con A (1 μg/ml), or αCD3 Ab, IL-2, Con A and TGF-β (10 ng/ml), or αCD3 Ab, IL-2, Con A, TGF-β and IL-1 (1 ng/ml). Total RNA from a human giant cell tumor of bone was used as a positive control for each RT-PCR. RANKL mRNA was undetectable in unstimulated T cells, whilst T cells stimulated with αCD3 Ab, Con A and IL-2 for 24 h demonstrated a high level of RANKL mRNA expression (Fig. 2B, lane 3). This steady state level of RANKL mRNA was not enhanced further by the addition of TGFβ (Fig. 2B, lane 4) or TGFβ and IL-1 (Fig. 2B, lane 5). The enhancement of osteoclast formation seen in response to the additional stimulus provided by TGFβ, or TGFβ and IL-1, may result from the action of these cytokines on osteoclastic precursors (manuscript in preparation). Alternatively, these additional cytokines may alter the profile of other factors secreted by the stimulated T cells. OPG mRNA levels were also slightly elevated in stimulated T cells, whilst the control failed to produce OPG mRNA (Fig. 2B). RANK mRNA was not detected in these cells (Fig. 2B).

Expression of RANKL in T cells in situ. Tissue sections with follicular lymphoid aggregates obtained from RA patients were used to confirm the expression of RANKL by T cells. Immunohistochemical detection of $CD3^+$ T cells was performed using αCD3 polyclonal antibody and was shown to localize to cells within the lymphoid aggregates (Figs. 3A and 3B). *In situ* hybridization was also performed on consecutive sections and a strong signal was obtained for RANKL mRNA in the $CD3^+$ cells (Figs. 3C and 3D). A small number of macrophage-like and fibroblast-like cells were also positive for RANKL mRNA expression in these sections.

DISCUSSION

The ability of osteoblastic stromal cells to support osteoclastogenesis from hemopoietic precursors is well established (10). In this report we have demonstrated that human PBMC-derived T cells were able to support osteoclast formation and activation from murine spleen cells. These cells were $TRAP^+$, expressed calcitonin receptors and were capable of resorbing bone (Fig. 1). Activation of the T cell receptor with αCD3 antibodies alone was insufficient to induce osteoclast formation from the murine spleen cells; however, the addition of Con A, a potent T cell mitogen, and cytokines such as IL-1 and TGFβ, provided the stimulus required to elicit osteoclast formation (Fig. 2A). As

previously reported, the addition of M-CSF was required for the process of osteoclastogenesis (1, 2). The RANKL −/− mice demonstrate the absolute requirement for RANKL in osteoclastogenesis (13), and in this *in vitro* assay system we have used the T cell as the only source of RANKL, therefore these cells must be mediating osteoclastogenesis.

Since IL-1 and TGFβ are abundantly expressed in RA, the effect of these cytokines in the *in vitro* coculture system was also assessed. IL-1 is a pivotal proinflammatory cytokine in RA and interestingly increases osteoclast formation and bone destruction both *in vivo* and *in vitro* (14, 15). TGFβ exhibits multipotential properties in RA where it acts as an immunoregulatory and anti-inflammatory agent inhibiting the production of proinflammatory cytokines such as IFNγ, yet it also possesses proinflammatory actions including the recruitment of leukocytes, macrophages and fibroblasts (7). The role of TGFβ in osteoclast development and bone homeostasis is also multifacetted, as transgenic TGFβ2 mice display progressive bone loss associated with increases in osteoblastic matrix deposition and osteoclastic bone resorption (16). In addition, TGFβ promotes the recruitment of osteoclast-like cells in giant cell tumor of bone, yet it has been recently shown to increase the mRNA expression of the osteoclastogenesis inhibitory factor, osteoprotegerin (17). Human PBMC-derived T cells, as used to promote $TRAP^+$ MNC formation, showed an increase in the expression of RANKL ten fold by 24 h post treatment with αCD3 and Con A compared to the control (Fig. 2B): no further increase in RANKL mRNA levels were observed with the addition of IL-1α and TGFβ. The role of these additional factors in the promotion of osteoclastogenesis in this coculture system results from their action on hematopoietic cells (manuscript in preparation). In agreement with the increase in the RANKL:OPG ratio in osteoblasts stimulated with osteotropic agents, such as PTH and IL-11 (3), the final RANKL:OPG mRNA ratio in treated T cells was 6:1. Both RANKL and OPG were undetectable in the control T lymphocytes. The expression of RANKL mRNA in infiltrating T cells in RA synovial tissue sections was confirmed *in situ* hybridization. RANKL expression by T cells *in vivo* is consistent with a role in periarticular bone loss associated with RA.

The involvement of T cells in normal bone physiology would appear to be minimal as both the nude and SCID mice, which have depleted T cell components, show no skeletal abnormalities. However, the ability of T cells to influence osteoclastogenesis has been reported by several groups. For instance, we have reported that T cells are a crucial component in the inhibition of osteoclast formation following interleukin-18 treatment *in vitro* (9), and by virtue of their IL-17 production may further influence bone destruction in RA (18). These data, together with our present study, suggest that the presence of a critical number of activated T cells may result in bone destruction. Stimulated T cells were able to support osteoclast formation, whereas resting T cells do not; however, the high rate of cell death necessitates a continuous supply of T cells. This can clearly be accommodated within a chronic disease state such as RA where, in some patients, there is T cell infiltration of the synovium and marked cytokine production.

The ability of T cells to support the generation of *bona fide* osteoclasts *in vitro* has been demonstrated for the first time in this series of experiments. Kong *et al.* (13) recently noted that RANKL-deficient mice exhibit severe osteoporosis, abnormal tooth eruption and defects in both T and B lymphocytes and suggested that T cells might play a role in osteoclastogenesis in disease states through the production of RANKL. The role of other cell types in rheumatoid arthritis, such as macrophages and fibroblasts, in relation to the production of RANK, RANKL and OPG remains to be elucidated. Our present study shows that appropriately stimulated T cells promote osteoclast formation and thus may directly contribute to the bone destruction observed in disease states such as RA.

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