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Title: Activated T-lymphocytes suppress osteoclastogenesis by diverting early monocyte/macrophage progenitor lineage commitment towards dendritic cell differentiation through downregulation of RANK and c-Fos

Short title: Activated T-lymphocytes suppress osteoclastogenesis and support dendritic cell differentiation

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Abbreviations used in this paper: AP-1, activator protein-1; BM, bone-marrow; ConA, concanavalin A; CsA, cyclosporin A; CtR, calcitonin receptor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; mAbs, monoclonal antibodies; M-CSF, monocyte-macrophage colony-stimulating factor; MLR, mixed lymphocyte reaction; NFAT, nuclear factor of activated T cells; OCL, osteoclast-like; OPG, osteoprotegerin; PI, propidium iodide; qPCR, quantitative PCR; RANK, receptor activator of NF-kappaB; RANKL, receptor-activator of NF-kappaB ligand; rm, recombinant mouse; TLR, Toll-like receptor; TRAIL, TNF-related apoptosis-inducing ligand; TRAP, tartrate-resistant acid phosphatase.

Abstract

Activated T-lymphocytes either stimulate or inhibit osteoclastogenesis from hematopoietic progenitors in different experimental models. To address this controversy, we used several modes of T-lymphocyte activation in osteoclast differentiation – mitogen-pulse, anti-CD3/CD28 stimulation, and *in vivo* and *in vitro* alloactivation. Osteoclast-like cells were generated from non-adherent immature hematopoietic monocyte/macrophage progenitors in murine bone-marrow in the presence of receptor activator of NF-κB ligand (RANKL) and monocyte-macrophage colony-stimulating factor (M-CSF). All modes of *in vivo* and *in vitro* T-lymphocyte activation and both CD4- and CD8-subpopulation produced similar inhibitory effects on osteoclastogenesis paralleled by enhanced dendritic cell differentiation. Osteoclast-inhibitory effect was associated with T-lymphocyte activation and not proliferation, and could be replaced by their culture supernatants. The stage of osteoclast differentiation was crucial for the inhibitory action of activated T-lymphocyte on osteoclastogenesis, since the suppressive effect was visible only on early osteoclast progenitors but not on committed osteoclasts. Inhibition was specifically associated with increased granulocyte-macrophage colony-stimulating factor (GM-CSF) expression by the mechanism of progenitor commitment toward lineages other than osteoclast because activated T-lymphocytes downregulated RANK, CD115, c-Fos and calcitonin receptor expression, and increased differentiation towards CD11c-positive dendritic cells. Activated T-lymphocyte inhibitory role in osteoclastogenesis, confirmed *in vitro* and *in vivo*, mediated through GM-CSF release, may be used to counteract activated bone resorption mediated by T-lymphocyte-derived cytokines in inflammatory and immune-disorders. We also demonstrated the importance of alloactivation in osteoclast

differentiation and the ability of cyclosporine A to abrogate T-lymphocyte inhibition of osteoclastogenesis, thereby confirming the functional link between alloreaction and bone metabolism.

Introduction

T-lymphocytes play an important role in the regulation of bone metabolism, particularly the bone resorption by osteoclasts [1-4]. However, the reports on activated T-lymphocyte effects on osteoclastogenesis are still controversial. Activation of T-lymphocytes in different experimental models or human diseases has mostly been shown to stimulate osteoclast differentiation and bone resorption by production of cytokines such as receptor activator of NF-κB ligand (RANKL), interleukin (IL)-6, IL-7, IL-17 and tumor necrosis factor (TNF)-α [2,5-9]. On the other hand, T-lymphocytes secrete interferon (IFN)-γ, IL-4, IL-10, IL-13, and granulocyte-macrophage colony-stimulating factor (GM-CSF), which mediate the inhibition of osteoclastogenesis from hematopoietic progenitors [2,5,10-14]. Although some of those inhibitory cytokines have been associated with T-lymphocyte activation, various *in vitro* modes of T-lymphocyte activation differentially affect osteoclastogenesis [2,8,10,12], and most *in vivo* models report the enhanced osteoclast formation upon T-lymphocyte activation [3,6,15,16].

T-lymphocytes have a role not only in osteoclast activation and bone resorption but also in modulation of multipotent monocyte/macrophage progenitor differentiation towards either bone or immune lineage, which is determined by ligand binding to cell-surface receptors, particularly receptor activator of NF-κB (RANK) for osteoclasts and Toll-like receptors (TLRs) for mononuclear phagocytes [17]. The interaction of RANK, expressed on osteoclast progenitors, with its ligand RANKL is crucial for osteoclastogenesis in the presence of monocyte-macrophage colony-stimulating factor (M-CSF) [6,7,18-20]. At the level of cell signaling, both RANK and TLRs activate the dimeric transcription factors NF-κB and activator protein (AP)-1. Transcription factor

c-Fos, a component of AP-1, plays a positive role in osteoclast but a negative role in macrophage and dendritic cell differentiation [17,21,22]. GM-CSF controls the bifurcation between osteoclasts and dendritic cells from common progenitors by regulating c-Fos expression [22,23].

To study the controversial effect of activated T-lymphocytes on osteoclastogenesis we used several activation protocols, including *in vivo* and *in vitro* alloactivation. Activation by any mode suppressed osteoclastogenesis stimulated by RANKL and M-CSF in murine hematopoietic progenitors. To elucidate the mechanism by which *in vitro* and *in vivo* activated T-lymphocytes suppress the differentiation towards the osteoclast lineage from bone-marrow (BM) hematopoietic progenitors we identified the stage at which the suppressive effect was achieved and the expression profile of the treated progenitors. In contrast to some other studies, our findings clearly show that activated T-lymphocytes have a net inhibitory effect on osteoclast differentiation by diverting early hematopoietic progenitors towards dendritic cell differentiation through downregulation of RANK and c-Fos.

Materials and methods

Subjects and Samples

Twelve-week old C57BL/6J (H-2^b) female mice were used in all experiments. Allogeneic CBA/J (H-2^k) mice was used for allostimulation. Maintenance of animals and all experimental procedures were approved by the Ethics Committee of the Zagreb University School of Medicine.

T-lymphocyte preparation

Murine lymph node cells were depleted of non-T-lymphocyte populations using anti-CD11b/anti-CD45R monoclonal antibodies (mAbs) (BD Pharmingen, San Jose, CA) and further separated by anti-CD4/anti-CD8 mAbs conjugated to magnetic beads (Dynel Biotech, Wirral, UK). Separated T-lymphocyte populations had the purity of >90% as confirmed by flow-cytometry.

Immunosuppressant pretreatment consisted of 1 hour-incubation at 37°C [24] with various concentrations (2.5 to 40 μg/mL) of cyclosporine A (CsA) (Sigma-Aldrich, Saint Louis, MO).

For activation, T-lymphocytes were pulsed during 15 minutes by concanavalin A (Con A) (Sigma-Aldrich), washed, and cultured for 24 hours. In some experiments T-lymphocytes were activated by a 24 hour-incubation with anti-CD3 (1 μg/mL; Caltag, Burlingame, CA) and anti-CD28 mAbs (0.5 μg/mL; BD Pharmingen). To inhibit proliferation, T-lymphocytes were treated with mitomycin C (Sigma-Aldrich) (20 min at 37°C) after Con A-pulse and 24 hour-culture. T-lymphocyte activation was confirmed by the expression of CD71, and changes in the cell-cycle using flow-

cytometry [25]. Proliferation was assessed by the colorimetric MTT (Sigma-Aldrich) assay. Activated T-lymphocytes were co-cultured with BM cells or cultured alone to obtain conditioned medium needed to treat osteoclastogenic cultures (see the paragraph *Osteoclast-like cell cultures*).

In vivo and in vitro alloactivation

For mixed lymphocyte reaction (MLR), T-lymphocytes were co-cultured with allogeneic mitomycin C-treated splenocytes (cell ratio 1:1) for 48 hours [24]. T-lymphocyte activation and proliferation were confirmed as described for mitogen pulse. Conditioned medium of *in vitro* alloactivated T-lymphocytes was used to treat osteoclastogenic cultures.

C57BL/6J mice were allostimulated *in vivo* by i.p. injection of allogeneic splenocytes (5×10^7/mouse) followed by foot-pad restimulation with the same cells (10^7/foot), 10 days after the first injection [25]. Untreated mice and mice treated with syngeneic splenocytes were used as controls. Allostimulated mice provided tibial BM for osteoclast cultures and regionally alloactivated (popliteal) lymph node for T-lymphocyte cultures. *In vivo* alloactivated T-lymphocytes were co-cultured with BM cells or additionally cultured for 24 hours to obtain conditioned medium needed to treat osteoclastogenic cultures.

Osteoclast-like cell cultures

For osteclast-like (OCL) cell generation, BM were cultured overnight (day -1) with 5 ng/mL rmM-CSF (R&D Systems, Abingdon, UK) in α-MEM/10% FCS (Sigma-Aldrich) to stimulate monocyte/macrophage lineage [25], followed by harvesting of

non-adherent cells as enriched hematopoietic monocyte/macrophage progenitors that are not yet committed towards a certain lineage of differentiation. Non-adherent BM cells (2×10^5/well) were plated (day 0) in 48-well plates with rmRANKL (a gift from Amgen, Thousand Oaks, Ca) and rmM-CSF (10 ng/mL for both) to stimulate osteoclast formation (termed *osteoclastogenic* cultures), with medium exchange at day 2.5. Different numbers of T-lymphocytes (1.2 to 5×10^5/well) or different volumes of T-lymphocyte conditioned medium (12.5 to 100%/well) were used to treat BM cultures at day 0 or 2.5. In some experiments rmGM-CSF (5 or 10 ng/mL; R&D), neutralizing anti-GM-CSF or anti- IFN-γ mAbs(1 μg/mL for both; BD Pharmingen) were added to osteoclastogenic cultures together with RANKL and M-CSF. At day 5, tartrate-resistant acid phosphatase (TRAP) positive multinucleated cells with ≥ three nuclei/cell were considered OCL and counted per well using light microscopy. Differentiated OCL cells highly expressed calcitonin receptor (CtR). There were no $TRAP^+$ OCL cells in cultures without addition of RANKL and M-CSF.

Flow-cytometry

Non-adherent BM cell differentiation in osteoclastogenic cultures (after 2.5 day-culture) was assessed by: PE-anti-CD115 (c-Fms), PE-anti-CD116 (GM-CSF receptor α) (eBioscience, San Diego, CA), FITC-anti-CD11b, APC-anti-CD11c (Caltag), and the combination of goat-anti-RANK (R&D Systems) with PE-anti-goat Abs. For intracellular cytokine staining, T-lymphocyte were treated with Brefeldin A (eBioscience), fixed/permeabilized, and stained with the combination of rabbit-anti-GM-CSF (eBioscience) and PECy5.5-anti-rabbit Abs. Apoptotic and dead cells were detected by Annexin V/propidium iodide (PI) staining (BD Pharmingen) according to

the manufacturer's recommendation. Results, using FACSCalibur (BD Pharmingen), were presented as histograms or dot-plots for 20,000 viable cells/sample. Dead and fragmented cells were excluded from the analysis by their properties on correlated forward/side scatter, and PI staining. Applied gates and quadrants were adjusted to non-stained cells and isotype-controls by delineating negative populations to approximately 10^1 fluorescence intensity (not shown).

Gene expression analysis

RNA was extracted using a commercial kit (TriPure; Roche) from harvested T-lymphocytes (freshly isolated or cultured) or osteoclastogenic cultures (2.5 or 5 day-culture). Each sample was obtained from ≥ three animals/group. RNA (1 μg) was converted to cDNA and amplified (20 ng/well) by quantitative (q)PCR, using specific amplimer sets designed by PrimerExpress software (Applied Biosystems, Foster City, CA) for mouse RANK, RANKL, osteoprotegerin (OPG), CD115, TNF-related apoptosis-inducing ligand (TRAIL), and β-actin with SYBRGreen chemistry (Applied Biosystems). Expression of c-Fos, GM-CSF, IFN-γ, CD178 (FasL), CtR and CD116 was analyzed using commercially available TaqMan assays (Applied Biosystems). qPCR reactions (25 μL/well) were conducted in an ABI Prism 7000 Sequence Detection System (Applied Biosystems), in quadruplicate, as described previously [26]. According to the standard curve, the relative amounts of RNA for target genes were calculated as the ratio of the quantity of target gene normalized to β-actin. RNA quantity for control sample in each experiment was set as 1 and the relative RNA quantities for other samples were calculated in accordance to this value.

Statistics

Experiments were performed at least three times and the representative data were shown (mean ± SD of four replicates per sample). Statistical analysis of $TRAP^+$ OCL cell number was performed using ANOVA and Student-Newman-Keuls post-hock test (MedCalc, Mariakerke, Belgium). Relative values of RNA quantity were statistically analysed using the comparison of the means t-test with Bonferroni correction for multiple-group comparison (MedCalc). For all experimemts, α-level was set at 0.05.

Results

Activated T-lymphocytes inhibit murine osteoclast differentiation

T-lymphocyte were activated by a brief mitogen pulse [27] in order to avoid the presence of Con A in the culture supernatants. Activation of T-lymphocytes consistently inhibited *in vitro* osteoclast differentiation in cultures of murine non-adherent BM cells supplemented with RANKL and M-CSF (Figure 1A). This was true both for co-cultures of activated T-lymphocytes and osteoclast progenitors and for cultures where osteoclast progenitors were treated with the supernatants of activated T-lymphocyte cultures (Figure 1B). The inhibitory effect was dose-dependent, i.e. reciprocal to the number of activated T-lymphocytes or the volume of activated T-lymphocyte conditioned medium. In further experiments, we used 0.25×10^6 T-lymphocytes per well or 50% conditioned medium per well, unless stated otherwise.

Furthermore, we added activated T-lymphocytes or conditioned medium at different time points of osteoclastogenic cultures to test if the stage of osteoclast differentiation was important for the inhibitory effect of activated T-lymphocytes. Only immature monocyte/macrophage progenitors (treatment 0-2.5 days) were sensitive to the inhibitory effect of activated T-lymphocytes, whereas committed osteoclast progenitors (treatment 2.5-5 days) did not respond to activated T-lymphocytes or conditioned medium (Figure 1C). In further experiments, the treatment by activated T-lymphocytes or conditioned medium was performed only for the first 2.5 days of osteoclastogenic culture.

To rule out that the effect of activated T-lymphocytes was a consequence of intensive T-lymphocyte proliferation and media exhaustion upon co-culture with BM cells, we

added mitomycin C-treated activated T-lymphocytes to cultures and found a similar inhibitory effect on osteoclastogenesis (Figure 1D). Inhibition of osteoclastogenesis was not specifically associated with mitogen stimulation, since osteoclastogenesis was blocked in BM cultures treated with the supernatant of T-lymphocytes activated by anti-CD28/CD3 Abs (Figure 1D). Interestingly, both $CD4^+$ and $CD8^+$ T-lymphocyte subpopulations were equally effective in the suppression of osteoclastogenesis, similar to unseparated T-lymphocytes (Figure 1E).

Inhibition of osteoclastogenesis is achieved by changes in cytokine gene expression by T-lymphocytes

As assessed by qPCR, activation of T-lymphocytes significantly increased the expression of several cytokines known to inhibit osteoclastogenesis such as GM-CSF, IFN-γ and OPG, with the parallel overexpression of the osteoclastogenic factor RANKL (Figure 2). OPG functions as a decoy receptor for RANKL and competes with RANK/RANKL binding [28]. Since we found the proportional increase in both RANKL and OPG expression upon T-lymphocyte activation (Figure 2), we do not believe that OPG is the important anti-osteoclastogenic factor in our model, especially because RANKL is exogenously added to the osteoclastogenic cultures and would override the inhibitory effect of OPG.

Treatment of BM cultures with GM-CSF produced a complete inhibition of osteoclastogenesis despite the presence of RANKL and M-CSF in the culture medium (Figure 3A). The addition of neutralizing anti-GM-CSF Abs in cultures treated with activated T-lymphocyte conditioned medium abrogated their inhibitory effect on osteoclastogenesis and restored the number of $TRAP^+$ OCL cells to approximately 90%

of the control cultures (Figure 3B) (ranging from 50 to 90% in the repeated experiments), whereas the addition of anti-IFN-γ Abs did not produce any changes in the OCL cell number (data not shown). Therefore we concluded that GM-CSF's osteoclast-inhibitory effect prevailed over the IFN-γ in our *in vitro* conditions. Intracellular cytokine staining confirmed an increased GM-CSF (Figure 3C) production by activated T-lymphocytes. Nevertheless, increased GM-CSF by T-lymphocytes was not paralleled by increased CD116 expression in osteoclastogenic cultures (data not shown).

The inhibitory effect was achieved by the significant GM-CSF overexpression in two major T-lymphocyte subpopulations $CD4^+$ and $CD8^+$ (RNA relative quantity 0.7 ± 0.1 in unstimulated and 3.8 ± 0.7 in activated $CD4^+$; 0.9 ± 0.2 in unstimulated and 3.4 ± 0.5 in activated $CD8^+$; compared with 1.0 ± 0.1 in unstimulated and 4.6 ± 0.3 in activated unseparated T-lymphocytes; $p < 0.01$, t-test with Bonferroni correction for multiple-group comparison), and both subpopulation have been previously shown to produce GM-CSF upon activation [29]. GM-CSF expression was specifically associated with T-lymphocytes, since we found increased GM-CSF RNA only in co-cultures of activated T-lymphocytes and non-adherent BM cells and not in non-adherent BM cell cultures treated with activated T-lymphocyte conditioned medium (data not shown).

Osteoclast precursors treated with mitogen activated T-lymphocytes or culture supernatants express lower level of RANK, CD115 and calcitonin receptor

We further assessed the expression of RANK and CD115, receptors for essential osteoclastogenic factors RANKL and M-CSF respectively [30], and found that the RNA for RANK and CD115 were significantly lower in 2.5 day-osteoclatogenic cultures

treated with activated T-lymphocytes or conditioned medium compared with control cultures (Figure 4). At day 5, the decrease in gene expression was more pronounced for RANK than for CD115, and was stronger in cultures treated with activated T-lymphocytes than with conditioned medium. Moreover, expression of c-Fos, transcription factor important for osteoclast lineage commitment [17], and CtR, marker of the differentiated osteoclast [21], was suppressed upon treatment with activated T-lymphocytes or conditioned medium (Figure 4). Flow-cytometry showed a downregulation of RANK and CD115 expression on day 2.5 after the addition of activated T-lymphocyte conditioned medium to osteoclastogenic cultures (Figure 5A).

Increased GM-CS expression mediates transdifferentiation between osteoclast and dendritic lineages

To test if activated T-lymphocytes could suppress osteoclast differentiation by GM-CSF-induced differentiation of common monocyte/macrophage progenitors away from the osteoclast towards the immune cell lineages, i.e. dendritic cells, we analyzed the expression of CD11c, a dendritic lineage marker [22], on non-adherent BM progenitors treated with activated T-lymphocyte conditioned medium. Flow-cytometry on day 2.5 revealed the presence of $CD11c^{high}$ population and parallel downregulation of RANK expression after the treatment with activated T-lymphocyte conditioned medium. Furthermore, we detected a decrease in macrophage $CD11b^{+}CD11c^{-}$ cell population, and an increase in dendritic $Cd11b^{-}CD11c^{+}$ and $Cd11b^{+}CD11c^{+}$ cell populations (Figure 5B).

Since T-lymphocytes may produce many pro-apoptotic molecules upon activation [31], we assessed the expression of TRAIL and CD178 in activated T-lymphocytes after 24

hour-culture but detected only a weak, insignificant upregulation by qPCR (data not shown). In addition, the apoptotic rate in osteoclastogenic cultures did not differ between control cultures and cultures treated with activated T-lymphocyte conditioned medium (19.5% Annexin V^+/PI^- cells in cultures treated with unstimulated vs. 18.8% in cultures treated with Con A-pulsed T-lymphocyte conditioned medium by flow-cytometry). Those results confirmed that progenitor commitment towards dendritic lineage and not the induction of osteoclast progenitor apoptosis was the major mechanism of osteoclast inhibition in our model.

Allogeneic stimulation of T-lymphocytes in vitro and in vivo produces similar effects on osteoclastogenesis as activation by mitogen or anti-CD28/CD3 Abs

In addition to mitogen and anti-CD3/CD28 stimulation, which has been commonly used to study the effects on osteoclastogenesis [6,8,12], we also performed T-lymphocyte allostimulation *in vivo* and *in vitro*. Conditioned medium of T-lymphocytes alloactivated in MLR during 48 hours produced factor(s) that decreased the number of $TRAP^+$ OCL cells in osteoclastogenic cultures stimulated with RANKL and M-CSF (Figures 6A and B). After *in vivo* allostimulation, we confirmed that the addition of *in vivo* alloactivated T-lymphocytes or conditioned medium to osteclastogenic cultures also suppressed osteoclastogenesis (Figure 6C). In addition, the number of $TRAP^+$ OCL cells differentiated from tibial non-adherent BM cells in osteoclastogenic cultures of *in vivo* allostimulated mice was significantly lower compared with cultures from control mice (Figure 6D). Pattern of RANK and CD115 expression was similar among experimental and control groups when allostimulation, anti-CD3/CD28 treatment or Con A-pulse were used for T-lymphocyte activation (see Figure 4). All modes of T-

lymphocyte activation were associated with an approximate 5-6 fold increase in GM-CSF expression (see Figure 2).

CsA abrogates inhibitory effect of T-lymphocytes on osteoclastogenesis

We further tested if immunosuppressants CsA, which inhibits T-lymphocyte activation, can overcome the suppression of osteoclastogenesis caused by Con A-pulsed T-lymphocytes. Since T-lymphocytes and osteoclasts share the sensitivity to many common cytokines [1,2,5] and suppressory signals, including CsA [32,33], we wanted to separate the effect of CsA on T-lymphocytes from the effect on osteoclast progenitors and, therefore, used CsA only for a brief pretreatment of T-lymphocytes and not for actual culture [24]. A brief 1 hour-preincubation of T-lymphocytes with different doses of CsA (2.5, 10, and 40 μg/mL) were performed prior to Con A-pulse and subsequent 24 hour-culture. CsA pretreatment of T-lymphocytes abrogated the suppression of osteoclastogenesis in a dose-dependent manner. The most pronounced reversal of inhibition of osteoclastogenesis was achieved when T-lymphocytes were first pretreated with the highest dose of CsA (40 μg/mL), pulsed with Con A, cultured for 24 hours and then added as a 50% conditioned medium to osteoclastogenic cultures (Figures 7A and B). The reversal of osteoclast inhibition was paralleled by changes in gene expression by Con A-pulsed T-lymphocytes, evidenced by an insignificant downregulation of RANKL ($p > 0.05$) and a significant downregulation of GM-CSF ($p = 0.0025$, ANOVA and Student-Newman-Keuls post-hock test) upon immunosuppressant pretreatment (Figure 7C).

Discussion

Our study demonstrated that activated T-lymphocytes suppressed hematopoietic cell differentiation towards the osteoclast lineage by affecting the lineage commitment of early monocyte/macrophage progenitors through the mechanism that involved RANK downregulation and c-Fos suppression. In addition to mitogen-pulse and anti-CD3/CD28 Abs, we used *in vitro* and *in vivo* T-lymphocyte alloactivation and showed for the first time that these methods also produce inhibition of osteoclastogenesis. The stage of osteoclast differentiation was crucial for the inhibitory action of activated T-lymphocyte on osteoclastogenesis, since the suppressive effect was visible only on early osteoclast progenitors when activated T-lymphocytes were added with RANKL and M-CSF at the beginning of the osteoclastogenic cultures but not on committed osteoclasts. The inhibitory effect was specifically associated with T-lymphocyte activation and not proliferation, and did not depend on cell-to-cell contact. The important mediator of osteoclast inhibition and dendritic lineage stimulation in our study was GM-CSF, known to regulate the bifurcation between osteoclast and dendritic cell lineages [22]. We found that activated T-lymphocytes upregulated GM-CSF expression and that the osteoclast-inhibitory effect of activated T-lymphocytes was abrogated by the addition of anti-GM-CSF Abs to osteoclatogenic cultures.

GM-CSF effect on osteoclastogenesis depends on the experimental model and the duration or timing of GM-CSF administration, and was differently reported in various animal models *in vivo* and osteoclast cultures *in vitro* [11-13,22,32,34-36]. In contrast to the study by Wyzga et al [12], who claimed that the mode of T-lymphocyte activation is crucial for the final effect on osteoclast differentiation, we confirmed that the stage of osteoclast precursors is more important by showing that GM-CSF in the presence of

RANKL at the beginning of the osteoclastogenic cultures inhibits osteoclast formation through cellular differentiation into dendritic cells, whereas is ineffective after the commitment of RANKL-induced differentiation towards osteoclasts. It has already been shown that GM-CSF treatment of early human osteoclast precursors blocks osteoclastogenesis [34], with a repression of major genes indicative of osteoclast functions [32], but those studies did not investigate activated T-lymphocytes as a possible source of GM-CSF in this regulation. Among other effects, GM-CSF inhibits c-Fos in osteoclast progenitors, which disrupts the RANK/c-Fos/nuclear factor of activated T cells (NFAT)c1 transcriptional cascade critical for osteoclastogenesis [17]. IFN-γ also suppresses osteoclastogenesis by disrupting RANKL-induced activation of NF-κB and JNK [1,5]. Nevertheless, increased IFN-γ expression by activated T-lymphocytes was not important in our model, since osteoclast inhibition was achieved at the level of decreased RANK expression (upstream of the described IFN-γ action) and could not be neutralized by anti-IFN-γ Abs. GM-CSF acts at earlier stages of osteoclast differentiation than IFN-γ [1,10,22], supporting our observation of the more important role of GM-CSF than IFN-γ in osteoclast inhibition.

In our experimental *in vitro* microenvironment, the treatment of early monocyte/macrophage BM progenitors with activated T-lymphocytes determined the differentiation towards dendritic cells away from the osteoclast lineage, despite the presence of RANKL and M-CSF in culture medium. A number of dendritic cell-like adherent clusters were observed in BM cultures treated with all types of activated T-lymphocytes or conditioned medium enriched in T-lymphocyte derived GM-CSF, similar to those observed after GM-CSF treatment. In addition, we detected a subpopulation of $CD11c^{high}$ cells characteristic for dendritic lineage, and a shift from

macrophage $CD11b^{+}CD11c^{-}$ towards dendritic $CD11b^{-}CD11c^{+}$ and $Cd11b^{+}CD11c^{+}$ populations in osteoclastogenic cultures treated with activated T-lymphocyte conditioned medium, which has not been found in control cultures. This supports a previous finding that GM-CSF stimulates osteoclast progenitor proliferation but inhibits differentiation, and in combination with RANKL promotes dendritic cell formation [22,23,34]. The mechanism underlying the differentiation switch between osteoclast and dendritic lineages certainly involves the regulation of transcription factors essential for osteoclast formation, including c-Fos [37]. c-Fos expression was downregulated in cultures treated by activated T-lymphocytes, showing that osteoclast differentiation is inhibited and dendritic cell differentiation reciprocally stimulated through the suppression of c-Fos, supporting our hypothesis that progenitor commitment toward lineages other than osteoclast is the underlying mechanism of osteoclast inhibition in our model.

By analyzing RANK and CD115 during *in vitro* differentiation [30], we found that the inhibitory effect of activated T-lymphocytes was more pronounced on RANK expression compared to CD115 expression. This suggests that downregulation of RANK signaling is the major mechanism of activated T-lymphocyte action, since RANK expression on hematopoietic progenitors is essential for osteoclast differentiation and activation [19-21]. CD115 gene was downregulated in cultures treated with activated T-lymphocytes at earlier stages of osteoclast differentiation, confirming that CD115 tyrosine kinase provides signals required for survival and proliferation of early osteoclast progenitors [21,30]. Studies using different osteoclast culture systems reported different findings regarding the expression of CD115, RANK, and CD116 in the presence of M-CSF, GM-CSF, or IL-3 [22,32,36,37]. In contrast to

showed upregulation of CD115 upon GM-CSF treatment of human mononuclear cell cultures [32], we detected a similar CD116 level irrespective of the treatment with activated T-lymphocytes, suggesting that the inhibition of osteoclastogenesis is not regulated by CD116 expression.

In addition to T-lymphocyte activation *in vitro* by mitogen-pulse or anti-CD3/CD28 Abs, we extended the importance of our findings by showing that T-lymphocytes alloactivated *in vitro* and *in vivo* also exerted a suppressive net-effect on osteoclastogenesis. These results have clinical significance, since bone homeostasis is often severely disturbed after organ transplantation that involves T-lymphocyte alloactivation [38,39]. Moreover, we confirmed that activated T-lymphocytes can suppress osteoclastogenesis in BM *in vivo* by the experiments where we used *in vivo* alloactivation by i.p. injection and regional foot-pad restimulation with allogeneic cells. When we cultured native non-adherent BM cells from these mice, osteoclastogenesis was significantly suppressed compared with cultures from unstimulated animals. These findings confirmed the complex function of cytokine expression patterns in the interplay between T-lymphocytes and osteoclast progenitors in BM environment despite a negligible presence of mature T-lymphocytes in BM [4,10].

The inhibitory effect of T-lymphocyte activation on osteoclastogenesis was abolished by T-lymphocyte pretreatment with immunosuppressant CsA. CsA action leads to the inhibition of NFAT and, hence, suppression of the transcription of IL-2 and other cytokine genes, including GM-CSF [24,33,40]. T-lymphocyte treatment with CsA prior to the Con A-pulse downregulated GM-CSF expression, which is consistent with the presence of NFAT response element in the enhancer part of GM-CSF transcription-control region [13,41]. In addition, we found only an insignificant decrease in RANKL

expression after CsA pretreatment, although a previous study reported that CsA potently blocked RANKL in T-cell hybridoma A1.1 [7]. Observed changes in the cytokine pattern produced by T-lymphocytes upon immunosuppressive treatment may contribute to the post-transplantation osteoporosis in patients receiving long term immunosuppressive treatment [38,39].

Although it is known that the immune system has powerful effects on bone resorption, and both immune cells resident in BM and immunocompetent cells in inflammatory tissue may interfere with bone metabolism, the exact nature of molecular effects still needs to be discovered before we fully understand the complexity of interactions between T-lymphocytes and osteoclasts. As a contribution to this aim, our study clearly demonstrated the inhibitory role of activated T-lymphocytes on osteoclastogenesis. The underlying mechanism involved increased production of GM-CSF, which drives the early monocyte/macrophage progenitor lineage commitment towards dendritic cell differentiation through the downregulation of RANK expression and c-Fos transcription. This may be a significant regulatory loop and a possible therapeutic target to counteract the activated bone resorption mediated by T-lymphocyte derived pro-osteoclastogenic cytokines in inflammatory and immune-disorders. In our study we also demonstrated the importance of *in vivo* and *in vitro* alloactivation in the regulation of osteoclast differentiation, thereby providing the novel evidence of the functional link between alloreaction and bone homeostasis. Ability of CsA to block T-lymphocyte activation and hence abrogate the inhibitory effect of T-lymphocytes on osteoclastogenesis may be important in clinical states associated with CsA treatment and contribute to the rapid bone loss that occurs after organ transplantation.

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Figure legends

Figure 1. Activated T-lymphocytes suppressed murine osteoclast differentiation stimulated by RANKL and M-CSF. *(A)* Photomicrographs of osteoclastogenic non-adherent bone-marrow cell cultures with α-MEM (control), unstimulated or activated (Con A-pulsed) 0.25×10^6 T-lymphocytes per well (lyT) and corresponding 50% T-lymphocyte conditioned medium (sup); ×100. Number of $TRAP^+$ osteoclast-like cells (OCLs) in non-adherent bone-marrow cell cultures stimulated by RANKL and M-CSF after addition of *(B)* different numbers of T-lymphocytes/well or different volumes of T-lymphocyte conditioned medium/well; *(C)* 0.25×10^6 T-lymphocytes/well or 50% T-lymphocyte conditioned medium/well at different time-points of culture (duration of treatment – days 0 to 2.5, 2.5 to 5 or 0 to 5); *(D)* 0.25×10^6 T-lymphocytes/well, 50% T-lymphocyte conditioned medium/well, or 0.25×10^6 mitomycin C-treated T-lymphocytes/well (lyT + MitC); or *(E)* 50% conditioned medium/well from unseparated (lyT), $CD4^+$ or $CD8^+$ T-lymphocytes. Values, mean ± SD (n=4). *, $p < 0.01$ vs. control culture and respective culture treated with unstimulated T-lymphocytes/conditioned medium. T-lymphocytes were activated by Con A-pulse, except for *(D)* where we used Con A-pulse or anti-CD3/CD28 treatment.

Figure 2. Activated T-lymphocytes increased the expression of GM-CSF, IFN-γ, RANKL and OPG by RT-qPCR. Relative quantity of RNA represents the ratio of RNA quantity for the respective gene normalized to the quantity of β-actin in freshly isolated T-lymphocytes (lyT nat), or in unstimulated and activated (Con A-pulsed) T-

lymphocytes cultured for 24 hours. Values, mean ± SD (n=4). *, $p < 0.01$ vs. control group and unstimulated T-lymphocytes.

Figure 3. GM-CSF mediates suppression of osteoclastogenesis by activated T-lymphocytes. *(A)* Photomicrographs of osteoclastogenic non-adherent bone-marrow cell cultures with α-MEM (control), activated T-lymphocytes conditioned medium (sup) or GM-CSF (5 or 10 ng/mL); ×100. *(B)* Number of $TRAP^+$ osteoclast-like cells (OCLs) in non-adherent bone-marrow cell cultures stimulated by RANKL and M-CSF after addition of 50% T-lymphocytes conditioned medium with or without 1 µg/mL neutralizing anti-GM-CSF Abs. Values, mean ± SD (n=4). *, $p < 0.01$ vs. control culture, culture treated with unstimulated T-lymphocyte conditioned medium and culture treated with activated T-lymphocyte conditioned medium plus anti-GM-CSF. *(C)* Flow-cytometric analysis of GM-CSF production by unstimulated T-lymphocytes or activated (Con A-pulsed) T-lymphocytes; dot-plots (FSC/PECy5.5-anti-rabbit + rabbit-anti-GM-CSF).

Figure 4. Addition of activated T-lymphocytes or conditioned medium to murine osteoclastogenic cultures stimulated by RANKL and M-CSF decreased the expression of RANK, CD115, c-Fos and calcitonin receptor (CtR) by RT-qPCR. Osteoclastogenic non-adherent bone-marrow cell cultures, with or without treatment by T-lymphocytes (lyT) or conditioned medium (sup) for the first 2.5 days of culture, were harvested for RNA extraction after 2.5 or 5 days of culture. Values, mean ± SD (n=4). *, $p < 0.01$ vs. respective time-point of control culture and respective culture treated

with unstimulated T-lymphocytes/conditioned medium. **, $p < 0.01$ vs. only the respective control culture.

Figure 5. Addition of activated T-lymphocyte conditioned medium to murine osteoclastogenic cultures stimulated by RANKL and M-CSF changed the expression of RANK, CD115, CD11c and CD11b by flow-cytometry. Non-adherent bone-marrow cells stimulated by RANKL and M-CSF were cultured for 2.5 days in α-MEM (control, left panel) or in 50% activated T-lymphocyte conditioned medium (lyT ConA sup, right panel). Cultures of bone-marrow cells treated with unstimulated T-lymphocyte conditioned medium (data not shown) were similar to control cultures. *(A)* Histograms (events/PE-anti-RANK and events/PE-anti-CD115); or *(B)* histograms (events/APC-anti-CD11c), arrow indicates the $CD11c^{high}$ population, and dot plots (PE-anti-RANK/APC-anti-CD11c, and FITC-anti-CD11b/APC-anti-CD11c).

Figure 6. *In vitro* and *in vivo* alloactivated T-lymphocytes suppressed murine osteoclast differentiation stimulated by RANKL and M-CSF. *(A)* Photomicrographs of osteoclastogenic non-adherent bone-marrow cell cultures with α-MEM (control), unstimulated (lyT) or alloactivated (MLR) 50% T-lymphocyte conditioned medium (sup); ×100. Number of $TRAP^+$ osteoclast-like cells (OCLs) in non-adherent bone-marrow cell cultures stimulated by RANKL and M-CSF after addition of *(B)* different volumes of unstimulated or *in vitro* allostimulated T-lymphocyte conditioned medium/well; or *(C)* 0.25×10^6/well or 50% conditioned medium/well of *in vivo* allostimulated popliteal T-lymphocytes, without or with *in vitro* restimulation for 24 hours. *(D) In vivo* allostimulation was performed by i.p. injection and foot-pad

restimulation with allogeneic splenocytes. Left pannel, photomicrographs of osteoclastogenic non-adherent bone-marrow cell cultures from unstimulated animals or from *in vivo* allostimulated animals, ×100; right pannel, number of $TRAP^+$ OCLs in non-adherent bone-marrow cell cultures stimulated by RANKL and M-CSF from unstimulated animals or *in vivo* allostimulated animals. Values, mean ± SD (n=4). *, $p < 0.01$ vs. control culture and respective culture treated with unstimulated T-lymphocytes/conditioned medium *(B* and *C)* or culture from unstimulated animals *(D)*. **, $p < 0.01$ vs. *in vivo* alloactivated but not *in vitro* restimulated cells.

Figure 7. Cyclosporine A (CsA)-treatment prior to Con A-pulse abrogates the inhibitory effect of T-lymphocytes on murine osteoclast differentiation stimulated by RANKL and M-CSF. *(A)* Photomicrographs of osteoclastogenic non-adherent bone-marrow cell cultures with α-MEM (control), unstimulated or activated (Con A-pulsed) T-lymphocyte (lyT) conditioned medium (sup) with or without CsA pretreatment; ×100. *(B)* Number of $TRAP^+$ osteoclast-like cells (OCLs) in non-adherent bone-marrow cell cultures stimulated by RANKL and M-CSF after the addition of 50% conditioned medium/well of T-lymphocytes pretreated with different concentrations of CsA. *(C)* RT-qPCR analysis of RANKL and GM-CSFexpression in unstimulated and activated (Con A-pulsed) T-lymphocytes, with or without CsA (40 μg/mL) pretreatment, after 24 hour-culture. Values, mean ± SD (n=4). *, $p < 0.01$ vs. respective unstimulated T-lymphocytes. **, $p < 0.01$ vs. ConA-pulsed T-lymphocytes without CsA-pretreatment.

Figure 1.

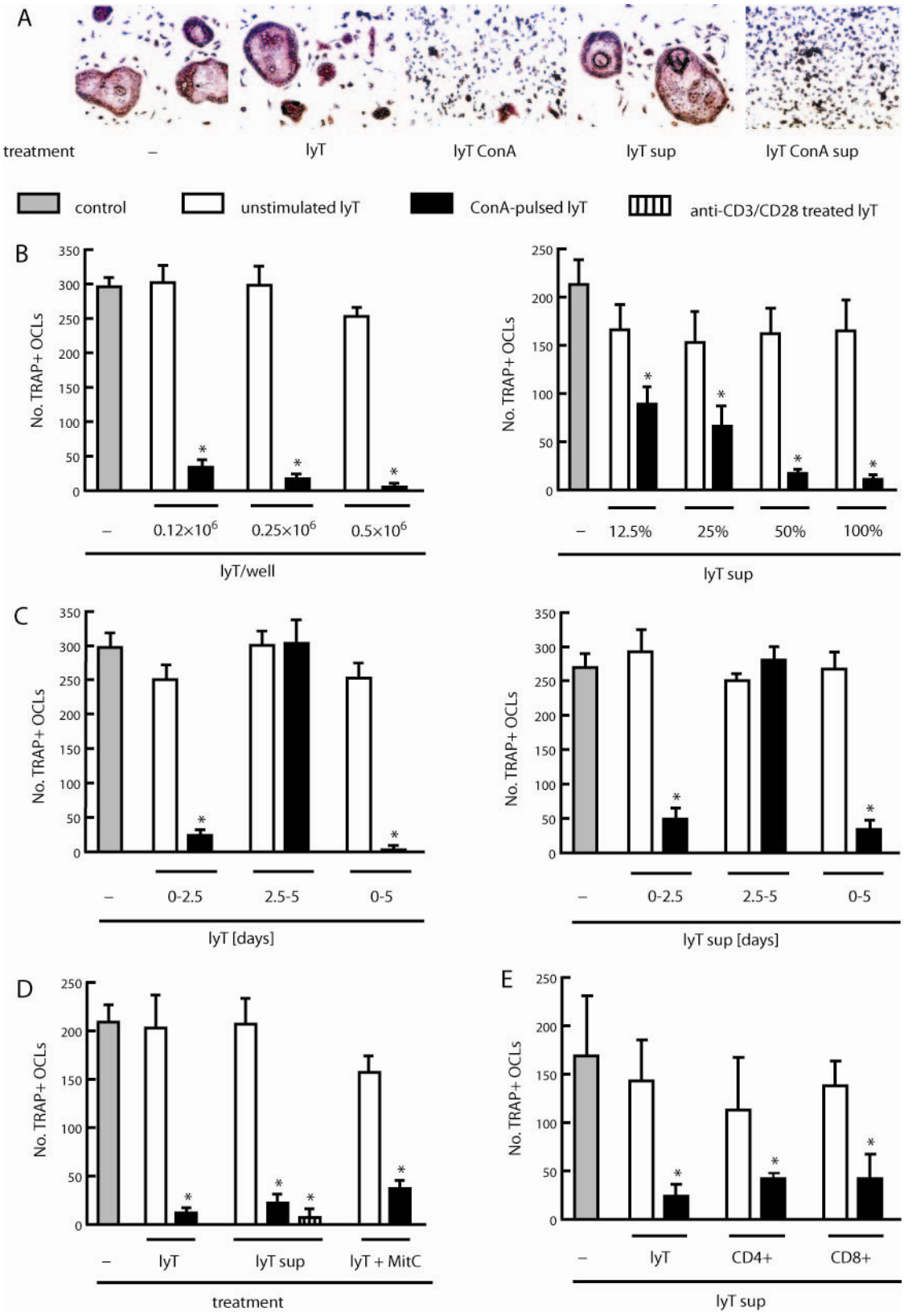

A
treatment
–
lyT
lyT ConA
lyT sup
lyT ConA sup
control
unstimulated lyT
ConA-pulsed lyT
anti-CD3/CD28 treated lyT
B
No. TRAP+ OCLs
0
50
100
150
200
250
300
350
–
0.12×10^6
0.25×10^6
0.5×10^6
lyT/well
No. TRAP+ OCLs
0
50
100
150
200
250
–
12.5%
25%
50%
100%
lyT sup
C
No. TRAP+ OCLs
0
50
100
150
200
250
300
350
–
0-2.5
2.5-5
0-5
lyT [days]
No. TRAP+ OCLs
0
50
100
150
200
250
300
350
–
0-2.5
2.5-5
0-5
lyT sup [days]
D
No. TRAP+ OCLs
0
50
100
150
200
250
–
lyT
lyT sup
lyT + MitC
treatment
E
No. TRAP+ OCLs
0
50
100
150
200
250
–
lyT
CD4+
CD8+
lyT sup

Figure 2.

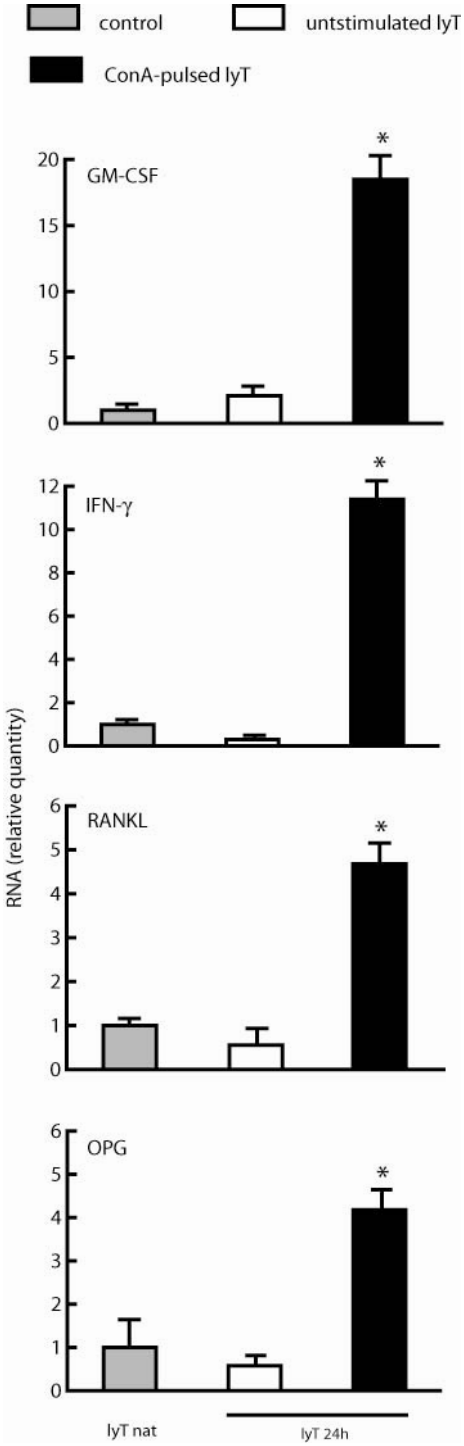

control
untstimulated lyT
ConA-pulsed lyT
GM-CSF
IFN-γ
RANKL
OPG
RNA (relative quantity)
lyT nat
lyT 24h

Figure 3.

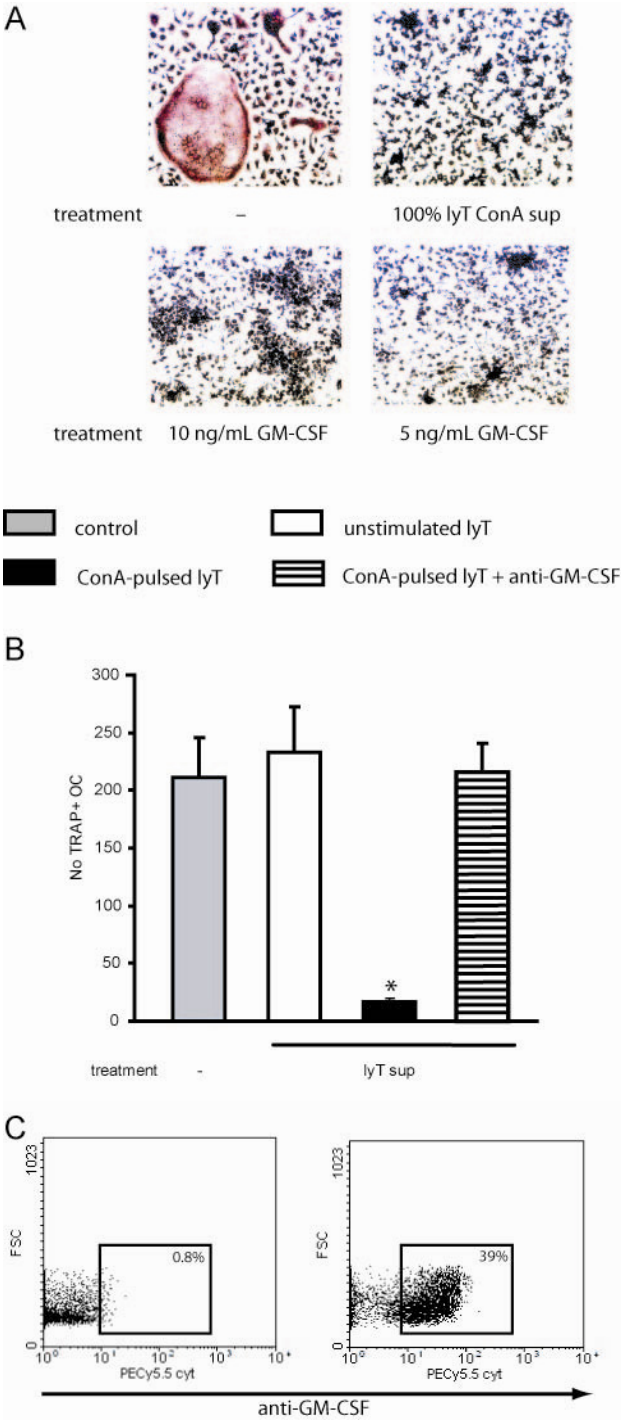

A
treatment
–
100% lyT ConA sup
treatment
10 ng/mL GM-CSF
5 ng/mL GM-CSF
control
unstimulated lyT
ConA-pulsed lyT
ConA-pulsed lyT + anti-GM-CSF
B
No TRAP+ OC
300
250
200
150
100
50
0
*
treatment
-
lyT sup
C
FSC
1023
0
0.8%
39%
PECy5.5 cyt
anti-GM-CSF

Figure 4.

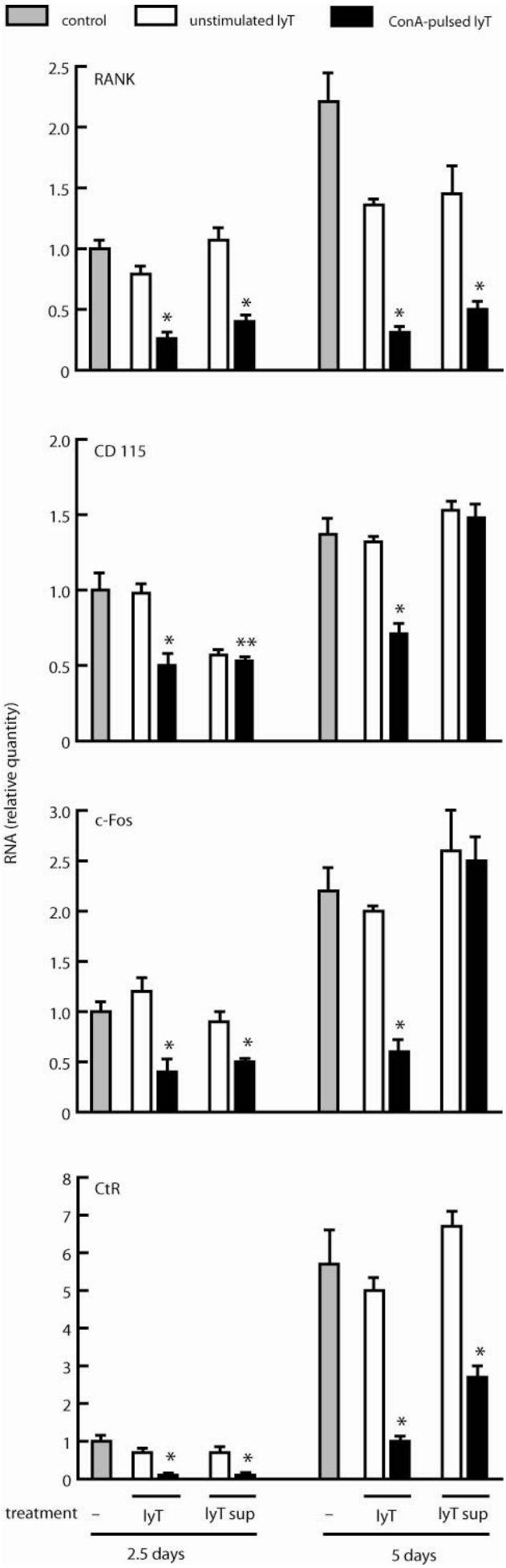

control
unstimulated lyT
ConA-pulsed lyT
RANK
CD 115
c-Fos
CtR
RNA (relative quantity)
treatment
–
lyT
lyT sup
2.5 days
5 days

Figure 5.

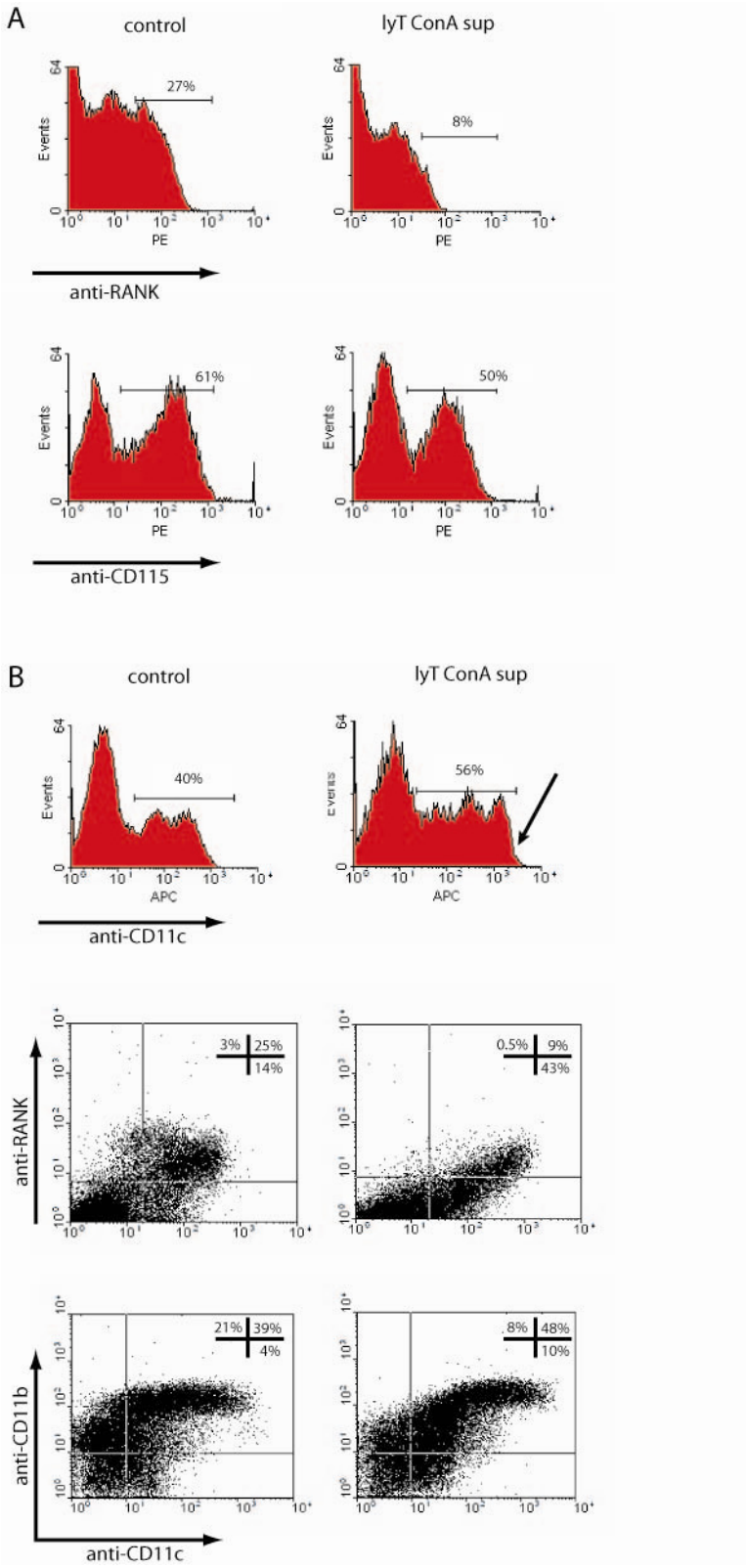

A
control
lyT ConA sup
27%
8%
Events
PE
anti-RANK
61%
50%
anti-CD115
B
control
lyT ConA sup
40%
56%
APC
anti-CD11c
3%
25%
14%
0.5%
9%
43%
anti-RANK
21%
39%
4%
8%
48%
10%
anti-CD11b
anti-CD11c

Figure 6.

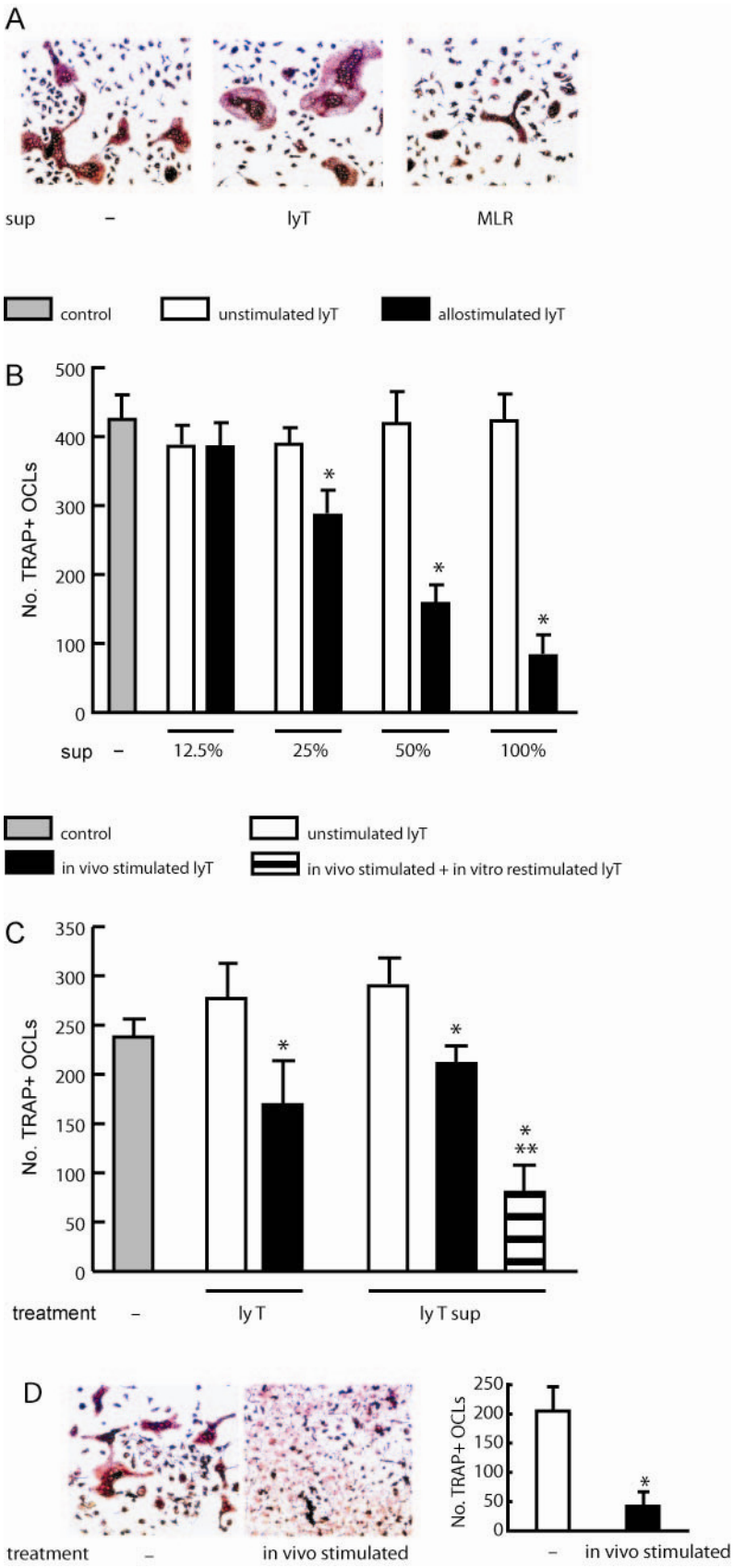
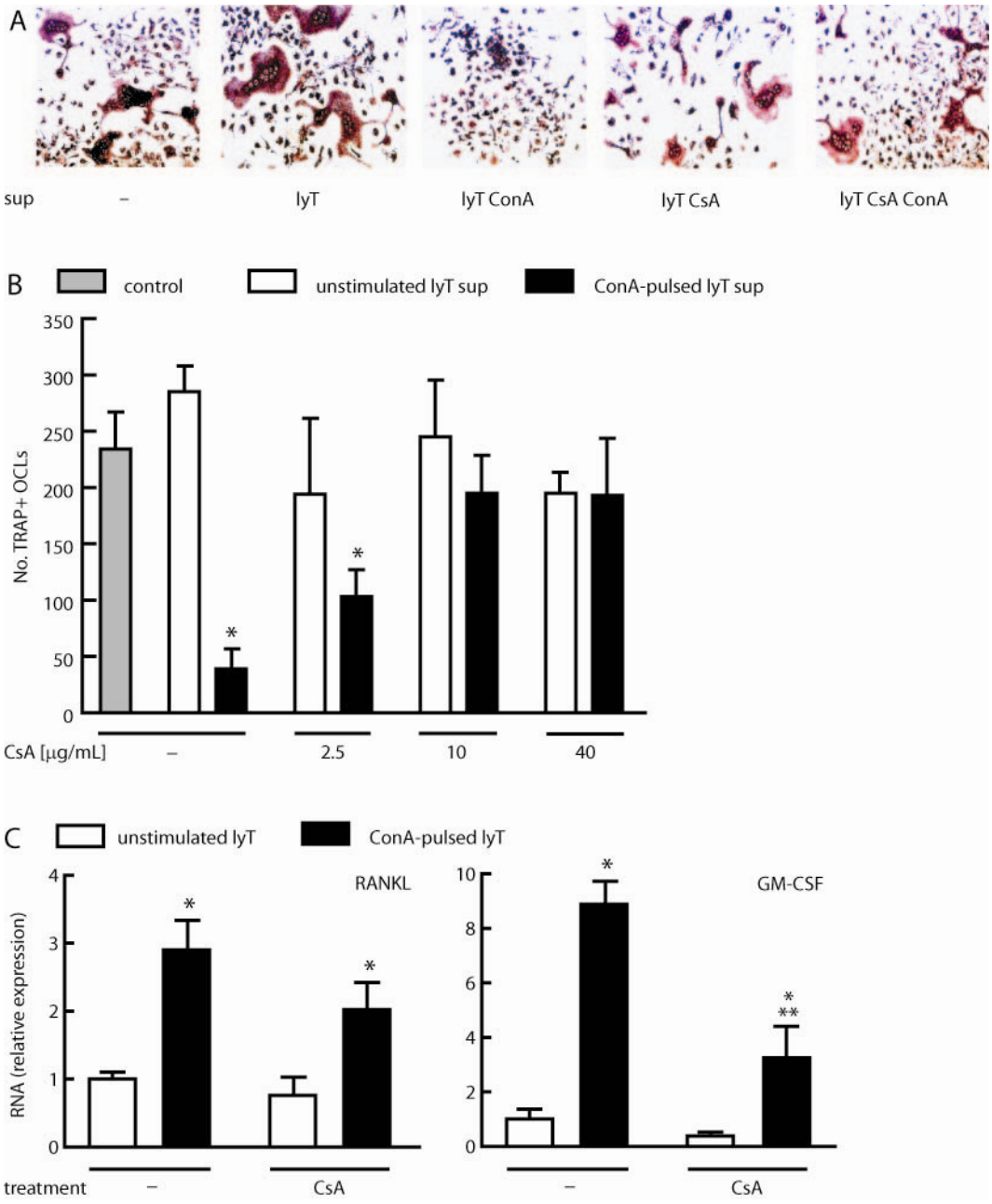

A
sup
–
lyT
MLR
control
unstimulated lyT
allostimulated lyT
B
No. TRAP+ OCLs
500
400
300
200
100
0
*
*
*
sup
–
12.5%
25%
50%
100%
control
in vivo stimulated lyT
unstimulated lyT
in vivo stimulated + in vitro restimulated lyT
C
No. TRAP+ OCLs
350
300
250
200
150
100
50
0
*
*
*
**
treatment
–
ly T
ly T sup
D
treatment
–
in vivo stimulated
No. TRAP+ OCLs
250
200
150
100
50
0
*
–
in vivo stimulated

Figure 7.


A
sup
–
lyT
lyT ConA
lyT CsA
lyT CsA ConA
B
control
unstimulated lyT sup
ConA-pulsed lyT sup
No. TRAP+ OCLs
350
300
250
200
150
100
50
0
CsA [μg/mL]
–
2.5
10
40
C
unstimulated lyT
ConA-pulsed lyT
RNA (relative expression)
RANKL
GM-CSF
treatment
–
CsA