

# M-CSF, TNF $\alpha$ and RANK ligand promote osteoclast survival by signaling through mTOR/S6 kinase

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## Abstract

Multinucleated bone-resorbing osteoclasts (Ocl) are cells of hematopoietic origin that play a major role in osteoporosis pathophysiology. Ocl survival and activity require M-CSF and RANK ligand (RANKL). M-CSF signals to Akt, while RANKL, like TNF $\alpha$ , activates NF- $\kappa$ B. We show here that although these are separate pathways in the Ocl, signaling of all three cytokines converges on mammalian target of rapamycin (mTOR) as part of their antiapoptotic action. Accordingly, rapamycin blocks M-CSF- and RANKL-dependent Ocl survival inducing apoptosis, and suppresses *in vitro* bone resorption proportional to the reduction in Ocl number. The cytokine signaling intermediates for mTOR/ribosomal protein S6 kinase (S6K) activation include phosphatidylinositol-3 kinase, Akt, Erks and geranylgeranylated proteins. Inhibitors of these intermediates suppress cytokine activation of S6K and induce Ocl apoptosis. mTOR regulates protein translation acting via S6K, 4E-BP1 and S6. We find that inhibition of translation by other mechanisms also induces Ocl apoptosis, demonstrating that Ocl survival is highly sensitive to continuous *de novo* protein synthesis. This study thus identifies mTOR/S6K as an essential signaling pathway engaged in the stimulation of cell survival in osteoclasts.

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**Keywords:** apoptosis; survival; S6 kinase; rapamycin; osteoclast

**Abbreviations:** Ocl, osteoclast; S6, ribosomal protein S6; S6K, ribosomal protein S6 kinase; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol-3 kinase

## Introduction

Osteoclasts (Ocls) are terminally differentiated multinucleated cells that resorb bone. High Ocl activity leads to bone loss associated with postmenopausal osteoporosis and Paget's

disease, among others. Ocl differentiation, activity and survival is regulated by the concerted action of cytokines and growth factors, synthesized by osteoblasts, stromal cells, or other cells of hematopoietic origin.<sup>1–3</sup>

Among the factors essential for Ocl generation, function and survival are M-CSF<sup>4–6</sup> and the ligand for the receptor activator of NF- $\kappa$ B RANK ligand (RANKL, also named ODF, OPG or TRANCE), a member of the TNF family.<sup>7–9</sup> Although less clear, a role for TNF $\alpha$  in Ocl differentiation and activation has also been shown.<sup>10–12</sup> The fine -tuning between bone resorption by Ocl and bone formation by osteoblasts is critical for the preservation of bone mass. Since Ocl lifespan could influence overall resorptive activity, regulation of Ocl apoptosis was proposed to be one of the mechanisms for controlling bone resorption.<sup>13,14</sup>

Apoptosis is a genetically programmed, morphologically distinct form of cell death that can be triggered by a variety of physiological and pathological stimuli. Regulation of Ocl survival is coordinated in part by proteins synthesized and presented by cells of the osteoblast lineage, including M-CSF and RANKL. Ocl differentiation *in vitro* depends on exogenous M-CSF,<sup>15</sup> and M-CSF removal from purified Ocl cultures results in caspase and MST1 kinase activation, leading to apoptosis.<sup>16</sup> Soluble RANKL (sRANKL) and TNF $\alpha$  have also been shown to support Ocl survival in addition to their effect on differentiation.<sup>17,18</sup> However, a common mechanism, if any, by which these prosurvival cytokines exert antiapoptotic effects in Ocl remains unknown.

Signaling by M-CSF has been shown to activate phosphoinositide 3-kinase (PI3K) and antiapoptotic Akt (also known as PKB) kinase in Ocl.<sup>19</sup> Akt activity is necessary and in some cases sufficient for cell survival and has been shown to target the apoptotic machinery by phosphorylating downstream molecules like BAD, caspase-9, glycogen-synthase kinase and forkhead family members.<sup>20,21</sup> RANKL and TNF $\alpha$  act primarily via NF- $\kappa$ B activation leading to the transcription and *de novo* synthesis of antiapoptotic proteins.<sup>22</sup> However, some studies suggest that RANKL and TNF $\alpha$  can stimulate the Akt pathway involving c-src kinase.<sup>18,23,24</sup> Additionally, although controversial, Akt activity has been suggested to be required for NF- $\kappa$ B induction by TNF.<sup>25,26</sup>

The mammalian target of rapamycin (mTOR, also known as FRAP or RAFT) is a serine/threonine kinase that plays a central role in the control of translation. mTOR might act as a master switch of cellular catabolism and anabolism.<sup>27</sup> Both mTOR and p70 ribosomal protein S6 kinase (S6K) are regulated via PI3K effectors, including Akt and phosphoinositide-dependent kinase 1 (PDK1).<sup>28–31</sup> Akt relieves mTOR from tuberous sclerosis complex (TSC1/2)-mediated suppression by phosphorylation of TSC2. Both PDK1 and mTOR have been shown to phosphorylate S6K at Thr389 and release the kinase from autoinhibition.<sup>31,32</sup> The penultimate activation step at this site, however, seems to be provided by NEK6, a newly identified S6K kinase.<sup>33</sup> Only after the release of autoinhibition does PDK1 efficiently phosphorylate and

activate S6K within the activation loop. The mTOR/S6K pathway has been shown to regulate translation via phosphorylation of 4E-BP1 and ribosomal protein S6 (S6);<sup>34</sup> and antiapoptotic signaling by phosphorylation of BAD<sup>35</sup> (reviewed in Castedo *et al.*<sup>27</sup>).

Guided by the observation that inhibitors of translation cause rapid apoptosis in Ocl, we investigated prosurvival signaling through the translational control pathway mTOR/S6K in these cells. We found an antiapoptotic pathway involving mTOR/S6K that is commonly engaged by M-CSF, TNF $\alpha$  and RANKL. Inhibition of mTOR results in Ocl apoptosis and suppression of bone resorption *in vitro*, suggesting that mTOR control of protein translation can be rate limiting in the control of the Ocl lifespan.

## Results

### Osteoclastogenic cytokines suppress both basal- and alendronate-induced apoptosis

Apoptosis was measured in purified Ocls in the absence or presence of alendronate (ALN), a bisphosphonate widely used in the treatment of osteoporosis. ALN-induced Ocl apoptosis (Figure 1a) is associated with the formation of apoptotic bodies (arrows) that form a ring-like array and/or retraction of the cell, accompanied by formation of pyknotic nuclei (inset), as we have previously described.<sup>16</sup> Apoptosis in Ocl is accompanied by caspase activation of proapoptotic MST1 kinase.<sup>16</sup> (Figure 1b–d). After caspase cleavage, 36 kDa MST1 kinase further increases caspase activity and promotes apoptosis.<sup>36–38</sup> The activity of caspase-cleaved MST1 can serve as a quantitative biochemical marker for apoptosis in the Ocl, and relative induction (*versus* control) is indicated below each in-gel kinase assay lane in Figure 1.

The mechanism of ALN-induced apoptosis is by suppression of protein geranylgeranylation,<sup>16</sup> thus coinhibition with geranylgeraniol (GGOH; Figure 1a and b) blocked ALN effects. Consistent with previous reports,<sup>16,39,40</sup> inhibition of type I geranylgeranyltransferase by a highly selective inhibitor geranylgeranyltransferase-1 inhibitor-2 (GGTI-2<sup>41</sup>) mimicked ALN effects on Ocl apoptosis (Figure 1b). GGTI-2- and ALN-induced morphological features of apoptosis and caspase-cleaved 36 kDa MST1 activities (3.6- and four-fold, respectively; Figure 1b) were essentially indistinguishable. Apoptosis induced by GGTI-2 and ALN could be suppressed by addition of GGOH, as seen with 36 kDa MST1 activity that was reduced to baseline levels (Figure 1b). This is consistent with the observation that GGTI-2 inhibits geranylgeranyltransferase in a geranylgeranyldiphosphate competitive manner (Hans Huber, personal communication).

Ocls require cytokines, especially M-CSF, for their survival *in vivo* and *in vitro*. In addition to M-CSF, RANKL is required for Ocl differentiation and bone resorption, and RANKL, TNF $\alpha$  and IL-1 $\alpha$  can help to suppress apoptosis in these cells. We therefore examined if these cytokines have additive effects with a minimal level of M-CSF (5 ng/ml) on suppression of Ocl apoptosis induced by ALN or GGTI-2. Both spontaneous and ALN-induced apoptosis could be suppressed to baseline

levels or below by additionally treating with sRANKL, TNF $\alpha$  or IL-1 $\alpha$ , as quantified by the activity of caspase-cleaved MST1 kinase (Figure 1b). Antiapoptotic effects extended to the prevention of morphological disruption (Figure 1a). Similar effects were seen in the suppression of GGTI-2-induced apoptosis (Figure 1b), with MST1 caspase cleavage suppressed to below baseline. Largest effects on caspase-cleaved 36 kDa MST1 kinase activity were observed when TNF $\alpha$ , IL-1 $\alpha$  and M-CSF were combined (Figure 1b), and kinase activities in control, ALN- or GGTI-2-treated Ocls were reduced to 70% below baseline. This was similar to the effect of GGOH on ALN-induced apoptosis, which resulted in a 10-fold reduction in 36 kDa MST1 kinase activity (Figure 1b, lane 12). The effects of RANKL on ALN-induced apoptosis were dose dependent (Figure 1c), resulting in up to an 80% reduction in activity of the 36 kDa MST1 kinase. Similar dose dependencies were obtained with TNF $\alpha$  or IL-1 $\alpha$  (data not shown).

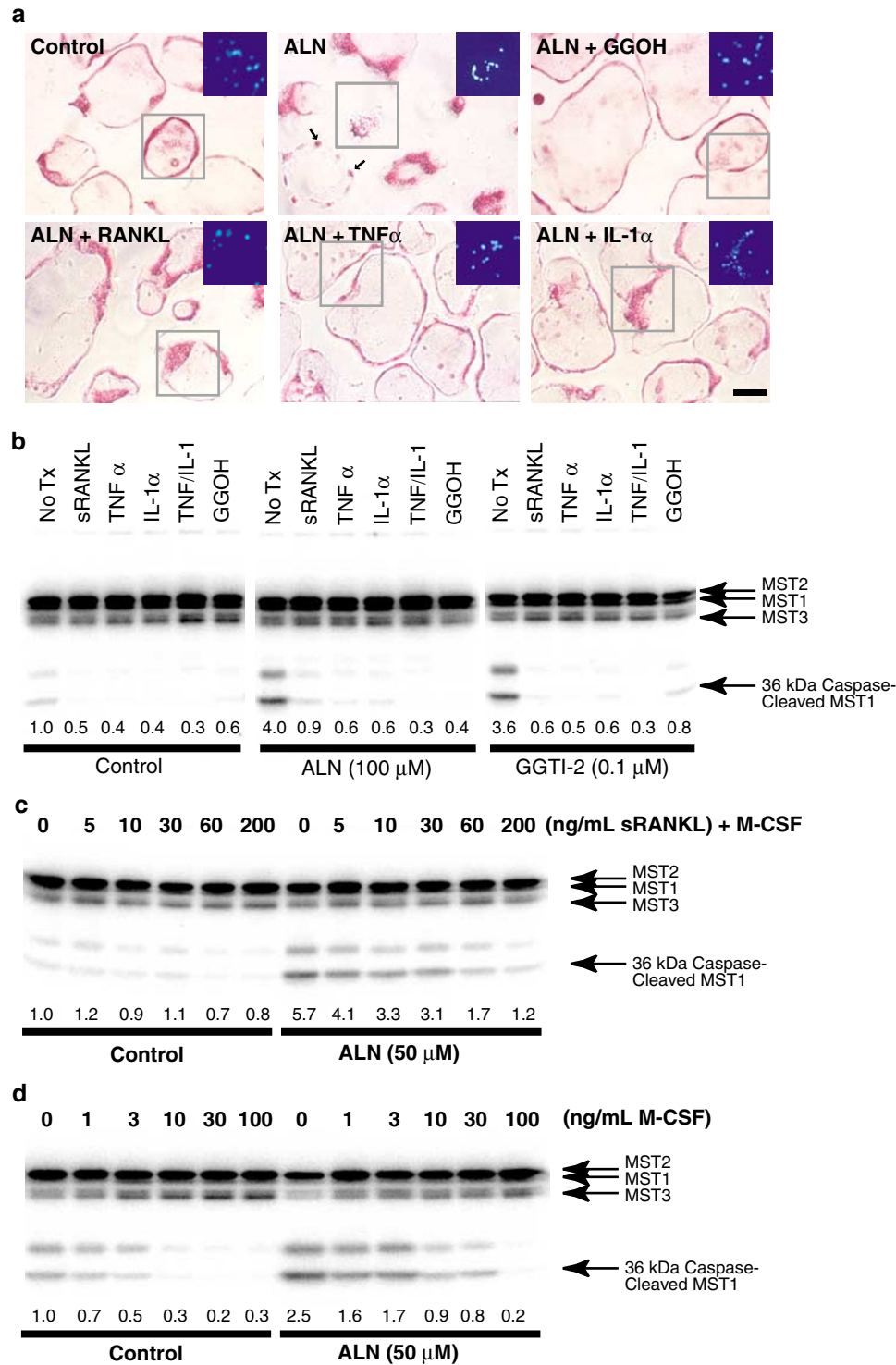
Under control conditions and in the absence of M-CSF (i.e. no exogenous apoptotic or antiapoptotic agent) Ocl exhibited spontaneous apoptosis as measured by detection of 36 kDa MST1 kinase (Figure 1d, lane 1), along with actin disruption, nuclear condensation, membrane blebbing and cellular lysis (data not shown). As expected, M-CSF dose-dependently suppressed spontaneous apoptosis and essentially completely blocked MST1 cleavage by up to five-fold when used at 30 ng/ml or above (Figure 1d, lanes 2–6). Interestingly, ALN-induced apoptosis was also dose-dependently suppressed by M-CSF (Figure 1d, lanes 7–12), whereby 36 kDa MST1 kinase activity was reduced up to 12-fold (100 ng/ml M-CSF) to levels seen in the absence of ALN (lanes 5 and 6).

Thus, M-CSF along with TNF $\alpha$ , sRANKL, and IL-1 $\alpha$ , which act via different signal transduction pathways, seem to have complementary and additive effects on suppression of Ocl apoptosis caused by inhibition of geranylgeranylation. Time-course experiments indicated that each of these cytokines delayed ALN- or GGTI-2-induced apoptosis by approximately 10–12 h (data not shown). These observations suggested the possibility of a common pathway in the prosurvival signaling of these agents.

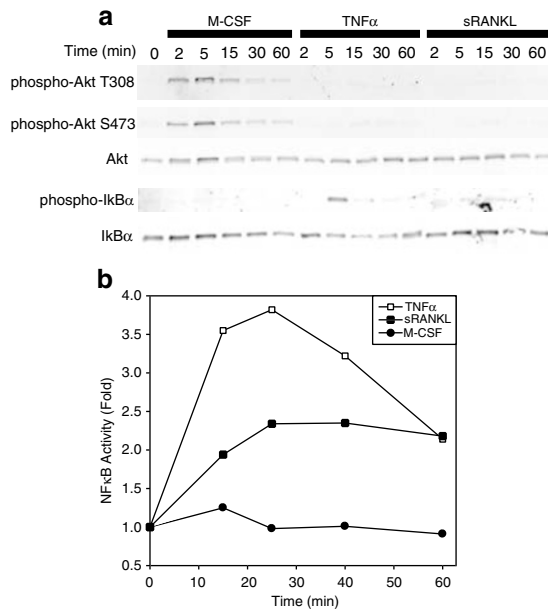
### Nonoverlapping Akt and NF- $\kappa$ B pathways induced by M-CSF and TNF $\alpha$ /sRANKL

We first examined in Ocl, potential overlap in the known signal transduction pathways of M-CSF, TNF $\alpha$  and sRANKL, starting with NF- $\kappa$ B and Akt. Using phospho-specific antibodies, we found that M-CSF rapidly induced activation of Akt by phosphorylating residues Thr308 and Ser473 (Figure 2a). M-CSF had no detectable effects on the NF- $\kappa$ B pathway, evaluated by phosphorylation and degradation of I $\kappa$ B $\alpha$  (Figure 2a) or binding of free NF- $\kappa$ B (i.e. not complexed with I $\kappa$ B) to DNA (Figure 2b).

TNF $\alpha$  and sRANKL induced very limited activation of Akt at Thr308 and Ser473, when compared to M-CSF. However, both cytokines induced phosphorylation of I $\kappa$ B $\alpha$  (Ser 32/36) at 5 min. For TNF $\alpha$ , its more potent induction of I $\kappa$ B $\alpha$  phosphorylation was followed by partial proteosomal degradation and



**Figure 1** Inhibition of spontaneous and ALN-, or GGTI-2 induced Ocl apoptosis by survival cytokines M-CSF,  $TNF\alpha$ , RANKL and IL-1 $\alpha$ . **(a)** Morphological effects of cytokines on ALN induction of apoptosis. In all groups, 5 ng/ml M-CSF was included to provide minimal Ocl survival. Ocl were untreated (Control) or further treated with ALN (50  $\mu$ M) alone or with added GGOH (10  $\mu$ M), sRANKL (100 ng/ml),  $TNF\alpha$  (50 ng/ml), IL-1 $\alpha$  (50 ng/ml) or  $TNF\alpha$  + IL-1 $\alpha$ . After 16 h, Ocl were stained for TRAP (red) and with Hoechst 33342 (blue). **(b)** In comparison to 5 ng/ml M-CSF alone (no Tx), effects on Ocl apoptosis of: sRANKL (100 ng/ml),  $TNF\alpha$  (50 ng/ml), IL-1 $\alpha$  (50 ng/ml),  $TNF\alpha$  + IL-1 $\alpha$ , or GGOH (10  $\mu$ M), each combined with 5 ng/ml M-CSF. Spontaneous apoptosis (Control) or that induced with ALN (100  $\mu$ M; b and c) or GGTI-2 (0.1  $\mu$ M) for 16 h was assessed. Ocl apoptosis was assessed by measuring the activity of the 36 kDa MST1 kinase caspase cleavage product by in-gel kinase assays as described in Materials and Methods. Fold activation of 36 kDa MST1 kinase (*versus* control) is noted beneath each phosphorimage. Arrows indicate full-length MST1 kinase and the 36 kDa cleavage product, as well as MST2 and MST3 protein kinases. **(c)** Effects of increasing concentrations of sRANKL on inhibition of spontaneous (Control) and ALN (50  $\mu$ M) induced Ocl apoptosis in the presence of 5 ng/ml M-CSF (in-gel kinase assay). **(d)** Effects of increasing concentrations of M-CSF on inhibition of spontaneous (Control) and ALN (50  $\mu$ M)-induced Ocl apoptosis (in-gel kinase assay)



**Figure 2** Selective effects of M-CSF,  $\text{TNF}\alpha$  and sRANKL on stimulation of the Akt and  $\text{NF-}\kappa\text{B}$  pathways. (a) Purified Ocl were treated with M-CSF,  $\text{TNF}\alpha$  and sRANKL for 0–60 min, as indicated. Cell lysates were immunoblotted with phospho-specific antibodies for Akt (Thr308 or Ser473) and  $\text{I}\kappa\text{B}\alpha$  (Ser32/36) as described in Materials and Methods. (b)  $\text{NF-}\kappa\text{B}$  activation was measured, as described in Materials and Methods, after treatment with  $\text{TNF}\alpha$  (open squares), sRANKL (closed squares) or M-CSF (closed circles) for 15–60 min, as indicated. For  $\text{NF-}\kappa\text{B}$  assays in (b), effective measurement required the pooling of cell lysates derived from multiple identically treated wells. A total of three pooled-sampling experiments were performed, with similar results

recovery of  $\text{I}\kappa\text{B}\alpha$  protein level. For sRANKL, we repeatedly found a modest level of  $\text{I}\kappa\text{B}\alpha$  phosphorylation that lasted up to 15 min, although little degradation of  $\text{I}\kappa\text{B}\alpha$  was observed. We therefore examined  $\text{NF-}\kappa\text{B}$  activation in separate time-course experiments (Figure 2b). Despite slightly differing in  $\text{I}\kappa\text{B}\alpha$  phosphorylation and degradation, both  $\text{TNF}\alpha$  and sRANKL induction of  $\text{NF-}\kappa\text{B}$  activation peaked at 25 min. Consistent with the more potent effect on  $\text{I}\kappa\text{B}\alpha$ , the magnitude of induction by  $\text{TNF}\alpha$  was greater than with sRANKL. However, this difference was transient and both effects were comparable at 60 min. As noted above, M-CSF failed to stimulate  $\text{NF-}\kappa\text{B}$  appreciably over this time course. Thus, M-CSF and  $\text{TNF}\alpha$ /sRANKL survival cytokines seem to induce different essentially nonoverlapping intracellular signaling pathways in Ocl.

### Ocl require continuous protein translation for survival

Identification of mTOR/S6K as critical for Ocl survival stemmed from analyses of  $\text{TNF}\alpha$  proapoptotic (caspase activation) *versus* antiapoptotic ( $\text{NF-}\kappa\text{B}$ -mediated) signaling.  $\text{TNF}\alpha$  induces apoptosis when cells are treated with a protein synthesis inhibitor such as cycloheximide (CHX) or when  $\text{NF-}\kappa\text{B}$  signaling is blocked.<sup>42,43</sup> Unexpectedly, we found that treatment of Ocl with translational inhibitors alone resulted in pronounced, rapid, apparently synchronous induction of Ocl apoptosis (Figures 3 and 4). In these experiments, translation was inhibited by three different mechanisms: (1) inhibition of

ribosomal translocase activity with CHX; (2) premature termination of the growing polypeptide chain by puromycin, a tRNA analog; and (3) blocking ribosome movement along mRNA with emetine. For comparison, the RNA-polymerase inhibitor actinomycin D was used to inhibit transcription. Within 2 h of inhibiting translation with cycloheximide (Figure 3b, b'), emetine (Figure 3c, c') and puromycin (data not shown; indistinguishable from CHX), we observed distinct changes in Ocl morphology, which were consistent with the induction of apoptosis. This included cell retraction, loss of actin ring structure, membrane blebbing and nuclear condensation. After 4 h, all tartrate-resistant acid phosphatases (TRAP)-positive cells were apoptotic. Actinomycin D treatment (Figure 3d, d') showed no morphological effects within this time frame. For all treatments, there was no evidence of cell survival after overnight exposure (16 h).

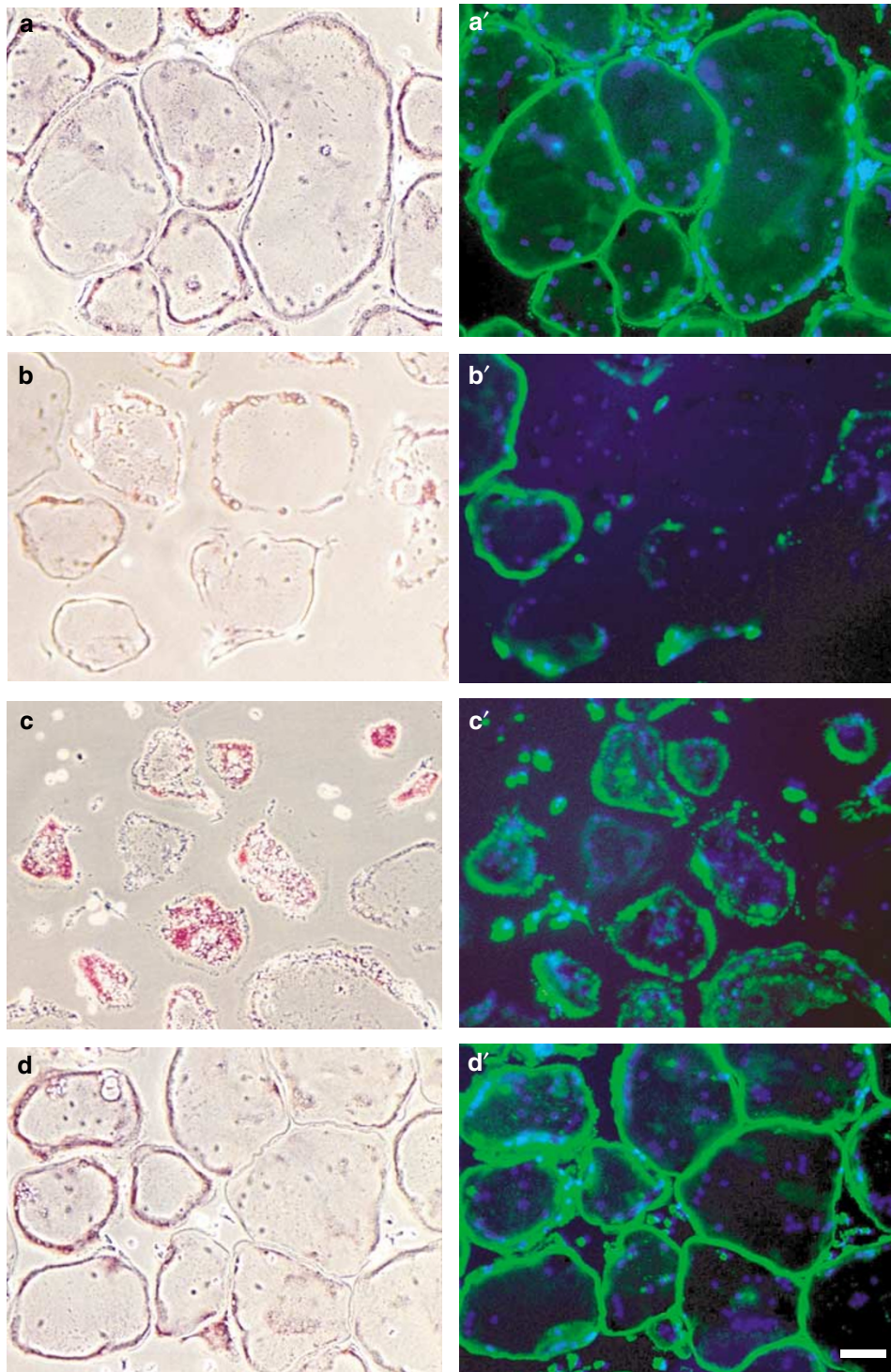
Apoptotic events induced by translation inhibitors were further characterized by measuring activities of initiator (caspase-9) and effector caspases (i.e. caspase-3) (Figure 4a) and the activity of the caspase cleavage product, 36 kDa MST1 (Figure 4b). With translation inhibitors, caspase-9 activity increased six- to eight-fold and caspase-3 by 20–30-fold after 1 h. Consistent with a highly synchronous induction of apoptosis, caspase-9 activity increased by 50–125-fold and caspase-3 by 100–200-fold, measured after 2–3 h. With a slower induction of apoptosis, the transcriptional inhibitor actinomycin D did not activate these caspases until 3 h, at which time caspase-9 and -3 were activated by 22- and 47-fold, respectively. This was consistent with the morphological appearance of apoptosis in actinomycin D-treated cultures at about 4 h (data not shown).

CHX, puromycin or emetine treatment also stimulated cleavage and activation of MST1 kinase by 14–35-fold, depending on the treatment (Figure 4b). At the same time point (2.75 h), control Ocls showed very little spontaneous apoptosis and actinomycin D had minimal effects on MST1 cleavage (1.2-fold). The actinomycin response was consistent with delayed caspase activation, as noted above. These data indicate that Ocl survival requires continuous *de novo* protein synthesis and a lack thereof results in rapid initiation and progression of the apoptotic program. We therefore investigated a possible role for a signaling pathway controlling translation in cytokine promotion of Ocl survival.

### Ocl antiapoptotic cytokines signal through the mTOR/S6K pathway

Based on the observation that translation plays a central role in Ocl survival, we examined the mTOR/S6K translational control pathway, as a possible common integrator for survival signaling in Ocl. Indeed, M-CSF,  $\text{TNF}\alpha$  and sRANKL all stimulated phosphorylation of S6K1 (p70) and S6K2 (p54) isoforms in Ocl (Figure 5a). Antibody recognition of an 85 kDa band, possibly p85 S6K1 and/or PKC, was unchanged. Consistent with an increase in mTOR activity, the cytokines also induced phosphorylation of its direct substrate, 4E-BP1 at Ser65.





**Figure 3** Inhibition of translation rapidly induces Ocl apoptosis. Ocl were prepared as in Figure 1 and left untreated (**a, a'**), or were treated with CHX (**b, b'**), emetine (**c, c'**), actinomycin D (**d, d'**) for 2.75 h. Effects of puromycin were indistinguishable from those of CHX (data not shown). Cells were fixed and stained for TRAP activity (**a–d**). Nuclear integrity was assessed using Hoechst No. 33342 (Blue), and FITC-phalloidin (Green) was used to visualize the actin cytoskeleton (**a'–d'**). Bar 100  $\mu$ M

Consistent with the activation of S6K by these cytokines, phosphorylation of the S6K substrate, S6 (Ser235/6), was also observed. By this measure, S6K activity increased several fold after 15 min exposure to M-CSF,  $\text{TNF}\alpha$  or sRANKL and remained elevated for up to 60 min (Figure 5b).

For M-CSF, peak phosphorylation was observed at 15 and 30 min, while with  $\text{TNF}\alpha$  and sRANKL, peak phosphorylation was observed at 30 and 60 min. Parallel measurements of these cell lysates showed that Akt phosphorylation was rapidly and transiently induced only by M-CSF.  $\text{TNF}\alpha$  and

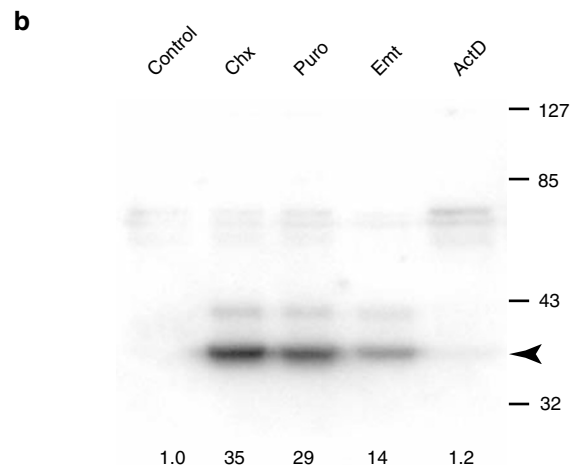
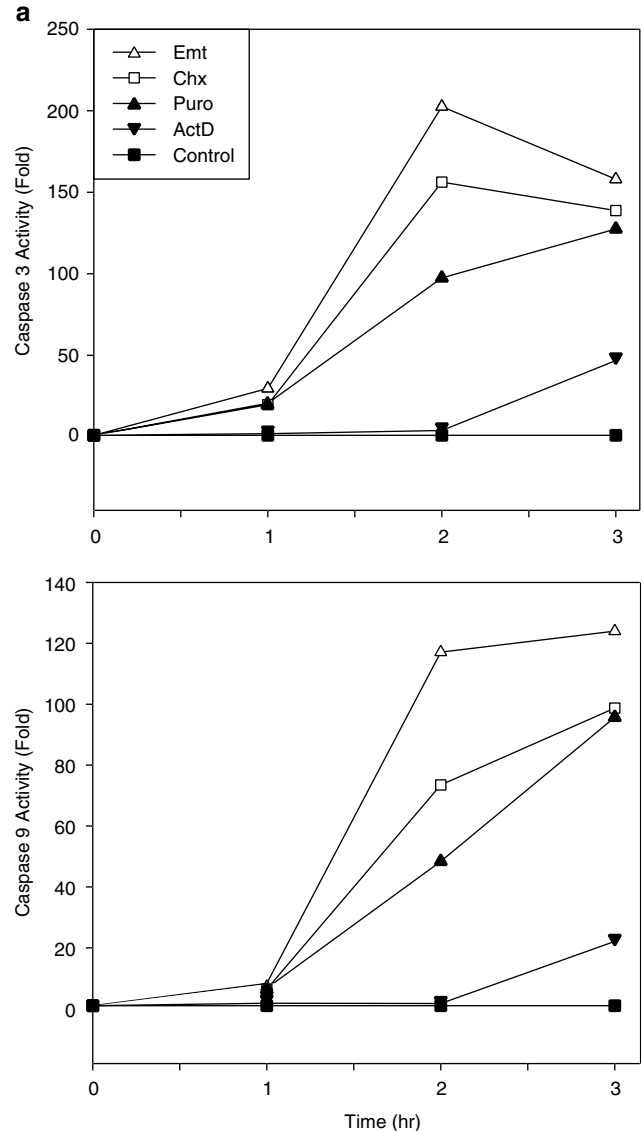
sRANKL similarly induced more rapid and transient effects on the phosphorylation of I $\kappa$ B $\alpha$  (Figure 2a). Thus, the S6 phosphorylation seen at 15–30 min appeared relatively delayed *versus*. Akt and I $\kappa$ B $\alpha$  responses. As expected, the mTOR inhibitor, rapamycin, inhibited all stimulatory effects of M-CSF, TNF $\alpha$  and sRANKL on S6K (at Thr389), S6 (Ser 235/6) and 4E-BP1 (Ser 65) phosphorylation (Figure 5a, c and d). No effect on p85 phosphorylation was observed. Based on the rapamycin suppression of S6K and S6 phosphorylation, stimulation of S6K in this system by M-CSF, TNF $\alpha$  and sRANKL requires the catalytic activity of mTOR. These findings indicate that mTOR is an essential component of the signaling pathways activated by these cytokines.

We also examined the effects of inhibiting PI3K (with LY294002), Akt (with Akt inhibitor) or MEK1/2 (with PD98054) on S6 phosphorylation. Activity of all three kinases is required for Ocl survival.<sup>18</sup> Inhibition of PI3K, mTOR or Akt resulted in complete inhibition of S6 ribosomal protein Ser235/6 phosphorylation in response to either cytokine (Figure 5c). Inhibition of MEK1/2 resulted in only partial reduction of S6 phosphorylation (Figure 5c and d). For inhibition of PI3K, we used LY294002 at a concentration of 5  $\mu$ M, which is approximately three-fold above its IC<sub>50</sub> for PI3K and equal to its reported IC<sub>50</sub> for mTOR autokinase activity *in vitro*.<sup>44</sup> Suppression of cytokine-induced S6 phosphorylation by LY294002 was complete (Figure 5c), suggesting that PI3K inhibition played a prominent role in the effect.

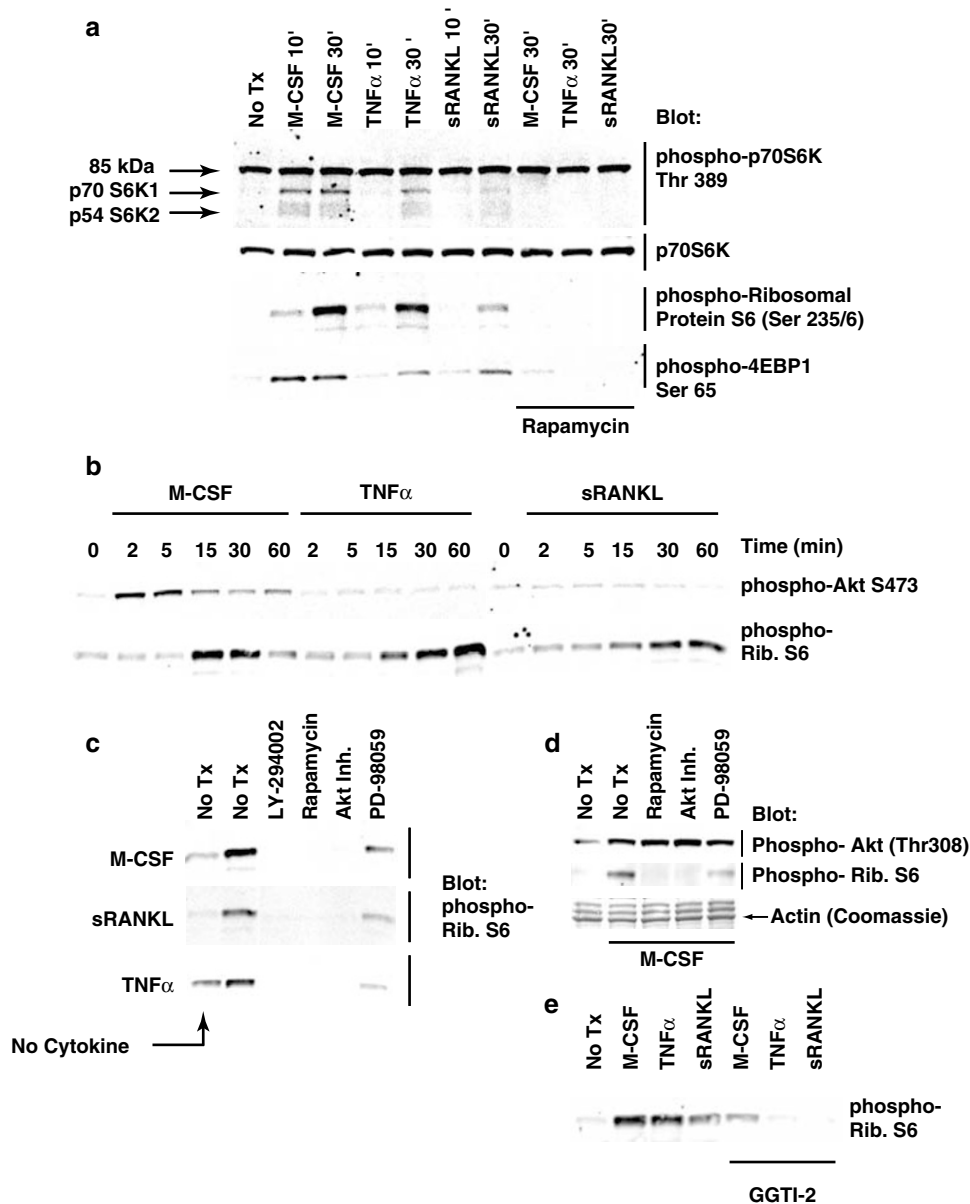
Downstream of PI3K, Akt acts to modulate mTOR via its effects on the TSC1/2 complex. Direct inhibition of Akt resulted in a complete loss of S6 ribosomal protein Ser235/6 phosphorylation in response to each cytokine (Figure 5c). This inhibitor did not interfere with phosphorylation of Akt itself (Figure 5d). The concentration of Akt inhibitor used (10  $\mu$ M) was two-fold above its IC<sub>50</sub> for suppression of Akt and eight-fold below its IC<sub>50</sub> for PI3K,<sup>45</sup> suggesting that this response was relatively Akt specific.

pp60<sup>C-src</sup> activity may also play a role in this part of the pathway, since it is required for signaling from PI3K to S6K and from RANKL to Akt.<sup>23,46</sup> Consistent with these reports the src family kinase inhibitor PP1 (10  $\mu$ M) completely suppressed cytokine-induced activation of S6K and phosphorylation of S6 (data not shown). Therefore, in addition to mTOR, activities of PI3K, Akt and pp60<sup>C-src</sup> seem necessary for the downstream effects of M-CSF, TNF $\alpha$  and RANKL on S6 phosphorylation in Ocl.

Geranylgeranylated proteins, specifically Cdc42 and Rac1 GTPases, have also been implicated in S6K activation.<sup>47</sup> Since M-CSF, sRANKL or TNF $\alpha$  delay apoptosis induced by GGTI-2 and ALN via inhibition of geranylgeranylation (see above), we investigated the effects of GGTI-2 on the



**Figure 4** Translational inhibition induces caspase activation and cleavage of MST1 kinase in Ocl. Purified Ocl were left untreated (Control, closed squares) or treated with emetine (Emt, open triangles), Chx (open squares), puromycin (Puro, closed triangles) or actinomycin D (ActD, closed inverted triangles). **(a)** Caspase-3 activity (upper panel) and caspase-9 activity (lower panel) in pooled Ocl cell lysates were quantified after 1–3 h of treatment, as in Materials and Methods. A total of three pooled-sampling experiments were performed with similar results. **(b)** After 2.75 h of Ocl treatment with translational or transcriptional inhibitors, MST1 kinase activities (arrowhead) in lysates were measured by in-gel kinase assay (as in Figure 1). One representative of three independent assays is shown



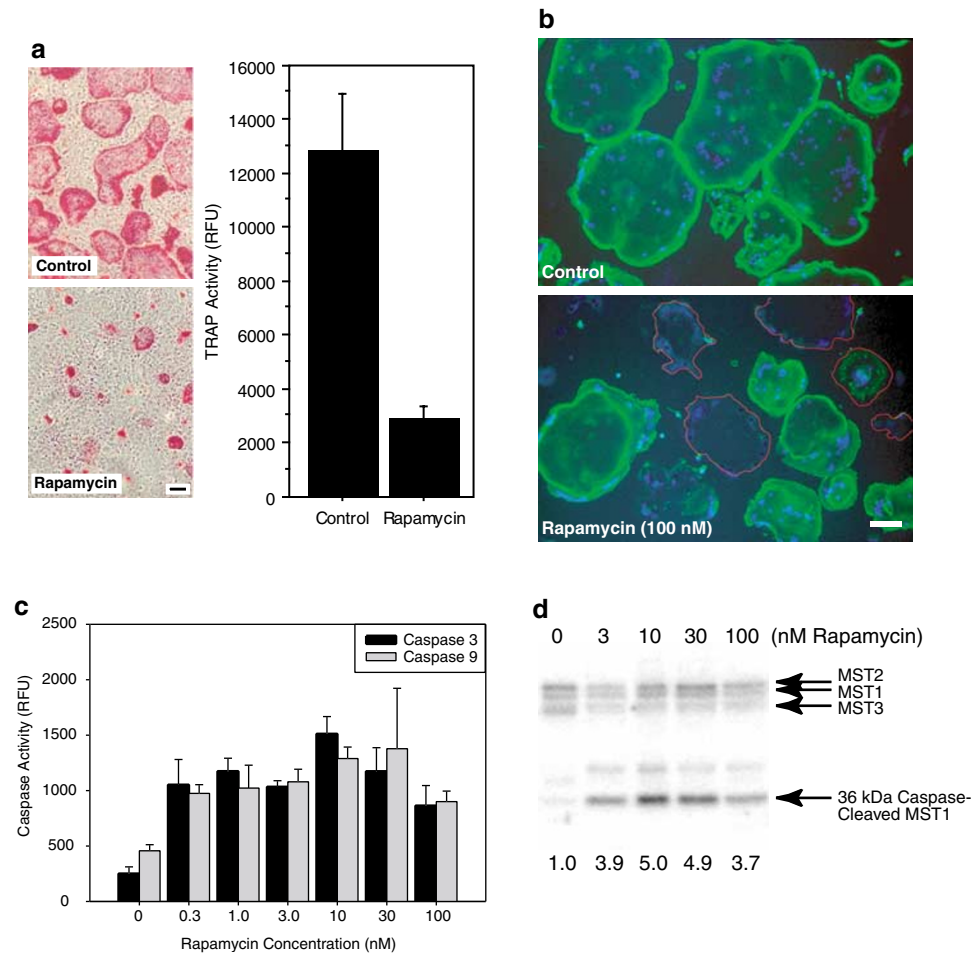
**Figure 5** Induction of mTOR and S6K by M-CSF, TNF $\alpha$  and sRANKL in Ocl. (a) Purified Ocl were treated with M-CSF, TNF $\alpha$  or sRANKL for 10 or 30 min and cell lysates were prepared and probed by immunoblotting with phospho-specific antibodies as indicated. Rapamycin (100 nM) was added 30 min prior to initiation of cytokine treatments. (b) Time course for induction of S6K activity in Ocl by cytokine was assessed using phospho-specific antibodies to S6 (phospho-Ser 235/6) and Akt (phospho-Ser 473). (c) S6 phosphorylation in response to M-CSF, sRANKL and TNF $\alpha$  at 30 min was assessed as in (b) in the absence (no Tx) or presence of inhibitors of: PI3K (LY-294002; 5  $\mu$ M), mTOR (Rapamycin; 100 nM), Akt (Akt Inh.; 10  $\mu$ M) and MEK1/2 (PD-98059; 20  $\mu$ M) or (e) GGTI-2 (2  $\mu$ M). (d) M-CSF (30 min) induction of Akt versus S6 phosphorylation in the presence or absence of rapamycin, Akt inhibitor or PD-98059. Inhibitors were added 30 min before cytokine treatments were started. One representative of three- to four independent assays is shown

phosphorylation of S6 ribosomal protein (Figure 5e). Pre-treatment of purified Ocl with GGTI-2 for 16 h reduced cytokine induced S6 ribosomal protein phosphorylation by 60–90%, depending on the cytokine tested. This suggests a possible role for geranylgeranylation in the regulation of the S6K pathway, likely through the established regulatory GTPases.<sup>47</sup> GGTI-2 and ALN had no consistent or substantial effect on signaling to Akt or NF- $\kappa$ B by any of the tested cytokines, as noted above. This suggests that the geranylgeranylation requirement is relatively specific for the mTOR/S6 kinase pathway downstream of M-CSF, RANKL and TNF $\alpha$ .

Overall, the suppressive effects of the signal transduction inhibitors on S6K were consistent for all three cytokines tested (M-CSF, TNF $\alpha$  and sRANKL), indicating that signaling by these prosurvival factors in Ocl intersects prior to activation of S6K and phosphorylation of S6.

### mTOR/S6K signaling is required for Ocl survival and function

Next, we investigated a possible role for mTOR activity in Ocl survival using the mTOR inhibitor, rapamycin. Rapamycin



**Figure 6** Rapamycin inhibition of mTOR induces Ocl apoptosis. (a) Osteoclastogenesis was assessed in the absence or presence of rapamycin (30 nM, days 2–7). TRAP staining (left panels) of mouse bone marrow/MB1.8 cell coculture (day 7) and quantification of TRAP activity (right panel) by a fluorescent assay as described in Materials and Methods. Values are mean  $\pm$  S.D. ( $n=8$ ); bar is 100  $\mu$ M. (b) Purified Ocl were treated with rapamycin (100 nM) for 20 h. Immunofluorescence image (overlay) of Ocl nuclei stained with Hoechst No. 33342 (Blue) together with FITC-phalloidin (Green) to visualize nuclei and the actin cytoskeleton. Using corresponding phase-contrast images, cell outlines were traced (Red) and superimposed for selected Ocl showing advanced induction of apoptosis. Bar is 100  $\mu$ M. (c) Caspase-9 and -3 activities were measured in cell lysates after 20 h treatment of Ocl with increasing concentrations of rapamycin, as indicated. Data are mean  $\pm$  S.D. ( $n=3$ ). (d) MST1 kinase activities in Ocl lysates were measured by in-gel kinase assay. The relative activity (*versus* control) of the 36 kDa caspase cleavage product of MST1 kinase is indicated beneath each lane

(30 nM) reduced the generation of mature TRAP-positive Ocl in cocultures of murine bone marrow cells and MB1.8 osteoblast-like cells by 75% (Figure 6a). This could be caused by suppression of the differentiation process or by the induction of Ocl apoptosis.

We examined induction of apoptosis using purified Ocls. Differentiated TRAP-positive Ocls generated in the coculture system were purified and maintained in the presence of M-CSF and sRANKL with increasing concentrations of rapamycin. Rapamycin selectively suppresses the translation of polypyrimidine mRNAs, which encode elongation factors (eEF1 $\alpha$  and eEF2) and ribosomal proteins. However, its overall effect on protein translation is more gradual, with an approximate 40% reduction in translation observed 21–24 h after onset of treatment.<sup>48,49</sup> In this study, there was no evidence of Ocl apoptosis at early time points; however, at 16–24 h rapamycin induced morphological features of apoptosis, including a loss of actin ring structure and membrane

integrity as well as the formation of pyknotic nuclei (Figure 6b). Consistent with a slower rate of translation suppression by rapamycin, a subset of Ocl still remained intact for up to 24 h. Thus, Ocl survival maintained by M-CSF and sRANKL could be blocked by the suppression of signaling through mTOR.

Rapamycin-induced apoptosis was further characterized by measuring caspase activities in cell lysates from treated and untreated Ocls (at 16 h). Most consistently, maximal effects were observed with rapamycin at 10 nM or above, whereby a six-fold induction in caspase-3 activity was seen both in individual experiments (Figure 6c) and in averages across several independent experiments (6.9-fold, data not shown). A concentration of 1 nM could induce caspase 3- up to five-fold (Figure 6c) with an average 4.1-fold induction seen across several independent experiments. Induction of caspase-9 was consistent with that of caspase-3 in all experiments. In separate analyses, the proapoptotic 36 kDa MST1 kinase was activated five-fold at 10 and 30 nM (Figure 6d). The lower



overall induction of caspase and 36 kDa MST1 activity by rapamycin compared to CHX treatment reflects the more asynchronous *versus* synchronous induction of apoptosis, respectively. These findings indicate that mTOR activity contributes to Ocl survival, and inhibition of its activity results in increased Ocl apoptosis.

Since rapamycin can shorten the lifespan of Ocl by inducing apoptosis, we examined if it inhibits bone resorption as a functional consequence of this effect. Rabbit bone marrow cells were grown and differentiated on bovine cortical bone slices for 3–4 days in the absence or presence of increasing concentrations of rapamycin (1–100 nM), which was added 4 h after seeding of marrow cells onto the bone slices. Rabbit Ocl bone resorption was measured by the release of collagen-I degradation product (C-terminal telopeptides, CTX) into the culture supernatants (Figure 7). Time-course analyses revealed that little bone resorption takes place during the first 24 h of culture and that ALN effects are not seen until 48 h or more.<sup>50</sup> We therefore examined rapamycin effects at 72 and 96 h, where it was observed to reduce bone resorption significantly at all tested concentrations. During the first 72 h, dose-dependent inhibition of up to 60% was observed,

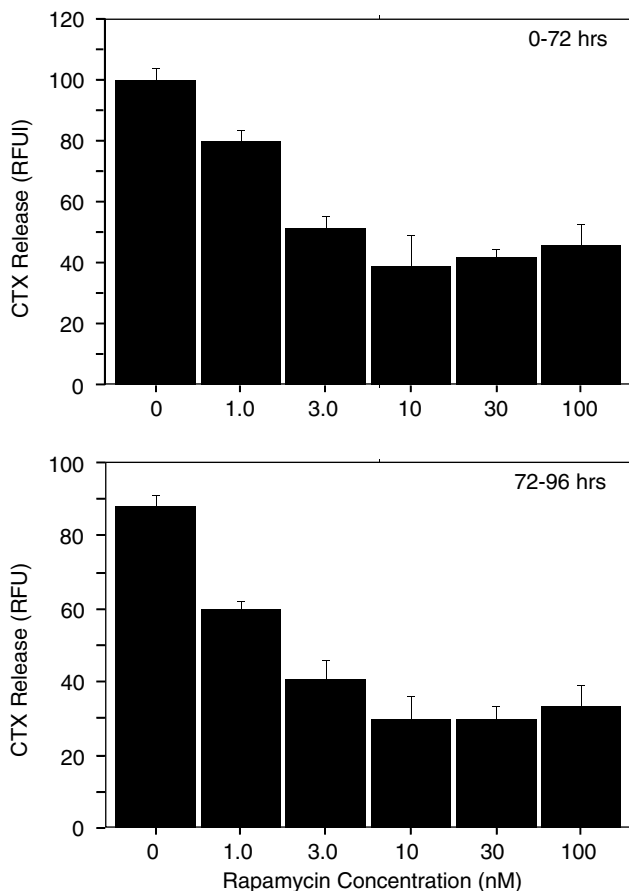
with an IC<sub>50</sub> of 1–3 nM. During the next 24 h (72–96 h time period), resorption was suppressed by about 70%. Similar dose dependence of apoptotic (Figure 6) and bone resorption effects (Figure 7) was observed in the respective mouse and rabbit models, with some slight variance at the lower end of the dosing range. As hypothesized, bone resorptive activity of rabbit Ocl at various concentrations of rapamycin correlated with the number of well-spread TRAP-positive cells on the bone slices ( $r^2=0.94$ ,  $P<0.0001$ ). Similar reductions in rabbit Ocl number were seen when cells were cultured on plastic instead of bone, comparable to the results from mouse Ocl cocultures described above.

## Discussion

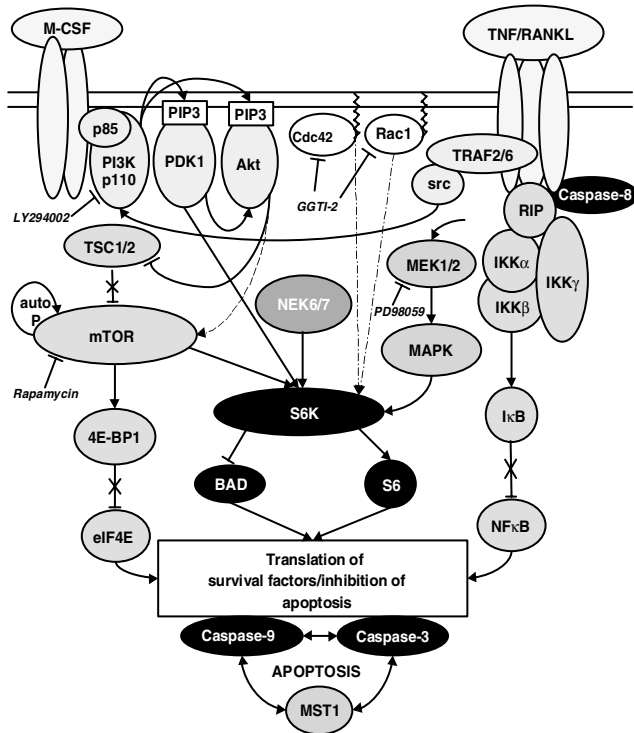
A delicate balance between bone resorption by Ocl and deposition of new bone by osteoblasts determines bone mass in the adult undergoing consistent adaptive bone remodeling. Imbalance of these coupled activities in favor of bone resorption results in bone loss and deterioration of its trabecular architecture leading to osteoporosis. Regulation of Ocl survival was suggested as a means to regulate resorptive activities.<sup>13,14,51</sup> Interestingly, cytokines produced by osteoblasts or stromal cells not only trigger differentiation, but also promote survival of mature Ocl. In this study, we describe for the first time an intersection of the signaling pathways induced in the Ocl by the antiapoptotic agents M-CSF, TNF $\alpha$  and RANKL at the level of mTOR/S6K activation. We also show that Ocl's have an acute requirement for ongoing protein translation for survival. mTOR/S6K regulation of protein translation may therefore play a critical role in regulating the lifespan of the Ocl, its bone-resorbing activity and, thus, play a role in regulating bone turnover.

The target of rapamycin, mTOR, is a Ser/Thr protein kinase and functions to regulate initiation and elongation phases of translation, as well as amino-acid import and the transcription of enzymes involved in multiple metabolic pathways.<sup>48,49,52</sup> mTOR can play both pro- and antiapoptotic roles, although the mechanisms remain largely elusive (see Castedo *et al.*<sup>27</sup> and references therein). In the present study, we demonstrate that mTOR is involved in the promotion of Ocl formation, survival and bone resorption. Interestingly, while suppressing apoptosis in multinucleated Ocl it also promotes apoptosis in HIV-env-fused cells. The latter response is linked to karyogamy, resulting in nuclear translocation of mTOR and subsequent p53 phosphorylation.<sup>53</sup> Here, we show that rapamycin treatment induces apoptosis in Ocl *in vitro*, as measured by morphological changes, by increased caspase activities and by cleavage and activation of the proapoptotic MST1 kinase. Further, inhibition of mTOR activation resulted in fewer TRAP-positive Ocls and a significant reduction in bone resorption *in vitro*. This defines mTOR, as a critical factor required for downstream effects of M-CSF, RANKL and TNF $\alpha$  on the Ocl.

Cytokine control of protein translation including mTOR activation does not seem to function within a linear signaling pathway, since PI3K activity is also necessary for further downstream signaling (Figure 8). A working model has been proposed, where the absence of either signal would prohibit activation of downstream targets.<sup>52</sup> In the Ocl, M-CSF was



**Figure 7** Inhibition of bone resorption by rapamycin. Rabbit bone marrow cells were seeded on bovine bone slices in 96-well plates and cultured in the absence or presence of rapamycin, at indicated concentrations, as described in Materials and Methods. Release of collagen-I C-terminal telopeptides (CTX) into the culture medium was measured after 72 h (upper panel) and between 72 and 96 h (lower panel). Data are mean  $\pm$  S.D. ( $n=3$ )



**Figure 8** Schematic illustration of mTOR/S6K intracellular signal transduction pathways in the Ocl. M-CSF, TNF $\alpha$  and RANKL induced signaling leading to activation of translation and inhibition of apoptosis. For simplicity, TNF $\alpha$  receptor and RANK are shown as one receptor

shown to signal through the PI3K pathway to activate the prosurvival kinase, Akt (see Nakamura *et al.*<sup>19</sup> and the present study). Signaling in Ocl to Akt by RANKL and TNF $\alpha$ , via p60<sup>C-src</sup> has also been reported.<sup>18,23</sup> However, although we found robust Akt activation by M-CSF in the Ocl, we did not observe substantial crossover signaling from either TNF $\alpha$  or RANKL. To this extent, the observation that all three cytokines converge on mTOR, as observed here in the Ocl, is unique. Only very recently has a link been found between Akt and S6K via TSC2 (tuberin) and mTOR.<sup>28–30</sup> Akt phosphorylation of TSC2, a negative regulator of mTOR, derepresses the mTOR-inhibitory function of the TSC1/2 complex and results in phosphorylation of both S6K and 4E-BP1. This is consistent with the negative effects of PI3K, Akt and Src-family inhibitors on S6K activity stimulated by M-CSF, sRANKL and TNF $\alpha$  seen here and previously for PI3K- and Src-family inhibitors in insulin-stimulated S6K activation in myoblasts and fibroblasts.<sup>46</sup> One discrepancy lies in the ability of sRANKL and TNF $\alpha$  to induce S6 phosphorylation in the Ocl without augmenting Akt activity. However, it is notable that measurable baseline Akt phosphorylation was detected at all time points. Indeed, all three cytokines stimulated phosphorylation of S6, with similar kinetics, with M-CSF-induced Akt activation rising and substantially declining prior to induction of S6K activity. This suggests that only a modest level of Akt activity is necessary and sufficient for S6K activation. As such, the TSC1/2 inhibition of mTOR may already be sufficiently derepressed in the Ocl, thus requiring only positive stimuli for S6K activation.

In addition to mTOR, S6K can be considered to be an integrator of diverse intracellular signal transduction pathways in the Ocl (Figure 8). Cytokine-mediated activation of S6K is a multistep process involving mTOR, NEK6 and PDK1. These kinases and mTOR-mediated suppression of a rapamycin-sensitive phosphatase have all been implicated in the phosphorylation of Thr389, a critical residue within the S6K autoinhibitory domain.<sup>31–34</sup> Phosphorylation of S6K at Thr389 releases the kinase from autoinhibition, enabling activation by PDK1 through phosphorylation of S6K (Thr229) within the kinase domain. We therefore used a phosphorylation state-specific antibody directed to Thr389 to assess cytokine activation in the Ocl. We found that M-CSF, TNF $\alpha$  and sRANKL all induce S6K phosphorylation in Ocl in a rapamycin-sensitive manner.

Erks also play a role in modulating S6K activity by targeting Thr421 and Thr424 in the COOH-terminal region. Previous work has shown that Erk1 and -2 are activated in Ocl by cytokine treatments, and Erk activation prevents Ocl apoptosis.<sup>18,23,54</sup> Consistent with this, we detected cytokine-induced phosphorylation of S6K at Thr421 and Thr424 using phosphorylation state-specific antibodies (data not shown). Furthermore, suppression of Erk activation with the Mek inhibitor, PD98059, resulted in partial suppression of S6K activity, as measured by S6 phosphorylation.

Other pathways upstream of S6K include geranylgeranylated small GTPases, such as Cdc42 and Rac1.<sup>47</sup> This seems important since M-CSF, TNF $\alpha$  and sRANKL, which activate S6K, also suppress induction of ALN- and GGTI-2-induced apoptosis at relatively high concentrations. This, together with the observation that GGTI-2 substantially reduces signaling of these cytokines through S6K, suggests a possible convergence of pro- and antiapoptotic signals of these agents in the Ocl. By inference, ALN and other nitrogen-containing bisphosphonates, which act in the Ocl via suppression of geranylgeranylation,<sup>16,39,40,55</sup> may induce apoptosis and suppress osteoclastic bone resorption, in part, via suppressing the mTOR/S6K pathway. This is also supported by the observation that rapamycin treatment induces apoptosis in Ocl with similar kinetics.

The antiapoptotic effect of mTOR identifies this enzyme as an essential participant in cytokine-induced prosurvival signal transduction in Ocl, in addition to Akt and NF- $\kappa$ B, probably acting via its positive impact on protein translation. Possible pathways for mTOR-dependent suppression of apoptosis in the Ocl include S6K-mediated phosphorylation and thus inactivation of the proapoptotic BCL-2 family member, BAD.<sup>35</sup> However, in Ocl, lysates we were unable to detect BAD protein and phosphorylation thereof, possibly due to very low levels of expression (data not shown).

A possible mechanism for rapamycin induction of Ocl apoptosis is via its suppressive effects on mTOR, S6K and, ultimately, 4E-BP1 and S6. Rapamycin inhibition of translation could lead to inadequate synthesis of a set of proteins that include survival factor(s), which according to their normal rate of turnover, become rate limiting. In this context, Li *et al.*<sup>56</sup> found a role for 4E-BP1 in the translational control of proteins critical for cell survival. We observed in this study the suppressed phosphorylation of 4E-BP1 by treatment of Ocl with rapamycin, which may account for part of the apoptotic

response. Expression of BCL-2-like proteins, such as MCL-1, can be attenuated by rapamycin.<sup>57</sup> Very recently Woltman *et al.*<sup>58</sup> reported that GM-CSF-mediated survival of dendritic cells indeed depends on PI3K/mTOR signaling and MCL-1. These findings along with those presented here suggest a broader involvement of mTOR in cell survival within the monocyte/macrophage-derived cell lineages. We therefore postulate that such proteins may play a critical role in the Ocl and in the responses described here.<sup>59</sup> Taken together, we can conclude that tight control of translation seems to be necessary to provide BCL-2-like proteins and prolong Ocl survival.<sup>60,61</sup> Identification of responsible candidate proteins is the subject of further research.

In summary, we describe for the first time a shared role for mTOR/S6K in the prosurvival signaling of osteoclastogenic cytokines. Inhibition of this translational control pathway results in initiation of apoptosis in Ocl *in vitro* and ultimately reduces bone resorption. The Ocl exhibits an acute dependence on continual protein translation for survival, a property not commonly found in most cells. This study therefore suggests downstream effectors of mTOR and S6K, including 4E-BP1, S6 and, possibly, BCL-2 family proteins to contribute to the survival of these bone-resorbing cells.

## Materials and Methods

### Cell culture and reagents

Ocl-like cells were generated by coculturing mouse bone marrow cells (from two mice) together with mouse MB1.8 cells in differentiation medium (MEM- $\alpha$  supplemented with 10% FBS and 10 nM  $1.25(\text{OH})_2$ -Vitamin D<sub>3</sub>) in six-well plates maintained in a humidified atmosphere with 10% CO<sub>2</sub>.<sup>62</sup> MB1.8 cells were removed by collagenase treatment<sup>63</sup> and purified Ocl were further cultured in the presence of 10–15 ng/ml M-CSF (R&D, Minneapolis, MN, USA) to suppress spontaneous apoptosis. For signal transduction experiments Ocl were cultured in 0.5% FBS without M-CSF for 1–2 h. Ocl were then treated either with M-CSF (40 ng/ml; R&D), TNF $\alpha$  (50 ng/ml; R&D), IL-1 $\alpha$  (50 ng/ml; R&D) or sRANKL (100 ng/ml; R&D) for various times in 0.5% FBS. The following reagents were used at indicated concentrations: cycloheximide (50  $\mu$ M; Biovision Inc., Mountain View, CA, USA), puromycin (50  $\mu$ g/ml, Sigma; St. Louis, MO, USA), emetine (50  $\mu$ g/ml, Sigma), actinomycin D (5  $\mu$ M; Biovision), rapamycin (3–100 nM, Calbiochem, San Diego, CA, USA), LY 294002 (5  $\mu$ M, Calbiochem), Akt inhibitor (10  $\mu$ M; Calbiochem), PD98059 (20  $\mu$ M; Alexis Corp., San Diego, CA, USA). GGT1-2 was a kind gift of Bill Lumma (Department of Medicinal Chemistry, Merck & Co., Inc.).

### Preparation of protein lysates and Western blotting

After treatments, cells were placed on ice and washed twice with ice-cold  $\beta$ -glycerophosphate-HEPES-buffered solution ( $\beta$ -HBS): 50 mM HEPES (pH 7.6),  $\beta$ -glycerophosphate (50 mM), EGTA (1 mM), NaCl (150 mM), and then lysed in  $\beta$ -HBS containing Triton X-100 (0.2%), microcystin LR (1  $\mu$ M), Na<sub>3</sub>VO<sub>4</sub> (1 mM), dithiothreitol (1 mM), and phenylmethylsulfonyl fluoride (1 mM) and a protease inhibitor mixture (Sigma) as described.<sup>16,40</sup> Protein concentrations were determined using a Bradford reagent kit (Bio-Rad, Hercules, CA, USA). Typically, 10–15  $\mu$ g of protein lysate could be generated from one well.

Equal amounts of protein lysates were loaded and separated on 12 or 4–15% gradient Tris-HCl gels (Bio-Rad). Proteins were transferred on

PVDF membranes, which were blocked with 5% nonfat milk/5% BSA in TBST (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% Tween-20). Primary antibodies were incubated overnight at 4°C with gentle agitation. Antiphospho-p70 S6 Kinase (Thr389) antibody, anti-p70 S6 kinase antibody, phospho-S6 ribosomal protein (Ser235/236) antibody, phospho-4E-BP1 (Ser65) antibody, phospho-Akt (Ser473), phospho-Akt (Thr 308), anti-Akt antibody, phospho-I $\kappa$ B $\alpha$  (Ser32/36) antibody (all from Cell Signaling Technology, Beverly, MA, USA), and anti-I $\kappa$ B $\alpha$  antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) were diluted 1 : 1000 in blocking solution. Detection was performed with alkaline phosphatase coupled secondary antibodies (1 : 10,000, Santa Cruz) and ECF substrate (Amersham, Piscataway, NJ, USA) using a Molecular Dynamics Storm 860 system (Sunnyvale, CA, USA).

### In-gel kinase assay

Kinase assays were performed in the gel using myelin basic protein (MBP) as a substrate, essentially as described with one modification: kinase buffer contained 20 mM MgCl<sub>2</sub> in place of 5 mM MnCl<sub>2</sub>.<sup>16</sup> Lanes were loaded with equivalent amounts of protein lysate (5–10  $\mu$ g/lane), and then gels were electrophoresed and processed for kinase assay. Dried gels were exposed to phosphorimaging screens and scanned and analyzed using the Storm 860 system.

### NF- $\kappa$ B activity assay

Ocl in six-well plates were incubated in regular growth medium supplemented with 10 ng/ml M-CSF overnight. Cells were starved in growth medium + 0.5% FBS without M-CSF for 1 h and then TNF $\alpha$  (50 ng/ml), RANKL (100 ng/ml) or M-CSF (80 ng/ml) was added for the times indicated. Whole-cell lysates were prepared and samples from three separate identically treated wells were pooled. This was necessary to generate sufficient material for assay. Using pooled samples, activated p65 NF- $\kappa$ B (RelA) was measured using a Trans-AM NF- $\kappa$ B Transcription Factor Assay Kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. For this assay, activity is measured in a 96-well format assessing both NF- $\kappa$ B-DNA binding and, simultaneously, antibody binding to a NF- $\kappa$ B epitope that is inaccessible when in a complex with I $\kappa$ B.

### Caspase activity assay

The fluorescent caspase-3 activity assay kit was from Biovision Inc.; the caspase-9 activity assay kit was from Oncogene Research Products (Boston, MA, USA). Cells were washed with Hank's-buffered salt solution (HBSS), and lysed and scraped in the lysis buffer supplied by the manufacturer. As with assays for NF- $\kappa$ B activation, lysates from two to three independently treated wells were pooled to generate sufficient material for assay. Lysates were cleared by centrifugation and supernatants were assayed according to instructions provided with each kit. Total protein (20–40  $\mu$ g) was used per assay, and activities were determined after 2 h incubation at 37°C. Values are in relative fluorescence units (RFUs)/ $\mu$ g protein.

### Fluorescent TRAP-activity assay

Cocultures of bone marrow cells and MB1.8 cells in 24-well plates were treated with 30 nM rapamycin for 7 days. Cells were washed with HBSS and lysed in 250  $\mu$ l solution (50 mM sodium acetate (pH 5.0), 30 mM sodium tartrate, 0.1% Triton X-100, 2.5 mg/ml naphthol AS-BI phosphate) for 30 min at 37°C. Reactions were stopped by adding 12.5  $\mu$ l 1 M NaOH

and TRAP activity was measured with a fluorescent plate reader (ex.360 nm/em.530 nm).

## Histochemistry

Ocl-like cells were washed with HBSS and fixed in 3.7% formaldehyde for 15 min and stained essentially as described.<sup>50</sup> Briefly, cells were stained for TRAP in 50 mM sodium acetate (pH 5.0), 30 mM sodium tartrate, 0.1% Triton X-100 with 0.3 mg/ml fast red violet LB (Sigma, St. Louis, MO, USA) and 0.1 mg/ml naphthol AS-MX phosphate (Sigma). Actin and nuclei were stained with 5 U/ml TRITC or FITC-labeled phalloidin (Molecular Probes, Eugene, OR, USA) together with 50 µg/ml Hoechst 33342 dye (Molecular Probes).

## Bone resorption assay

Bone resorption by Ocl's differentiated from New Zealand White rabbit tibiae (Covance Research Products, Denver, PA, USA) was measured as described.<sup>63</sup> Briefly, tibiae were minced and bone fragments rocked gently in  $\alpha$ -MEM with 10 mM HEPES (pH 7.1). A total of  $10^6$  cells/well in  $\alpha$ -MEM supplemented with 10% FBS and 10 nM  $1,25(\text{OH})_2\text{-Vitamin D}_3$  were seeded on bovine bone slices in 96-well plates and cultured for 4 h. Thereafter, rapamycin (3–100 nM) was added in triplicate and cultures were continued for additional 68 or 92 h (72 or 96 h total in culture). Collagen fragments released into the medium were measured by the CrossLaps ELISA assay (Osteometer Biotech, Herlev, Denmark). Statistical analysis was performed with StatView (SAS Institute Inc., Cary, NC, USA).

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