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The two homologous chaperonin 60 proteins of *Mycobacterium tuberculosis* have distinct effects on monocyte differentiation into osteoclasts

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Summary

Mycobacterium tuberculosis **produces two homologous chaperonin (Cpn)60 proteins, Cpn60.1 and Cpn60.2 (Hsp65). Both proteins stimulate human and murine monocyte cytokine synthesis but, unlike Cpn60 proteins from other microbial species, fail to stimulate the breakdown of cultured murine bone. Here, we have examined the mechanism of action of these proteins on bone remodelling and osteoclastogenesis, induced *in vitro* in murine calvarial explants and the murine monocyte cell line RAW264.7. Additionally, we have determined their effect on bone remodelling *in vivo* in an animal model of arthritis. Recombinant Cpn60.1 but not Cpn60.2 inhibited bone breakdown both *in vitro*, in murine calvaria and *in vivo*, in experimental arthritis. Analysis of the mechanism of action of Cpn60.1 suggests that this protein works by directly blocking the synthesis of the key osteoclast transcription factor, nuclear factor of activated T cells c1. The detection of circulating immunoreactive intact Cpn60.1 in a small number of patients with tuberculosis but not in healthy controls further suggests that the skeleton may be affected in patients with tuberculosis. Taken together, these findings reveal that *M. tuberculosis* Cpn60.1 is a potent and novel inhibitor of osteoclastogenesis both *in vitro* and *in vivo* and a potential cure for bone-resorptive diseases like osteoporosis.**

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Introduction

Chaperonin (Cpn)60, or heat shock protein (Hsp)60, is the prototypic intracellular molecular chaperone and much of our knowledge of chaperone-induced protein folding has emerged from the study of the *Escherichia coli* (GroEL) protein (Kerner *et al*., 2006). Over the past decade, it has also become evident that, in addition to folding proteins intracellularly, molecular chaperones can be secreted from cells and can exist on the cell surface, or in the extracellular fluid, where they can act as intercellular signalling proteins, or even agonist receptors (Henderson and Pockley, 2005).

The first report that Cpn60 proteins had intercellular signalling activity used the Cpn60.2 (Hsp65) protein from the major human pathogen, *Mycobacterium tuberculosis*. This protein was shown to activate human monocytes to release pro-inflammatory cytokines (Friedland *et al*., 1993). Several years later, Henderson's group reported that a potent bone-resorbing protein released by the oral bacterium, *Aggregatibacter (Actinobacillus) actinomycetemcomitans*, was the Cpn60 protein of this organism (Kirby *et al*., 1995). *A. actinomycetemcomitans* is the causative agent of a form of periodontal disease, known as localized aggressive periodontitis, in which there is a rapid loss of the jaw bone supporting the molars and premolars (Henderson *et al*., 2003). GroEL was also shown to be a potent inducer of bone resorption *in vitro* (Kirby *et al*., 1995). It was established that GroEL was able to cause bone resorption because it stimulated the proliferation and differentiation of the myeloid precursor cells that form the bone-resorbing osteoclast population of bone (Reddi *et al*., 1998). Surprisingly, the highly homologous Cpn60.2 proteins from *M. tuberculosis* and *Mycobacterium leprae* failed to stimulate bone breakdown *in vitro* (Kirby *et al*., 1995).

Mycobacterium tuberculosis H37Rv is one of a small proportion of bacteria that have more than one gene coding for Cpn60 proteins. The bacterium has two *cpn60*

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genes, which code for proteins that are known as Cpn60.2 (GENBANK locus tag Rv0440) or Hsp65 and Cpn60.1 (GENBANK locus tag Rv3417c), the latter gene only being identified in 1993 (Kong *et al*., 1993). These two proteins share 60% sequence identity. We assumed that the Cpn60.1 protein would exhibit the ability to promote osteoclast formation and induce bone resorption. Contrary to our expectations, the Cpn60.1 protein was totally inactive as an inducer of bone resorption. The major bone-resorbing component of *M. tuberculosis* was identified as the cochaperone, Cpn10 (Meghji *et al*., 1997).

The myeloid cell populations of the mammal are the first line of defence against bacterial infection and we are only now beginning to understand how bacteria can interact and influence myeloid cell function. A good example is *M. tuberculosis* which can survive within the macrophage and induce a granulomatous response in which novel forms of myeloid cell differentiation, in the form of epithelioid cells and multinucleate giant cells, occur (Russell, 2007). There is increasing interest in the processes by which osteoclasts are formed and activated. Osteoclasts, along with muscle cells, are the only two naturally multinucleated cells present in the mammal. In recent years, the mechanism of osteoclast differentiation has begun to be elucidated with three tumour necrosis factor (TNF) family members, termed receptor activator of NF-κB ligand (RANKL), RANK and osteoprotegerin (OPG), acting in a complex feedback system to control osteoclast formation (osteoclastogenesis) (Asagiri and Takayanagi, 2007).

All the early studies of the Cpn60 proteins from various bacteria and mammals supported the hypothesis that these proteins were pro-inflammatory molecules able to activate myeloid cells, vascular endothelial cells and epithelial cells (Maguire *et al*., 2002; Henderson, 2003). However, we have found that recombinant *M. tuberculosis* Cpn60.1, but not Cpn60.2, has direct anti-inflammatory effects in an animal model of asthma (Riffo-Vasquez *et al*., 2004). This has prompted us to re-examine the interaction of the *M. tuberculosis* Cpn60 proteins with bone and bone cells, and has revealed that the Cpn60.2 protein has no positive or negative effects on these cells. In contrast, we have established that the *M. tuberculosis* Cpn60.1 protein is a potent inhibitor of osteoclastogenesis both *in vitro* and *in vivo*.

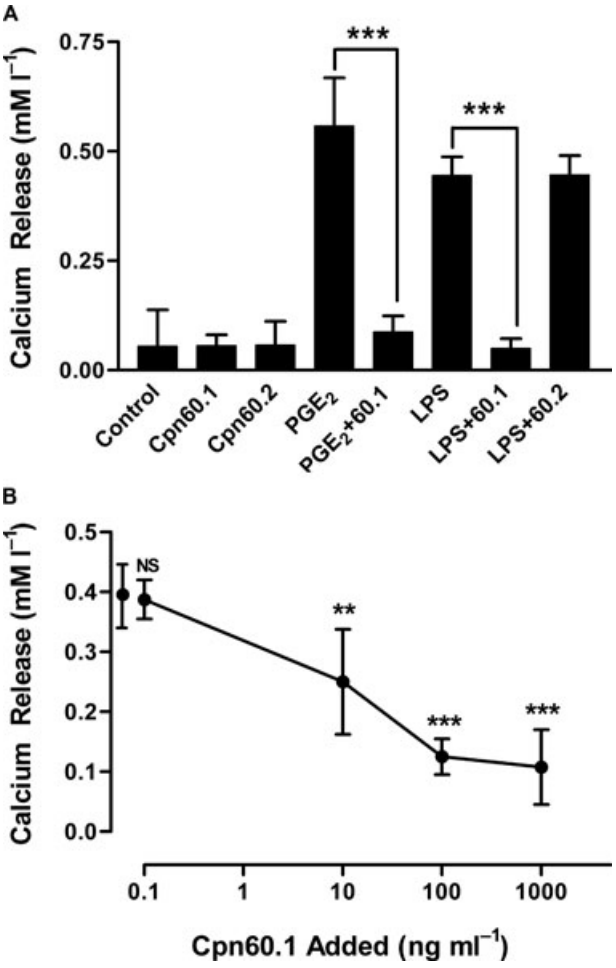

Fig. 1. A. The inhibitory effect of *M. tuberculosis* Cpn60.1 on agonist-induced murine calvarial bone resorption. The figure shows from left to right: (i) the spontaneous release of calcium from unstimulated bone (control); (ii) and (iii) the release of calcium from bone exposed to 1 μg ml^{-1} Cpn60.1 or 1 μg ml^{-1} Cpn60.2 respectively (Cpn60.1, Cpn60.2); (iv) calcium release stimulated by 1 μM PGE_2 (PGE_2); (v) the inhibition of PGE_2-stimulated calcium release by 1 μg ml^{-1} Cpn60.1 (PGE_2 + 60.1); (vi) calcium release stimulated by 10 μg ml^{-1} *P. gingivalis* LPS (LPS); (vii) the inhibition of LPS-induced calcium release by 1 μg ml^{-1} Cpn60.1 (LPS + 60.1) and (viii) the lack of inhibition of LPS-induced calcium release by 1 μg ml^{-1} Cpn60.2 (LPS + 60.2). Results are expressed as the mean and standard deviation of five replicate cultures from a typical experiment (***$P < 0.001$).
B. Dose-dependent inhibition of RANKL-induced murine calvarial bone resorption by Cpn60.1. Murine calvarial explants have been stimulated to resorb by addition of 30 ng ml^{-1} RANKL. The addition of recombinant *M. tuberculosis* Cpn60.1 over the concentration range 10 ng ml^{-1}–1 μg ml^{-1} caused a dose-dependent inhibition of bone resorption. Results expressed as mean and standard deviation of five replicate cultures from a typical experiment and significance relative to the control (far left spot; no addition of Cpn60.1) denoted accordingly (NS, not significant; **$P = 0.01$; ***$P = 0.001$).

Results

Influence of M. tuberculosis *Cpn60 proteins in the murine calvarial bone resorption assay*

The measurement of the release of calcium from cultured neonatal murine calvaria is a standard assay for identifying and quantifying osteolytic (bone-destroying) molecules (Meghji *et al*., 1998). Addition of Cpn60.1 or Cpn60.2 [1 μg ml^{-1} – equivalent to an 8 nM solution assuming the native state of these proteins is dimeric (Qamra *et al*., 2005)] to murine calvaria stimulated no release of calcium (Fig. 1A). In other experiments, we

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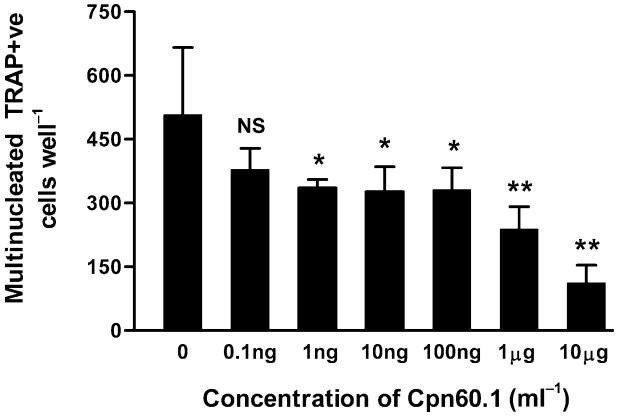

Fig. 2. Dose-dependent inhibition of the formation of TRAP-positive multinucleate cells from mononuclear RAW264.7 precursors. Wells were seeded with 4×10^3 RAW264.7 cells and, after 24 h, RANKL (50 ng ml^{-1}) was added with either no Cpn60.1 or 10-fold increasing concentrations of this protein. Cells were cultured for a further 6 days and then the numbers of TRAP-positive multinucleate cells per well were enumerated. In the absence of RANKL, no osteoclasts formed. Results are expressed as the mean and standard deviation of six replicate cultures from a typical experiment and significance relative to the control (0) denoted accordingly (NS, not significant; *$P = 0.05$; **$P = 0.01$).

have added between 10 and 100 μg ml^{-1} of these chaperonins without evidence of calcium release. To determine if either of the mycobacterial Cpn60 proteins had the ability to inhibit agonist-induced calcium release from murine calvaria, they were added at 1 μg ml^{-1} to cultures stimulated with 1 μM prostaglandin (PG)E_2 or 10 μg ml^{-1} *Porphyromonas gingivalis* lipopolysaccharide (LPS). With both agonists, addition of Cpn60.1 at 1 μg ml^{-1} completely inhibited the release of calcium ($P < 0.001$). In contrast, Cpn60.2 had no inhibitory activity. To determine the potency of Cpn60.1 as an osteolytic inhibitor, it was added over the molar concentration range from 8 pM to 8 nM (10 ng ml^{-1} to 1 μg ml^{-1}) to calvaria stimulated to resorb by the addition of 30 ng ml^{-1} RANKL. Significant inhibition of bone breakdown was seen at concentrations of Cpn60.1 of between 80 and 800 pM (Fig. 1B). The murine calvarial bone resorption assay is stimulated by low concentrations of LPS. The lack of osteolytic activity of the two recombinant *M. tuberculosis* Cpn60 proteins demonstrates that they are free of LPS contamination. This is supported by the use of the *Limulus* assay for LPS which shows that the *M. tuberculosis* Cpn60 proteins contain < 10 pg LPS μg^{-1} protein.

Influence of M. tuberculosis *Cpn60 proteins on osteoclast production from RAW cells and murine bone marrow*

In the presence of 50 ng ml^{-1} RANKL, the murine monocyte cell line, RAW264.7 will form large numbers of tartrate-resistant acid phosphatase (TRAP)-positive multinucleate cells that are conventionally identified as mature osteoclasts (Cuetara *et al.*, 2006). In the absence of RANKL, no osteoclasts form over the 7 day period of the assay. Addition of *M. tuberculosis* Cpn60.1 or Cpn60.2 to RAW cell cultures failed to induce osteoclast formation. Addition of a range of concentrations of Cpn60.2 to RAW cells stimulated to form osteoclasts by addition of 50 ng ml^{-1} RANKL had no effect on osteoclast formation (results not shown). In contrast, Cpn60.1 caused a dose-dependent inhibition of osteoclast formation (Fig. 2). Routine measurement of cell viability revealed no increase in cell death in cultures exposed to Cpn60.1, ruling out this as an explanation for decreased osteoclast numbers. Similar results were obtained with cultures of murine bone marrow-derived cells.

Time-course of the inhibition of osteoclast formation by Cpn60.1

The osteoclast induction assay takes 5–7 days and this encompasses the various steps in the formation of mature osteoclasts, including the proliferation, differentiation and fusion of the osteoclast precursor cells (Cuetara *et al.*, 2006). To determine if Cpn60.1 influenced the early or later stages of osteoclastogenesis, RAW cells were activated by the addition of RANKL, and Cpn60.1 was added to such cultures either immediately or after 1, 2, 3 or 4 days of culture. Surprisingly, the time of addition of Cpn60.1 was irrelevant and there was the same significant degree of inhibition of osteoclast formation at each time point analysed (Fig. 3).

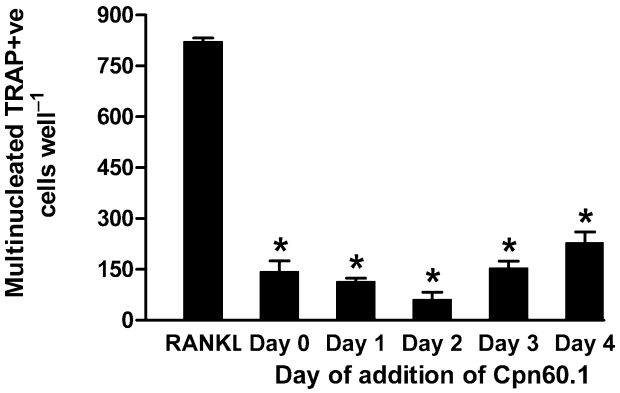

Fig. 3. Effect of time of addition of *M. tuberculosis* Cpn60.1 to RAW264.7 cells on osteoclast formation. Wells were seeded with 4×10^3 RAW264.7 cells and, after 24 h, RANKL (50 ng ml^{-1}) was added to promote osteoclast formation. The addition of Cpn60.1 was either concurrent with the RANKL or was added at 1, 2, 3 or 4 days after addition of RANKL. After 7 days, the numbers of TRAP-positive multinucleate cells per well were enumerated. No osteoclasts formed in the absence of exogenous RANKL. Results are expressed as the mean and standard deviation of four replicate cultures from a typical experiment. Inhibition of osteoclast formation by Cpn60.1 was highly significant (*$P < 0.00001$), irrespective of the day of addition.

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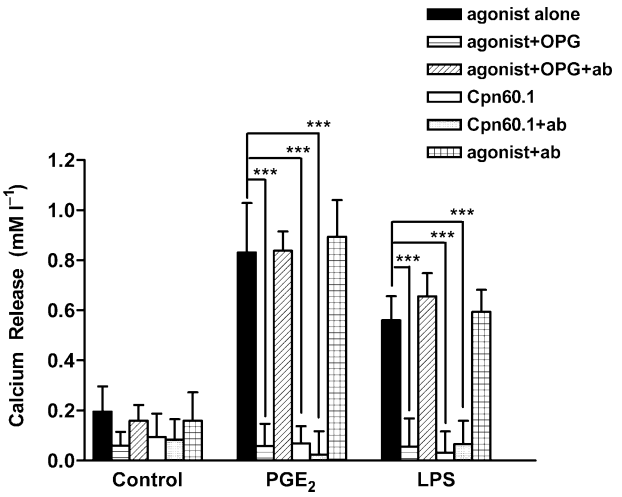

Fig. 4. Failure of Cpn60.1 to induce the production of OPG. Murine calvaria were exposed to either no osteolytic stimulant or to 1 μM PGE_2 or 10 μg ml^{-1} LPS. In each set of columns, the following treatments were made: (i) agonist alone, (ii) agonist plus 2 μg ml^{-1} OPG, (iii) agonist plus OPG 2 μg ml^{-1} and 1 μg ml^{-1} neutralizing antibody to OPG, (iv) 1 μg ml^{-1} Cpn60.1, (v) 1 μg ml^{-1} Cpn60.1 plus 1 μg ml^{-1} neutralizing antibody to OPG and (vi) agonist plus 1 μg ml^{-1} anti-OPG antibody. Results are expressed as mean and standard deviation of five replicate cultures from a typical experiment. Failure to block Cpn60.1 inhibition of bone resorption by anti-OPG demonstrates that the chaperonin is not upregulating synthesis of OPG. Statistical significance within groups relative to the control (agonist alone) is shown on the figure (***$P < 0.001$).

Mechanism of action of Cpn60.1: extracellular effects

The simplest explanations for the inhibitory actions of Cpn60.1 are that it inhibits RANKL binding to RANK or that it induces the synthesis of OPG. To determine if Cpn60.1 could interfere with RANKL binding to RAW264.7 cells, we used FITC-labelled RANKL and assessed binding by flow cytometry. Concentrations of Cpn60.1 able to inhibit osteoclastogenesis failed to inhibit RANKL binding to cells (results not shown). To determine if Cpn60.1 bound directly to either RANKL or RANK to influence their function, we set up a direct binding enzyme-linked immunosorbent assay (ELISA). This failed to show any binding of Cpn60.1 to RANKL or RANK (results not shown). To determine if Cpn60.1 can induce the synthesis of OPG, we used a neutralizing antibody to OPG which was added to murine calvarial explants stimulated to resorb by the addition of LPS. Addition of Cpn60.1 inhibited LPS-induced bone resorption and this osteolysis was not blocked by the addition of sufficient anti-OPG antibody to block the effects of 2 μg ml^{-1} OPG (Fig. 4).

Mechanism of action of Cpn60.1: intracellular effects

The interaction of RANKL with RANK induces a complex intracellular signalling network involving the NF-κB system, nuclear factor of activated T cells (NFAT)c1 and MAP kinases, particularly c-Jun N-terminal kinase (cJNK). An obvious hypothesis was that Cpn60.1 would inhibit the activation of one or more of these intracellular signalling components. In the case of NF-κB and NFATc1, RANKL stimulated the rapid phosphorylation of I-κB and NFATc1 respectively. Addition of Cpn60.1 acutely (5–120 min) and chronically (24 h) failed to inhibit such phosphorylation (results not shown). In the case of cJNK, Cpn60.1 actually stimulated the phosphorylation and activation of this key kinase (results not shown). Thus these particular signalling systems do not seem to be the target of Cpn60.1. We also measured the levels of mRNA for NFATc1 in RAW264.7 cells stimulated with RANKL using quantitative RT-PCR. At 24 h after the addition of Cpn60.1, there was significant inhibition of the amplifiable levels of NFATc1 mRNA in the cytosols of RAW264.7 cells. The levels of NFATc1 mRNA were abrogated almost to control levels in cells treated with Cpn60.1 for 48 h. However, cells incubated with Cpn60.1 for 96 h had no significant decrease in NFATc1 mRNA. In contrast to Cpn60.1, the addition of Cpn60.2 produced no significant inhibition in the levels of NFATc1 mRNA (Fig. 5).

Influence of M. tuberculosis *chaperonins on adjuvant arthritis*

Adjuvant arthritis in the rat is a chronic inflammatory condition of the joints which, in afflicted joints, is characterized by excessive osteoclastic bone resorption. In this study, we compared the anti-inflammatory and anti-resorptive actions of the three recombinant *M. tuberculosis* chaperonins. In replicate experiments, the administration of Cpn10 to rats with adjuvant arthritis caused a statistically significant inhibition of the clinical joint score ($P < 0.001$), suggesting that this protein was having an anti-inflammatory effect. In contrast, Cpn60.2 exacerbated the inflammation in the joints ($P < 0.001$). Administration of Cpn60.1 had absolutely no effect on the joint score. The results from a typical experiment are shown (Fig. 6).

Influence of M. tuberculosis *chaperonins on adjuvant arthritis bone pathology*

In animals treated with vehicle (arthritis control), radiographic imaging of the feet showed clearly that joint architecture was lost with a narrowing of the joint spaces, the formation of bone erosions and the induction of periarticular osteoporosis. Although significantly enhancing disease severity, therapeutic administration of *M. tuberculosis* Cpn60.2 had no effect on osteoclastic bone remodelling while Cpn10, in spite of its apparent anti-inflammatory effects (reduction in clinical score), moderately exacerbated the joint destruction, but just failed to reach statistical significance. In complete contrast, the administration

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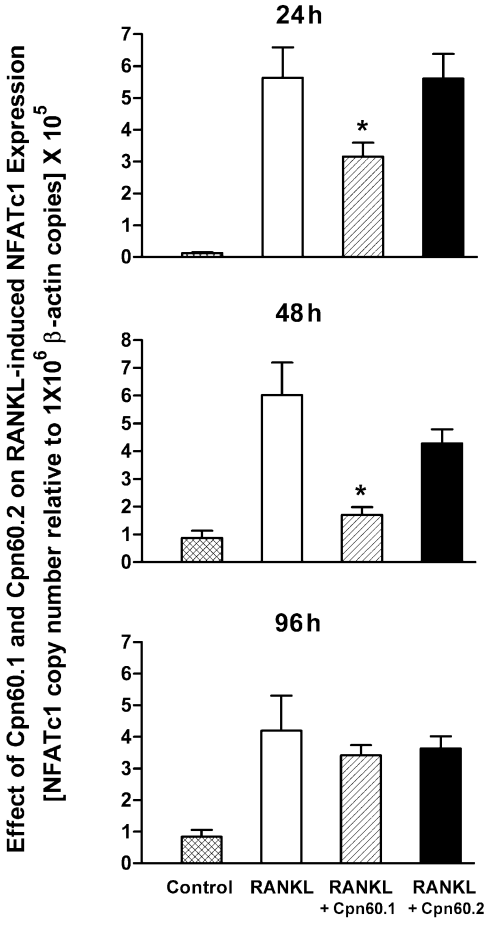

Fig. 5. *M. tuberculosis* Cpn60.1 decreases the levels of intracellular NFATc1 mRNA at 24 and 48 h. Addition of 50 ng ml^{-1} RANKL to RAW264.7 cells for 24, 48 or 96 h resulted in a significant increase in intracellular levels of mRNA for NFATc1. The effect of adding 10 μg ml^{-1} recombinant *M. tuberculosis* Cpn60.1 or Cpn60.2 to these cultures on NFATc1 mRNA levels was monitored. Cpn60.1 significantly inhibited this increase in mRNA levels at 24 (*$P < 0.02$) and 48 h (*$P < 0.0008$), but not at 96 h. Addition of Cpn60.2 had no significant effect on NFATc1 mRNA levels at any time point. Results are expressed as the mean and standard deviation of six replicate cultures from a typical experiment.

of *M. tuberculosis* Cpn60.1 almost completely abrogated the marked osteoclastic bone resorption seen in vehicle-treated animals as measured by juxtaarticular osteoporosis and the numbers of erosions ($P < 0.01$). The results from a typical experiment are shown in Fig. 7A and B.

Identification of Cpn60.1 in serum of patients with tuberculosis

To determine if intact *M. tuberculosis* Cpn60.1 is secreted and appears in biological fluids, plasma from a small number of patients with acute tuberculosis and a number of healthy controls was subject to surface-enhanced laser desorption/ionisation-time of flight (SELDI-TOF) mass spectroscopic analysis using a monospecific antiserum to Cpn60.1. This identified the presence of a single 60 kDa protein peak with an estimated concentration in the range of 1–5 μg ml^{-1} in the patient samples. No such peak was found in the healthy controls. To confirm that this protein peak was *M. tuberculosis* Cpn60.1, one plasma sample was immunoprecipitated with the antiserum to *M. tuberculosis* Cpn60.1 (which did not cross-react with *M. tuberculosis* Cpn60.2, human Hsp60 or *E. coli* GroEL) and the adsorbed 60 kDa protein was subject to peptide mass fingerprinting using matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF MS). This revealed that the 60 kDa protein was genuine *M. tuberculosis* Cpn60.1 (Fig. 8).

Discussion

The term, mononuclear phagocyte, now encompasses a very wide range of distinct cell populations distributed throughout the mammalian body and includes one of the only two naturally occurring multinucleated cells – the osteoclast (Hume, 2006). Most tissue macrophages are thought to be sentinels of infection and it is becoming appreciated that, in addition to the geographical diversity of macrophages, they also exhibit significant phenotypic plasticity when they are activated. Depending on the stimulus, it is now appreciated that macrophages can enter a number of distinct activation states which prime them for antibacterial or anti-parasite killing (Gordon, 2003). Of course, pathogens have evolved a variety of means to cope with the macrophage (Henderson and Oyston, 2003). The bacterial pathogen of *Homo sapiens* with, arguably, the most complex interaction with mono-

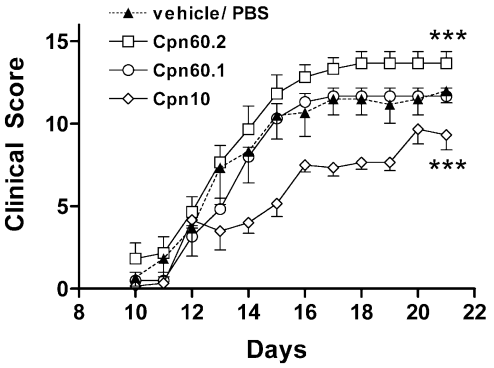

Fig. 6. The effect of administration of recombinant chaperonins on joint inflammation. The time-course of the clinical scores of joint inflammation in groups of six Wistar rats dosed with recombinant *M. tuberculosis* chaperonin proteins. (▲) Animals receiving vehicle. (◇) Animals administered with Cpn10. (○) Animals receiving Cpn60.1. (□) Animals receiving Cpn60.2. Only the Cpn10 protein shows inhibition of joint inflammation. The results are from a typical experiment and are expressed as the mean and standard deviation ($n = 6$). ***$P < 0.001$.

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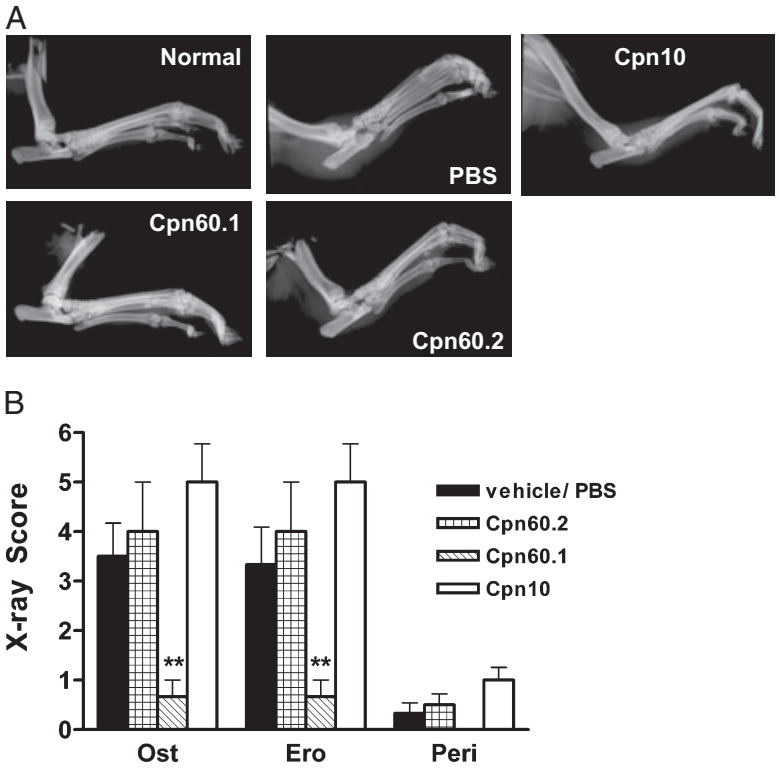

Fig. 7. Cpn60.1 prevents bone destruction *in vivo* in arthritic rats.
A. To assess radiological disease progression, both hind paws of each animal were fixed, mounted and imaged on high-speed radiographic film and graded for three parameters of bone destruction, namely osteoporosis (Ost), erosions (Ero) and periostitis (Peri), each scoring a maximum of 4/paw, 8/animal. Representative radiographs are included. The radiograph marked Normal shows a non-diseased joint. In sham-treated animals (marked PBS), joint architecture is lost (narrowing of joint spaces), erosions are visible and the bones are osteopaenic. In contrast, animals administered recombinant *M. tuberculosis* Cpn60.1 show a joint architecture that is normal (joint spaces are visible), erosions are absent or minimal and bones have a normal density. Animals administered Cpn60.2 exhibit moderate bone destruction (all three parameters). Spaces in the larger joints are just visible, but there is extensive osteopaenia and erosion. Animals receiving Cpn10 have the greatest degree of bone destruction (all three parameters). There is almost total loss of joint spaces with extensive erosions and osteopaenia.
B. Cumulated X-ray scores for six animals clearly show that administration of *M. tuberculosis* Cpn60.1 suppressed all three parameters of bone destruction in Wistar rats with adjuvant arthritis. Results are from a typical experiment and are expressed as mean and standard deviation ($n = 6$). $^{**}P < 0.01$.

nuclear phagocytes is *M. tuberculosis*. This organism is associated with the formation of the tuberculoid granuloma, a complex structure containing additional mononuclear phagocyte phenotypes including the epithelioid cell and the multinucleated giant cell (Ulrichs and Kaufmann, 2006; Russell, 2007). The formation of the granuloma protects the infected individual, and failure to produce such structures is generally lethal (Altare *et al*., 1998). However, the granuloma also provides *M. tuberculosis* with a long-term refuge, and the interplay between this bacterium and its human host to drive granuloma formation is still not understood in molecular detail. A recent study of the interaction of mycobacteria with human blood leucocytes has revealed that only virulent *M. tuberculosis* is capable of inducing the formation of the Langhan's multinucleate giant cell. Non-virulent mycobacteria could not induce such formation. Of interest, these Langhan's cells were unable to ingest bacteria and it has been suggested that they may have functions similar to dendritic cells (Lay *et al*., 2007).

As recently reviewed, evidence is starting to accumulate to support the hypothesis that bacterial molecular chaperones play an important role in bacterial virulence (Henderson *et al*., 2006). It is now established that bacterial molecular chaperones, such as Cpn10, peptidyl prolyl isomerase, Cpn60 and Hsp70, can act as intercellular signalling proteins with the capacity to modulate the behaviour of mammalian cells (Henderson *et al*., 2006). The authors (B.H., A.R.M.C.) have been studying the chaperonins of *M. tuberculosis* for some years (Qamra *et al*., 2005) and have shown that both Cpn60 proteins can induce human peripheral blood leucocytes to produce a range of pro-inflammatory cytokines (Lewthwaite *et al*., 2001). To gain more insight into the role these proteins play in the pathogenesis of *M. tuberculosis* infection, we have attempted to inactivate the three genes encoding the

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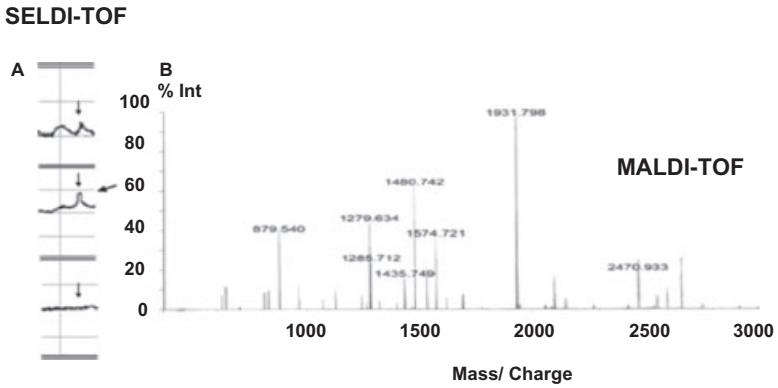

Fig. 8. Identification of *M. tuberculosis* Cpn60.1 in plasma from tuberculous patients.
A. From top to bottom is the SELDI-TOF spectrum of material bound to a PS20 chip to which monospecific antibody to *M. tuberculosis* Cpn60.1 had been covalently attached. The top spectrum is of recombinant Cpn60.1 added to the chip at 100 μg ml^{-1}. Next down is a plasma sample from a patient with tuberculosis. The bottom spectrum is from a laboratory volunteer. The latter has no 60 kDa peak (denoted by red line). This is a typical spectrum obtained from four patients and eight controls.
B. The MALDI-TOF MS spectrum of Cpn60.1 immunoprecipitated using the antibody coated on the PS20 chip. The peptide fingerprint is of *M. tuberculosis* Cpn60.1.

chaperonins. To our surprise, we could only inactivate the gene encoding Cpn60.1, with the resultant isogenic mutant showing no major changes in behaviour or in its global transcriptome. The *cpn60.1* gene could not complement an *E. coli* mutant lacking the *E. coli cpn60* gene (*groEL*) but the *M. tuberculosis cpn60.2* gene could, revealing that *M. tuberculosis* Cpn60.1 is not a functional molecular chaperone. When we infected mice or guinea pigs with the Δcpn60.1 isogenic mutant, which grew at a similar rate to the wild type, or complemented strain, there was only minimal inflammation in the lungs, suggesting that the Cpn60.1 protein was acting as a major granuloma-inducing virulence factor of *M. tuberculosis* (Hu *et al*., 2008).

In the present study, we have discovered that the recombinant Cpn60.1 protein from *M. tuberculosis* inhibits the formation of osteoclasts in murine cell and bone explant culture (in the latter blocking bone resorption) and in a rat model of arthritis (adjuvant arthritis) characterized by its marked production of osteoclasts and loss of bone matrix (Schett *et al*., 2005). In contrast, the highly homologous Cpn60.2 protein from the same bacterium appears to have no stimulatory or inhibitory effect on osteoclast generation or bone breakdown.

Osteoclasts are relatively rare cells, which act to resorb the calcified matrix of bone and are essential for normal skeletal turnover. These cells are attracting increasing interest because of their participation in chronic diseases, such as: (i) osteoporosis, rheumatoid arthritis, Paget's disease (Cohen, 2006) and (ii) bacterially driven conditions, such as periodontitis and osteomyelitis (Henderson and Nair, 2003), and because of the recent discovery of a novel group of cytokines and intracellular signalling proteins involved in the control of osteoclast formation and activation (Asagiri and Takayanagi, 2007; Teitelbaum, 2007). The cell surface TNF family member RANKL, which can be expressed on activated osteoblasts or on other cell populations, such as T cells (Theill *et al*., 2002), interacts with RANK on the surface of osteoclast precursor cells. This results in the recruitment of members of the TNF receptor associated factor (TRAF) family of adapter molecules, principally TRAF-6 (Darnay *et al*., 1999). This pathway is also claimed to be modulated by pathogen-associated molecular patterns, such as LPS and peptidoglycan (Asagiri and Takayanagi, 2007). TRAF-6 recruitment results in the activation of NF-κB, which in turn, in a complex manner involving MAP kinases (Wada *et al*., 2006), results in the formation of an AP-1/NFATc1 transcription complex which activates a range of 'osteoclastic genes' (Asagiri and Takayanagi, 2007; Teitelbaum, 2007). Osteoclastic bone resorption, both *in vitro* and *in vivo,* can be blocked by inhibition of (or lack of) components of the NF-κB, MAP kinase and NFATc1 signalling/transcription pathways (Asagiri and Takayanagi, 2007; Teitelbaum, 2007). However, such inhibition is generally conditional. Thus NF-κB-inducing kinase-deficient mice, in which IKKα would not be phosphorylated and thus the alternative NF-κB pathway is inoperative, are resistant to RANKL (Novack *et al*., 2003). In contrast, mice with an inactivating mutation in IKKα are indistinguishable from wild-type mice in their osteolytic response to LPS (Novack *et al*., 2003).

Addition of Cpn60.1 or Cpn60.2 to explants of murine calvarial bone stimulated to resorb by addition of the potent osteolytic agents, LPS or PGE_2 revealed that the recombinant Cpn60.1 was able to block resorption of the bone matrix at concentrations in the pico- to nanomolar range, assuming that the recombinant chaperonins are dimers (Qamra *et al*., 2005). This inhibition by

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Cpn60.1 could be explained by the protein acting directly with osteoblasts, or with osteoclasts or through interaction with both cell populations. To determine if Cpn60.1 acted directly on osteoclasts, we used the murine RAW264.7 monocyte cell line, which will differentiate into osteoclasts in the presence of RANKL (Collin-Osdoby *et al*., 2003; Cuetara *et al*., 2006). We found that Cpn60.1 was able to dose-dependently inhibit osteoclast formation from RAW264.7 cells. This demonstrated that Cpn60.1 is acting at the level of the osteoclast precursor and interfering with the complex process of osteoclastogenesis. In contrast, recombinant Cpn60.2 was inactive either as a stimulator or an inhibitor of osteoclast formation.

Osteoclast formation is a complex process involving cell division, fusion and differentiation (Teitelbaum, 2007), and generally the RAW264.7 cultures show the presence of osteoclasts only after 5–7 days in culture (Collin-Osdoby *et al*., 2003; Cuetara *et al*., 2006). It was assumed that the Cp60.1 would only inhibit if added to cells at the beginning of the culture process. However, to our surprise, Cpn60.1 was able to block osteoclast formation when added up to 4 days after initiation of cell culture. This is an unexpected finding and supports the hypothesis that Cpn60.1 is able to interfere with multiple steps in the process of osteoclastogenesis.

Two general mechanisms could account for the inhibition of osteoclastogenesis. The Cpn60.1 protein could be interfering in some way with RANKL binding to RANK or it could be modulating intracellular signalling pathways, including upregulating the synthesis of the natural inhibitor of the RANKL/RANK signalling system, OPG (Teitelbaum, 2007). However, we found no evidence that Cpn60.1: (i) inhibited RANKL binding to RANK, (ii) bound to RANKL or RANK and interfered with their interaction or (iii) increased the synthesis of OPG. We examined the three most likely cell signalling components to see if Cpn60.1 could interfere with any of them. There was no evidence over the first 24 h of culture that Cpn60.1 blocked the activation of NF-κB or the phosphorylation of NFATc1. The MAP kinase cJNK has been found to be a key factor in osteoclastogenesis and the selective inhibitor, SP600125, can prevent osteoclast formation *in vitro* and *in vivo* (Han *et al*., 2001). However, we found that Cpn60.1 actually stimulated cJNK. We also measured the levels of mRNA for NFATc1 in RAW264.7 cells. After 24 h exposure to RANKL, there was significant elevation in the level of NFATc1 mRNA in RAW264.7 cells. However, in the presence of Cpn60.1, at this time point, the levels of NFATc1 mRNA were significantly decreased. In cells activated by RANKL for 48 h in the presence of recombinant Cpn60.1, the levels of NFATc1 mRNA were reduced to that in unstimulated cells. However, at 96 h, the NFATc1 mRNA levels in stimulated cultures exposed to Cpn60.1 were similar to those in cells exposed only to RANKL. In contrast, recombinant Cpn60.2 did not significantly inhibit intracellular levels of NFATc1 mRNA at any time point. Thus, we propose that an explanation for the inhibition of osteoclast formation by Mtb Cpn60.1, over the first 48–72 h of culture, is the ability of this protein to selectively block the increase in NFATc1 mRNA caused by exposure to RANKL, possibly by inhibition of the transcription of the gene encoding NFATc1. There was no inhibition of NFATc1 in RAW264.7 cells exposed to Cpn60.1 for 96 h in culture. Yet Cpn60.1 was able to block osteoclast formation when added at this time point. This suggests that Cpn60.1 has more than one mode of action in respect of the process of osteoclastogenesis. In the early part of the process, it is the inhibition of the elevation of NFATc1 mRNA that blocks osteoclast formation. However, at later stages, we speculate that other, possibly as yet undiscovered, processes are being blocked.

Is the effect of Cpn60.1 on osteoclastogenesis direct or indirect (via induction of the synthesis of inhibitory proteins)? We have shown that Cpn60.1 does not induce OPG. Other possible inhibitory factors include the cytokines: IFNγ, IL-4 and IL-10 (Gillespie, 2007). Cpn60.1 does not induce human peripheral blood leucocytes to secrete IL-4 or IFNγ and both Cpn60.1 and Cpn60.2 are equivalent, and very weak, inducers of IL-10 synthesis (Lewthwaite *et al*., 2001). Moreover, microarray analysis of human peripheral blood leucocyte responses to both mycobacterial Cpn60 proteins reveals identical patterns of induction of cytokine mRNAs (B. Henderson and A.R.M. Coates, unpublished). Given that Cpn60.2 does not block osteoclast formation and does not inhibit NFATc1 mRNA levels, we would suggest that the *M. tuberculosis* Cpn60.1 is having a direct effect on the production of NFATc1 mRNA.

While of interest, the finding that *M. tuberculosis* Cpn60.1 inhibits the formation of osteoclasts from a murine cell line (and bone marrow cultures) may have been just an interesting artefact. Could this protein inhibit osteoclast formation in the whole animal? Adjuvant arthritis in the rat is a very well-understood model in which there is massive upregulation of the processes of osteoclastogenesis in affected joints and in which it has been shown that such upregulation is due to increased RANKL/RANK interaction (Kong *et al*., 1999). Adjuvant arthritis is induced by intradermal injection of dead *M. tuberculosis* and it is known that Cpn60.2 is involved in the control of this disease (Prakken *et al*., 2003). However, an identical disease can be induced by pristane in which there is no involvement of mycobacterial components (Hopkins *et al*., 1984). Adjuvant arthritis can be inhibited if animals are vaccinated by *M. tuberculosis* Cpn60.2. This means that the Cpn60.2 is given long before induction of disease. In our protocol, the rats receive recombinant *M. tuberculosis* chaperonins during the disease induction phase and so

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any effects seen are not due to prior immune modulation. In this model, Cpn60.2 exacerbated joint inflammation (swelling), yet had little effect on the underlying bone destruction. Cpn10 was anti-inflammatory in that it suppressed soft tissue inflammation (synovitis) but, although it had no significant overall effect, tended to enhance parameters of bone destruction. Cpn60.1 had no clinically measurable anti-inflammatory activity, but it almost completely inhibited the local osteoporosis seen in inflamed joints and blocked the periarticular joint erosions, both events being due to the increased formation of osteoclasts. Thus *M. tuberculosis* is able to inhibit osteoclast formation and prevent bone destruction bot*h in vitro* and *in vivo*.

An intriguing question is whether the osteoclasts in patients with tuberculosis are exposed to Cpn60.1. For this to happen, the Cpn60.1 protein would have to be released. To answer this question, we have used mass spectrometric techniques with a specific antiserum which we raised to recombinant *M. tuberculosis* Cpn60.1 to seek for the presence of Cpn60.1 in the blood of patients with acute tuberculosis. This revealed the presence of an estimated 1–5 μg ml^{-1} of intact *M. tuberculosis* Cpn60.1 in the plasma of patients with tuberculosis but not in healthy controls. The mechanism of release of this Cpn60.1 has not been identified. However, the finding that *M. tuberculosis* Cpn60.1 is released suggests that it may influence skeletal bone dynamics in tuberculosis.

In conclusion, in this paper we have established that the *M. tuberculosis* Cpn60.1 protein is a potent inhibitor of the formation of the multinucleate osteoclast population, which is involved in bone remodelling and appears to inhibit such formation (at least over the first 48–72 h) by blocking the synthesis of the key transcription factor NFATc1. Surprisingly, the highly homologous Cpn60.2 protein from the same organism has no effect on osteoclast generation or bone remodelling. Given the 60% sequence identity between these proteins, this suggests that the osteoclast inhibitory epitope is a limited sequence of residues. Administration of recombinant Cpn60.1 to rats with adjuvant arthritis blocked the bone destruction without affecting joint inflammation. This brings into focus the bipolar activity of *M. tuberculosis* Cpn60.1. The inactivation of the *cpn60.1* gene in *M. tuberculosis* (Hu *et al*., 2008) has highlighted the fact that this protein is a major stimulator of tuberculoid granuloma formation in animals with the concomitant stimulation of giant cell formation. At the same time, this same protein can inhibit the formation of osteoclasts. This suggests that Cpn60.1 functions at a bifurcation in the developmental pathway of giant cells and osteoclasts, stimulating the formation of the former and inhibiting the formation of the latter from a precursor cell population. In a world which is desperately seeking active inhibitors of bone resorption, *M. tuberculosis* Cpn60.1, or peptides derived from it, could be utilized as a novel anti-resorptive agent provided it was possible to block the ability of this protein to induce giant cell formation.

Finally, intact *M. tuberculosis* Cpn60.1 protein has been identified in the blood of tuberculous patients. What are the consequences of these findings? The capacity of Cpn60.1 to modulate monocyte differentiation has been amply demonstrated although not fully characterized, and the finding of this protein in blood suggests that it may have actions on bone and other monocyte/macrophage cell populations which are deleterious to the infected patient. In a small proportion of patients with tuberculosis, infection is of the bone and these individuals can show severe bone destruction and this is, in part, due to another molecular chaperone, Cpn10 (Meghji *et al*., 1997). If Cpn60.1 is an inhibitor of osteoclasts, would one not expect to see protection of bone in this disease? The answer must depend on the local ratios of osteoclast-stimulating and -inhibiting bacterial factors in infected bone and, in such circumstances, the osteolytic factors obviously mask the inhibitors. However, in non-skeletal infections, there may be influences on bone that have not been examined because the effect of Cpn60.1 on bone has only now been recognized.

Experimental procedures

Reagents

The RANKL and OPG were a kind gift from Amgen (Thousand Oaks, California, USA). LPS was prepared from *P. gingivalis* and was a gift of Professor Michael Curtis, London University. All other reagents were either produced in-house or were obtained from commercial sources.

Cloning and expression of M. tuberculosis *chaperonin genes and purification of proteins*

The virulent *M. tuberculosis* strain H37Rv was the source of the genomic DNA for cloning both the *cpn60.1* gene (GENBANK locus tag Rv3417c) and the *cpn60.2* gene (GENBANK locus tag Rv0440). The gene coding for Cpn60.1 was amplified and cloned in the expression vector pET22b (Novagen) and expressed using standard methods in *E. coli* strain, Top10. The Cpn60.1 was expressed as inclusion bodies. To produce folded proteins, the inclusion bodies were re-suspended in 6 M guanidine hydrochloride, and this was quickly diluted by adding a 10-fold volume of PBS. Refolding was continued overnight at 4°C, and the protein was clarified by centrifugation at 30 000 *g* and subsequent filtration of the cleared protein through a 0.25 μm filter. The recombinant protein was then bound to Probond nickel-chelating resin (Invitrogen), and bound protein was washed with 5 column volumes of a 2 mg ml^{-1} solution of polymyxin B (Sigma) in PBS to remove any contaminating LPS. Protein was eluted from the column by application of a 10–300 mM imidazole gradient in 10 column volumes, and fractions containing the recombinant Cpn60.1 were pooled and dialysed against 20 mM sodium phos-

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phate buffer, pH 6.8. The recombinant Cpn60.1 was further purified by anion exchange chromatography using a Porous HQ column on a BioCad Sprint chromatography system (Applied Biosystems, Warrington, UK).

The *cpn60.2* gene was cloned and the recombinant protein purified from *E. coli* by standard ion exchange chromatography followed by dialysis against 10 mM ammonium bicarbonate (Thole *et al*., 1987). The *cpn10* gene was cloned, expressed and purified as described (Fossati *et al*., 1995). All three recombinant proteins were > 95% pure as assessed by SDS-PAGE, each displaying a single band on overloaded gels stained with colloidal Coomassie blue.

Measurement of LPS contamination in recombinant proteins

This was done using a commercially available *Limulus* amoebocyte lysate assay which was used according to the manufacturer's instructions (Associates of Cape Cod). LPS contamination varied between 1 and 10 pg μg^{-1} of recombinant protein.

Murine calvarial bone resorption assay

Bone resorption was assayed by measurement of calcium release from 5-day-old murine calvariae in culture (Meghji *et al*., 1998). Briefly, after removal of any adherent connective tissue, the calvariae were halved along the sagittal suture and each bone was cultured individually at 37°C on a 1 cm^2 stainless steel grid in 1.5 ml of BGJ-modified medium (ICN-Flow) supplemented with antibiotics, ascorbate (100 mg ml^{-1}: Sigma) and 5% complement-inactivated rabbit serum (Sigma). After 24 h, the medium was removed and replaced with medium containing either a known osteolytic agent (PGE_2; 1 μM or *P. gingivalis* LPS 10 μg ml^{-1}) or that agent in combination with a range of concentrations of *M. tuberculosis* Cpn60 proteins (10 ng ml^{-1}–1 μg ml^{-1}). These assays are always done in the presence of 20 μg ml^{-1} polymyxin B (Sigma) (except when LPS was being tested) to inhibit the potential activity of contaminating LPS. Each osteolytic agent was tested in five replicate cultures. Replicate calvariae were cultured in unsupplemented culture media to determine the calcium release from unstimulated bone and another set of calvariae were incubated with 1 μM PGE_2 to show that the bone was responsive and to provide information on the maximum response of the system. After 48 h of culture, the media were removed and measured for calcium content by automated colorimetric assay (Gitelman, 1967). In some experiments, a neutralizing antibody to OPG (R and D Systems) was added at a concentration of 1 μg ml^{-1}. Experiments were repeated twice with similar results and representative experiments are presented.

Osteoclast induction assays

The murine myeloid cell line RAW264.7 was used as a reproducible source of osteoclast precursor cells. Cells were seeded into 48-well plates (NUNC) at either 2000 or 4000 cells per well in growth medium (α-MEM with 10% FCS; Gibco/BRL), and incubated at 37°C in 95% air/5% CO_2 overnight to allow the cells to adhere. After 24 h, the medium was changed and cells were exposed to 50 ng ml^{-1} RANKL (Amgen) alone, or with graded concentrations of Cpn60.1 or Cpn60.2 (1 ng ml^{-1} to 10 μg ml^{-1}). As a positive control, separate treatment groups were exposed to 10 μg ml^{-1} OPG (Amgen). The cells were incubated for a further 6 days with a medium change on day 4. Cells were subsequently fixed and stained for TRAP using an acid phosphatase staining kit (Sigma Diagnostics). Cells containing three or more nuclei and positive for TRAP were enumerated and assumed to be osteoclasts. Cell viability was routinely checked by measuring uptake of Trypan blue.

Murine bone marrow was removed from the long bones and cultured as described (Reddi *et al*., 1998). After the overnight incubation, only cells that had adhered to the plastic were retained. These cells were carefully taken off the plastic using a cell scraper, centrifuged at 1500 r.p.m. for 5 min to pellet them, and re-suspended and placed into T-25 tissue culture flasks in DMEM with 5% heat-inactivated FCS, 10 ng ml^{-1} RANKL and 30 ng ml^{-1} M-CSF to promote osteoclastogenesis. Parallel flasks were similarly treated but various concentrations of Cpn60.1 were also added. The flasks were then incubated at 37°C in an atmosphere of 95% O_2/5% CO_2 for 10 days, prior to being fixed and stained for TRAP and cells counted.

Cell signalling analysis

To attempt to identify the mechanism of the inhibitory activity of Cpn60.1, the three most likely intracellular signalling pathways were examined. To determine if Cpn60.1 was inhibiting NF-κB activation, RAW cells were stimulated with 30 ng ml^{-1} RANKL in the presence or absence of various concentrations of Cpn60.1. Cells were then lysed on ice for 10 min in 250 μl of lysis buffer (76.5 mM Tris-HCl, 10% v/v glycerol, 2% w/v SDS in dH_2O supplemented with 1 mM $NaVO_3$, 1 mM AEBSF and 50 μg ml^{-1} leupeptin) and lysates separated by SDS-PAGE and Western blotted with antibodies to either unphosphorylated or phosphorylated I-κB (Santa Cruz Biotechnology, Santa Cruz, USA). To ascertain if Cpn60.1 was blocking the phosphorylation of NFATc1, cell lysates were produced using the same methodology and Western blotted with an antibody to phosphorylated NFATc1 (Santa Cruz). Western blots were then analysed using ImageJ. To determine if Cpn60.1 was inhibiting the phosphorylation of cJNK, human synovial fibroblasts were isolated from osteoarthritic or rheumatoid synovial linings taken from patients undergoing joint replacement. Appropriate local ethical approval was granted for using these tissues. Rat synovial fibroblasts were prepared from the synovial linings of normal and arthritic Wistar rats. Briefly, tissues were minced, collagenase-treated and filtered and, after extensive washing, cells were plated out in DMEM with 10% FCS (Gibco/BRL) and incubated at 37°C in 95% air/5% CO_2. At confluence, cells were trypsinized and split in a 1:3 ratio and replated. Cells were used for cJNK assays between passages 2 and 6. Kinase assays were carried out as described by Han *et al*. (2001). The time-course of c-JNK phosphorylation in response to IL-1β (2 ng ml^{-1}) was determined. To determine if Cpn60.1 inhibited c-JNK activation induced by IL-1β, synovial fibroblasts were incubated with Cpn60.1 (1 or 5 μg ml^{-1}) for 30 min prior to addition of IL-1β (2 ng ml^{-1}) and further incubated for 60 min. Appropriate controls (no stimulation or IL-1β and Cpn60.1 alone) were run. After incubation with activators/inhibitors, cells were lysed, boiled in sample buffer and separated by SDS-PAGE on 12% gels. Proteins were transferred onto nitrocellulose using standard techniques. Immunodetection of phos-

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phorylated c-JNK made use of the mouse IgG1 phospho-SAPK/JNK (Thr183/Tyr185) G9 monoclonal antibody (Cell Signalling Technology) according to the manufacturer's instructions. After appropriate washing, the membrane was incubated with HRP-conjugated (adsorbed) rabbit anti-mouse immunoglobulin (Dako). After a further wash cycle, the HRP activity (a measure of c-JNK activation) was detected by enhanced chemiluminescence using a commercial kit (Amersham-Biosciences) on Kodak X-OMAT-AR film.

To measure the influence of Cpn60 proteins on NFATc1 mRNA levels, RAW264.7 cells (ATCC UK, Teddington, UK) 2.5×10^5 ml^{-1} were incubated for 24, 48 or 96 h with combinations of RANKL (50 ng ml^{-1}) and Cpn60.1 or Cpn60.2 (10 μg ml^{-1}). Total RNA was extracted from these cultures and reversed transcribed with M-MLV according to manufacturer's instructions (Sigma). Real-time PCR was conducted with a Techne Quantica machine and Quansoft software using the DNA-binding dye SYBR green for the detection of PCR products. Two microlitres of external plasmid standard or cDNA was added to a final reaction volume of 25 μl containing 20 mM Tris-HCl pH 8.3, 100 mM KCl, 7 mM $MgCl_2$, 0.4 mM dNTPs, 0.05 U μl^{-1} Taq DNA polymerase, SYBR green and specific primers (0.2 μM). Primers used were as follows: murine NFATc1 sense, 5′-CCGTTGCTTCCAGAAAATAACA-3′; NFATc1 antisense, 5′-TGTGGGATGTGAACTCGGAA-3′; β-actin sense, 5′-GTCATCACTATTGGCAACGAG-3′; and β-actin antisense, 5′-CCTGTCAGCAATGCCTGGTACAT-3′, which yield product lengths of 152 and 197 base pairs respectively. Reaction conditions were 95°C for 3 min, followed by 35 cycles of 95°C for 20 s, 59°C for 20 s and 72°C for 20 s. At the end of each PCR run, a melt curve analysis was performed to show the absence of non-specific bands. For each sample, NFATc1 mRNA levels were expressed as relative copy number normalized to 10^6 β-actin mRNA copies.

Flow cytometric measurement of RANKL binding to RAW264.7 cells

Recombinant RANKL was fluoresceinated with FITC using a standard protocol and isolated from free FITC on a desalting column. RAW 264.7 cells were harvested and pelleted and washed using PBS. The cell pellet was then washed twice in wash buffer (2% FCS in PBS), before being fixed in 3 ml of 1% paraformaldehyde (Sigma) in PBS for 30 min at room temperature. Fixative was discarded and the cells were re-suspended in wash buffer. One-millilitre aliquots of this suspension were then placed in 5 ml FACS tubes (Becton Dickinson) and centrifuged for 5 min at 350 r.p.m. Wash buffer was then carefully discarded, and 100 μl of the appropriate concentration of FITC-labelled RANKL in wash buffer, or the same concentration of RANKL either with excess unlabelled RANKL or with various concentrations of Cpn60.1 was added and incubated for 1 h at room temperature. Cells were then washed three times and then re-suspended in 0.5 ml of wash buffer. These suspensions were then sorted using a flow cytometer and analysed using CellQuest software.

ELISA for detection of Cpn60.1 binding to RANKL or RANK

The 96-well plates (NUNC Immunoplate, VWR International) were sensitized by incubation for 1 h with a 1 μg ml^{-1} solution of recombinant human RANKL (Amgen) or with recombinant human RANK (R and D Systems) in PBS. Plates were then washed in wash/dilution buffer (NaCl 0.5 M, NaH_2PO_4 2.5 mM, Na_2HPO_4 7.5 mM, Tween 20 0.1%w/v, pH 7.4) and blocked with 10 mg ml^{-1} milk powder (Sigma) for 1 h at room temperature. Plates were washed extensively in wash/dilution buffer. To determine binding of recombinant Cpn60.1 to RANKL or RANK, graded concentrations of Cpn60.1 were added to wells in quadruplicate and incubated for 2 h at room temperature. After washing, wells were incubated with a rabbit polyclonal antibody to Cpn60.1 prepared in-house (1:1000 dilution). Appropriate controls were included to show that this antibody did not bind to RANKL or RANK or to the milk powder or plastic. After incubation for 1 h at room temperature, the wells were washed and a goat anti-rabbit HRP-conjugated antiserum (Sigma 1:20 000 dilution) was incubated with the wells for 1 h at room temperature. Wells were washed again and substrate/H_2O_2 was added and, after an appropriate reaction time, the absorbance of the wells was measured on a Dynex plate reader at 450 nm.

Induction of adjuvant arthritis, chaperonin treatment and clinical monitoring

Heat-killed human strains C, DT and PN of *M. tuberculosis* (Veterinary Laboratories Agency, DEFRA, Weybridge, UK) were finely ground in a pestle and mortar and suspended in light paraffin oil at a final concentration of 10 mg ml^{-1}. In each experiment, female Wistar rats were randomly assigned to groups of six animals and maintained to a weight of 180–200 g. Four groups of six rats were inoculated with 100 μl of the *M. tuberculosis* suspension in the base of the tail (day 0) as described (Andrews *et al.*, 1987). One group of six rats was maintained as healthy controls (group 1: naïve). On days 4–6, the four groups receiving adjuvant were treated as follows. In group 2, each rat was injected with 50 μl of PBS in the tail (vehicle control). Similarly, groups 3–5 were treated with 50 μl of *M. tuberculosis* Cpn10, Cpn60.1 or Cpn60.2 respectively, at a concentration of 1 mg ml^{-1} in PBS. Animals were observed daily for 3 weeks and clinical signs of arthritis monitored blindly in all four paws using a standardized scoring system (Currey and Ziff, 1968; Clarke *et al.*, 1979) of 0–4 where 0 is no inflammation and 4 is gross inflammation and deformity of the joint (maximum score is 16). Animals were killed after 3 weeks, or when scores reached maximum values. This experiment was repeated twice with similar results.

Radiographic monitoring of bone damage

The right and left hind paws of each animal were imaged on high-speed X-ray film (Kodalith Ortho Film, Type 3; Kodak) using a Faxitron machine (Field Emission Corp). Using a modification of the method of Clarke *et al.* (1979), assessment was made on the basis of three parameters: osteoporosis, erosions and periostitis, with each parameter being scored 0–4, according to severity by a blinded observer.

Statistical analysis

Data from adjuvant arthritis clinical scores used a repeated-measures ANOVA with Dunnett's post-test which takes into

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account repeated measurements on multiple occasions on individual animals. For analysis of the bone destruction in adjuvant arthritis, an unpaired *t*-test with Welch correction was used. For explant and cell culture studies, Student's *t*-test was applied.

Measurement of circulating M. tuberculosis *Cpn60.1*

The four patients studied were either inpatients or outpatients at St George's Hospital. They all had laboratory-confirmed active tuberculosis and were being treated with anti-tuberculous drugs. The eight healthy controls were volunteers from the Department of Medical Microbiology at St George's University of London. They had no features of active tuberculosis, and remained healthy for at least 5 years after the blood samples were taken. Their skin tuberculin status is unknown. Plasma was used for all the measurements. The polyclonal antibody against full-length recombinant *M. tuberculosis* Cpn60.1 (prepared in house) protein was raised by Cambio (Cambridge, UK) using Freund's incomplete adjuvant. This antibody did not cross-react with *M. tuberculosis* Cpn60.2, *E. coli* GroEL or with human Cpn60.

To detect intact *M. tuberculosis* Cpn60.1 in human plasma, A PS20 chip (Ciphergen) (Epoxide surface which binds primary amines) was coated with 5 μl of the rabbit polyclonal antibody against *M. tuberculosis* Cpn60.1. The antibody was cross-linked to the surface of the chips during a 16 h incubation period at 4°C. Excess antibody was then removed and 5 μl of a 500 mM ethanolamine solution was added. The chips were incubated for 20 min at room temperature, followed by three 15 minute washes in 0.5% Triton X-100 in PBS and then one 15 min wash in PBS. The chips were allowed to air-dry and 5 μl of the plasma samples was added to each of the prepared chips and again left at room temperature for 120 min. The chips were then washed, as detailed previously, then finally they underwent three 5 min washes in 5 mM Hepes. The chips were air-dried and 5 μl of EAM suspension (50% acetonitrile, 0.2% trifluoroacetic acid, 10 μl 10 mg ml^{-1} sinapinic acid) added followed by air-drying. The chips were then analysed using a SELDI-TOF mass spectrometer (Ciphergen). The laser intensity set at 290, the detector sensitivity at 7 and the optimization range at 30–70 kDa. The spectra were obtained following a 100 shot average.

To confirm the identity of the proteins identified by SELDI-TOF, the antibody coated on the PS20 chip was used to immunoprecipitate Cpn60.1 using a Seize-X immunoprecipitation kit (Perbio). Briefly, the anti-Cpn60.1 antibody was cross-linked to a ProteinA column, which was incubated with plasma from the patients. The Cpn60.1 was then eluted from the column using the low-pH elution buffer supplied with the Seize X kit and the eluate was neutralized by the addition of 10 μl of Tris-HCl pH 9.5.

Immunoprecipitated proteins were run on a 10% SDS-PAGE gel and were stained using Brilliant blue G colloidal concentrate (Sigma). The band which corresponded to the size of the Cpn60 protein was excised from the gel and was destained using 3×15 min washes using 50% acetonitrile, 25 mM ammonium bicarbonate solution, pH 8. The gel slice was then washed with 100% acetonitrile for 5 min and was dried under vacuum. The gel slice was re-hydrated with 10 μg ml^{-1} Trypsin (Promega, Southampton, UK) diluted in 25 mM ammonium bicarbonate (Sigma) and left on ice for 30 min. Further trypsin was added until the gel slice was covered and then incubated for 16 h at 37°C. Peptides were recovered from the gel slices by extraction twice with 50 μl of 0.1% trifluoroacetic acid (TFA) (Perbio) for 1 h. The extracted peptides were lyophilized in a vacuum centrifuge for 1 h, re-suspended in 10 μl of 0.1% TFA and purified with ZipTip Microcolumns according to the manufacturers instructions (Millipore). The peptides were recovered from the ZipTip columns by elution with 5 μl of 10 mg ml^{-1} α-cyano-4-hydroxycinnamic acid matrix (Sigma) in 50% ACN/0.1% TFA. A 1 μl volume of each eluted sample was placed on the mass spectrometer sample plate. Mass spectra were obtained on a Voyager DE-PRO (PE Biosystems). All spectra were obtained as 200 shot averages and the peptide peaks were calibrated either internally using the trypsin peaks (842.5099 and 1049.1062) or by close external calibration using a peptide mixture (PE Biosystems) containing angiotensin 1 (monoisotopic mass of 1296.6853 Da), ACTH 1–17 (monoisotopic mass of 2093.0867 Da), ACTH 18–39 (monoisotopic mass of 2465.1989 Da) and ACTH 7–38 (monoisotopic mass of 3657.9294 Da). MS-Fit (University of California San Francisco Mass Spectrometry Facility), installed locally, was used to identify proteins from peptide mass fingerprints by comparing the monoisotopic peptide masses from individual spots with theoretical tryptic digests of translated sequence data. All searches were performed against the National Center for Biotechnology Information non-redundant sequence database for *M. tuberculosis* with the mass tolerance set at 50 p.p.m., and scoring for missed cleavages set at one. Protein identities were assigned based on the highest molecular weight search (MOWSE) score.

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References

Altare, F., Jouanguy, E., Lamhamedi, S., Doffinger, R., Fischer, A., and Casanova, J.L. (1998) Mendelian susceptibility to mycobacterial infections in man. *Curr Opin Immunol* **10:** 413–417.

Andrews, F.J., Morris, C.J., Kondratowicz, G., and Blake, D.R. (1987) Effect of iron chelation on inflammatory joint disease. *Ann Rheum Dis* **46:** 327–333.

Asagiri, M., and Takayanagi, H. (2007) The molecular understanding of osteoclast differentiation. *Bone* **40:** 3251–3264.

Clarke, R.L., Cuttino, J.T., Anderle, S.K., Cromartie, W.J., and Schwab, J.H. (1979) Radiologic analysis of arthritis in rats after systemic injection of streptococcal cell walls. *Arthritis Rheum* **22:** 25–35.

Cohen, M.M. (2006) The new bone biology: pathologic, molecular, and clinical correlates. *Am J Med Genet A* **140:** 2646–2706.

Collin-Osdoby, P., Yu, X., Zheng, H., and Osdoby, P. (2003) RANKL-mediated osteoclast formation from murine RAW 264.7 cells. *Methods Mol Med* **80:** 153–166.

Cuetara, B.L.V., Crotti, T.N., O'Donoghue, A.J., and McHugh, K.P. (2006) Cloning and characterization of osteoclast precursors from the RAW264.7 cell line. *In Vitro Dev Cell Biol* **42:** 182–188.

Currey, H.L.F., and Ziff, M. (1968) Suppression of adjuvant

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disease in the rat by heterologous antilymphocyte globulin. *J Exp Med* **127:** 185–203.

Darnay, B.G., Haridas, V., Ni, J., Moore, P.A., and Aggarwal, B.B. (1999) Characterization of the intracellular domain of the receptor activator of NF-κB (RANK). Interaction with tumor necrosis factor receptor associated factors and activation of NF-κB and c-Jun N-terminal kinase. *J Biol Chem* **273:** 20551–20555.

Fossati, G., Lucietto, P., Giuliani, P., Coates, A.R.M., Harding, S., Colfen, H., *et al.* (1995) *Mycobacterium tuberculosis* chaperonin 10 forms stable tetrameric and heptameric structures: implications for its diverse biological activities. *J Biol Chem* **270:** 26159–26167.

Friedland, J.S., Shattock, R., Remick, D.G., and Griffin, G.E. (1993) Mycobacterial 65-kD heat shock protein induces release of proinflammatory cytokines from human monocytic cells. *Clin Exp Immunol* **91:** 58–62.

Gillespie, M.T. (2007) Impact of cytokines and T lymphocytes upon osteoclast differentiation and function. *Arthritis Res Ther* **9:** 103.

Gitelman, H.J. (1967) An improved automated procedure for the determination of calcium in biological specimens. *Anal Biochem* **18:** 520–531.

Gordon, S. (2003) Alternative activation of macrophages. *Nat Rev Immunol* **3:** 23–35.

Han, Z., Boyle, D.L., Chang, L., Bennett, B., Karin, M., Yang, L., *et al.* (2001) c-Jun N-terminal kinase is required for metalloproteinase expression and joint destruction in inflammatory arthritis. *J Clin Invest* **108:** 73–81.

Henderson, B. (2003) Chaperonins: chamelion proteins that influence myeloid cells. In *Heat Shock Proteins and Inflammation*. van Eden, W. (ed.) Basle: Birkhauser, 175–192.

Henderson, B., and Nair, S.P. (2003) Hard labour: bacterial infection of the skeleton. *Trends Microbiol* **11:** 570–577.

Henderson, B., Allan, E., and Coates, A.R.M. (2006) Stress wars: the direct role of host and bacterial molecular chaperones in bacterial Infection. *Infect Immun* **74:** 3693–3706.

Henderson, B., and Oyston, P. (2003) *Bacterial Evasion of Host Immune Responses*. Cambridge: Cambridge University Press.

Henderson, B., and Pockley, A.G. (2005) *Molecular Chaperones and Cell Signalling*. Cambridge: Cambridge University Press.

Henderson, B., Ward, J.M., Nair, S.P., and Wilson, M. (2003) Molecular pathogenicity of the oral opportunistic pathogen *Actinobacillus actinomycetemcomitans*. *Annu Revs Microbiol* **57:** 29–55.

Hopkins, S.J., Freemont, A.J., and Jayson, M.I. (1984) Pristane-induced arthritis in BALB/c mice. I. Clinical and histological features of the arthropathy. *Rheumatol Int* **5:** 21–28.

Hu, Y., Henderson, B., Lund, P.A., Tormay, P., Liu, H.L., Gurcha, S.S., *et al.* (2008) *A Mycobacterium tuberculosis* mutant lacking the *groEL* homologue *cpn60.1* is viable but fails to induce an inflammatory response in animal models of infection. *Infect Immun* **76:** 1535–1546.

Hume, D.A. (2006) The mononuclear phagocyte system. *Curr Opin Immunol* **18:** 49–53.

Kerner, M.J., Naylor, D.J., Ishihama, Y., Maier, T., Chang, H.C., Stines, A.P., *et al.* (2006) Proteome-wide analysis of chaperonin-dependent protein folding in *Escherichia coli*. *Cell* **122:** 209–220.

Kirby, A.C., Meghji, S., Nair, S.P., White, P.A., Reddi, K., Nishihara, T., *et al.* (1995) The potent bone resorbing mediator of *Actinobacillus actinomycetemcomitans* is homologous to the molecular chaperone, GroEL. *J Clin Invest* **96:** 1185–1194.

Kong, T.H., Coates, A.R., Butcher, P.D., Hickman, C.J., and Shinnick, T.M. (1993) *Mycobacterium tuberculosis* expresses two chaperonin-60 homologs. *Proc Natl Acad Sci USA* **90:** 2608–2612.

Kong, Y.Y., Feige, U., Sarosi, I., Bolon, B., Tafuri, A., Morony, S., *et al.* (1999) Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. *Nature* **402:** 304–309.

Lay, G., Poquet, Y., Salek-Peyron, P., Puissegur, M.P., Botanch, C., Bon, H., *et al.* (2007) Langhans giant cells from *M. tuberculosis*-induced human granulomas cannot mediate mycobacterial uptake. *J Pathol* **211:** 76–85.

Lewthwaite, J.C., Coates, A.R.M., Tormay, P., Singh, M., Mascagni, P., Poole, S., *et al.* (2001) *Mycobacterium tuberculosis* chaperonin 60.1 is a more potent cytokine stimulator than chaperonin 60.2 (hsp 65) and contains a CD14-binding domain. *Infect Immun* **69:** 7349–7355.

Maguire, M., Coates, A.R.M., and Henderson, B. (2002) Chaperonin 60 unfolds its secrets of cellular communication. *Cell Stress Chaperones* **7:** 317–329.

Meghji, S., White, P.A., Nair, S.P., Reddi, K., Heron, K., Henderson, B., *et al.* (1997) *Mycobacterium tuberculosis* chaperonin 10 stimulates bone resorption: a potential contributory factor in Pott's disease. *J Exp Med* **186:** 1241–1246.

Meghji, S., Hill, P.A., Harris, M. (1998) Bone organ cultures. In *Methods in Bone Biology*. Arnett, T.R., and Henderson, B. (eds). London: Chapman and Hall, 106–126.

Novack, D.V., Yin, L., Hagen-Stapleton, A., Schreiber, R.D., Goeddel, D.V., Ross, F.P., and Teitelbaum, S.L. (2003) The IκB function of NF-κBp100 controls stimulated osteoclastogenesis. *J Exp Med* **198:** 771–871.

Prakken, B.J., Roord, S., Ronaghy, A., Wauben, M., Albani, S., and van Eden, W. (2003) Heat shock protein 60 and adjuvant arthritis: a model for T cell regulation in human arthritis. *Springer Semin Immunopathol* **25:** 47–63.

Qamra, R., Mande, S.C., Coates, A.R.M., and Henderson, B. (2005) The unusual chaperonins of *Mycobacterium tuberculosis*. *Tuberculosis* **85:** 385–394.

Reddi, K., Meghji, S., Nair, S.P., Arnett, T.R., Miller, A.D., Preuss, M., *et al.* (1998) The *Escherichia coli* chaperonin 60 (groEL) is a potent stimulator of osteoclast formation. *J Bone Miner Res* **13:** 1260–1266.

Riffo-Vasquez, Y., Spina, D., Page, C., Desel, C., Whelan, M., Tormay, P., *et al.* (2004) Differential effects of *Mycobacterium tuberculosis* chaperonins on bronchial eosinophilia and hyperresponsiveness in a murine model of allergic inflammation. *Clin Exp Allergy* **34:** 712–719.

Russell, D.G. (2007) Who puts the tubercle in tuberculosis. *Nat Rev Microbiol* **5:** 39–47.

Schett, G., Stolina, M., Bolon, B., Middleton, S., Adlam, M., Brown, H., *et al.* (2005) Analysis of the kinetics of osteoclastogenesis in arthritic rats. *Arthritis Rheum* **52:** 3192–3201.

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Teitelbaum, S.L. (2007) Osteoclasts: what do they do and how do they do it? *Am J Pathol* **170:** 427–435.

Theill, L.E., Boyle, W.J., and Penninger, J.M. (2002) RANK-L and RANK: T cells, bone loss, and mammalian evolution. *Annu Rev Immunol* **20:** 795–823.

Thole, J.E., Keulen, W.J., De Bruyn, J., Kolke, A.H., Groothuis, D.G., Berwald, L.G., *et al.* (1987) Characterization, sequence determination and immunogenicity of a 64-kilodalton protein of *Mycobacterium bovis* BCG expressed in *E. coli* K-12. *Infect Immun* **55:** 1466–1475.

Ulrichs, T., and Kaufmann, S. (2006) New insights into the function of granulomas in human tuberculosis. *J Pathol* **208:** 261–269.

Wada, T., Nakashima, T., Hiroshi, N., and Penninger, J.M. (2006) RANKL-RANK signaling in osteoclastogenesis and bone disease. *Trends Mol Med* **12:** 17–25.

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