

## REPORT

## Role of RANKL and RANK in bone loss and arthritis

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The tumour necrosis factor family molecule RANKL (RANKL, TRANCE, ODF) and its receptor RANK are key regulators of bone remodelling and regulate T cell/dendritic cell communications, and lymph node formation. Moreover, RANKL and RANK are expressed in mammary gland epithelial cells and control the development of a lactating mammary gland during pregnancy and the propagation of mammalian species. Importantly, RANKL and RANK are essential for the development and activation of osteoclasts and bone loss in response to virtually all triggers tested. Therapeutically, inhibition of RANKL function via the decoy receptor osteoprotegerin completely prevents bone loss at inflamed joints and has partially beneficial effects on cartilage destruction in all arthritis models studied. Modulation of these systems provides a unique opportunity to design novel treatments to inhibit bone loss and crippling in arthritis.

Morphogenesis and remodelling of bone entail the synthesis of bone matrix by osteoblasts and the coordinate resorption of bone by osteoclasts.<sup>1</sup> It has been estimated that about 10% of the total bone mass in humans is being remodelled each year. Osteoblasts and osteoclasts arise from distinct cell lineages and maturation processes—that is, osteoclasts arise from mesenchymal stem cells while osteoclasts differentiate from haematopoietic monocyte/macrophage precursors.<sup>2</sup> Imbalances between osteoclast and osteoblast activities can arise from a wide variety of hormonal changes or perturbations of inflammatory and growth factors, resulting in skeletal abnormalities characterised by decreased (osteoporosis) or increased (osteopetrosis) bone mass.

Increased osteoclast activity is seen in many osteopenic disorders, including postmenopausal osteoporosis, Paget's disease, lytic bone metastases, or rheumatoid arthritis, leading to increased bone resorption and crippling bone damage.<sup>1</sup> Various factors have been described including CSF1 (M-CSF), IL1, TGF $\beta$ , TGF $\alpha$ , TNF $\alpha$ , TNF $\beta$ , IL6, vitamin 1,25-dihydroxyvitamin D<sub>3</sub>, IL11, calcitonin, PGE<sub>2</sub>, or parathyroid hormone (PTH) that affect osteoclastogenesis at distinct stages of development.<sup>2</sup> However, genetic ablation experiments have shown that these factors are not essential for osteoclast development *in vivo*. Because of the enormous social and economic impacts of bone loss and crippling to human welfare and the search to increase human life span without the "side effects" of old age, it was of paramount importance to identify essential factors involved in osteoclast development and bone remodelling. The essential molecules have been recently identified to be the TNF-TNFR superfamily proteins RANKL, RANK, and OPG.

The TNF family molecule RANKL (receptor activator of NF $\kappa$ B ligand; also known as osteoprotegerin ligand (RANKL); TNF related activation induced cytokine (TRANCE), osteoclast differentiation factor (ODF), and TNFSF11) and its receptor RANK (TNFRSF11A) are key regulators of bone remodelling and essential for the development and activation of osteoclasts.<sup>3–7</sup> RANKL also regulates T cell/dendritic cell

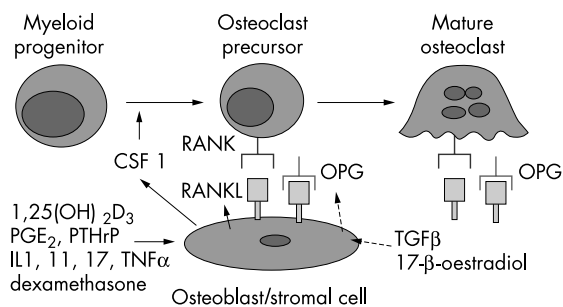
communications, dendritic cell survival,<sup>7,8</sup> and lymph node organogenesis.<sup>4</sup> Moreover, production of RANKL by activated T cells directly controls osteoclastogenesis and bone remodelling and explains why autoimmune diseases, cancers, leukaemias, asthma, chronic viral infections, and periodontal disease result in systemic and local bone loss.<sup>9</sup> In particular, RANKL seems to be the pathogenetic principle that causes bone and cartilage destruction in arthritis. Inhibition of RANKL function via the natural decoy receptor osteoprotegerin (OPG, TNFRSF11B) prevents bone loss in postmenopausal osteoporosis and cancer metastases and completely blocks bone loss and crippling in various rodent models of arthritis. Intriguingly, RANKL and RANK play essential parts in the formation of a lactating mammary gland in pregnancy.<sup>10</sup> This system provided a novel and unexpected molecular paradigm that links bone morphogenesis, T cell activation and the organisation of lymphoid tissues, and mammary gland formation required for the survival of mammalian species. Inhibition of RANKL function via its natural decoy receptor OPG or small molecules might be the future treatment of choice to abolish osteoporosis, tooth loss, or crippling in arthritis.

**RANKL**

RANKL/OPGL/TRANCE/ODF/TNFSF11 was cloned simultaneously by four independent groups.<sup>3–6,8</sup> The *rankl* gene encodes a TNF superfamily molecule of 316 amino acids (38 kDa) and three RANKL subunits assemble to form the functional trimeric molecule. Trimeric RANKL is made as a membrane anchored molecule and can then be released from the cell surface as a soluble homotrimeric molecules after proteolytic cleavage by the metalloprotease disintegrin TNF $\alpha$  convertase (TACE).<sup>11</sup> It remains to be seen whether TACE is indeed the critical protease required for the release of RANKL from the cell surface. Although it has been proposed that soluble and membrane bound RANKL might have different biological functions, for example, membrane bound RANKL might work more efficiently than soluble RANKL,<sup>12</sup> both soluble and membrane bound RANKL can function as potent agonistic ligands for osteoclastogenesis *in vitro*.<sup>3</sup> The potential differences between soluble and membrane bound RANKL should be investigated further.

RANKL is highly expressed in osteoblast/stromal cells, primitive mesenchymal cells surrounding the cartilaginous anlagen and hypertrophying chondrocytes.<sup>3</sup> RANKL mRNA has also been observed in prehypertrophic and hypertrophic chondrocytes at day 15 of embryogenesis and extraskelatal tissues such as the brain, heart, kidneys, skeletal muscle, and skin throughout mouse development.<sup>13</sup> RANKL expression can be upregulated by bone resorbing factors such as glucocorticoids, vitamin D<sub>3</sub>, IL1, IL6, IL11, IL17, TNF $\alpha$ , PGE<sub>2</sub>,

**Abbreviations:** TNF, tumour necrosis factor; RANKL, receptor activator of NF $\kappa$ B ligand; OPG, osteoprotegerin; TACE, TNF $\alpha$  convertase; ODF, osteoclast differentiation factor; TRANCE, TNF related activation induced cytokine; TRAF, tumour necrosis factor receptor associated factor; RA, rheumatoid arthritis



**Figure 1** Regulation of osteoclast formation. Calcitropic factors such as vitamin D<sub>3</sub>, prostaglandin E<sub>2</sub>, IL1, IL11, TNF $\alpha$  and glucocorticoid induce RANKL expression on osteoblasts. RANKL binding to the RANK expressed on haematopoietic progenitors activates a signal transduction cascade that leads to osteoclast differentiation in the presence of the survival factor CSF1. Moreover RANKL stimulates bone resorbing activity in mature osteoclasts via RANK. OPG produced by osteoblasts acts as a decoy receptor for RANKL and inhibits osteoclastogenesis and osteoclast activation by binding to RANKL. TGF $\beta$  released from bone during active bone resorption has been suggested as one feedback mechanism for upregulating OPG. Oestrogen can increase OPG production on osteoblasts, which is a possible explanation of postmenopausal osteoporosis after oestrogen withdrawal.

or PTH.<sup>1,3,6</sup> Using *in vitro* culture systems, RANKL can both activate mature osteoclasts and mediate osteoclastogenesis in the presence of CSF1.<sup>3,6</sup> As it was unclear whether RANKL was indeed essential for osteoclast development and function *in vivo*, our group created the first *ranks* mutant mice using gene targeting.<sup>4</sup> *Rankl*<sup>-/-</sup> mice display severe osteopetrosis, stunted growth, and a defect in tooth eruption, and *ranks*<sup>-/-</sup> osteoblasts cannot support osteoclastogenesis. These mice still contain haematopoietic precursors that can differentiate into phenotypically and functionally mature osteoclasts *in vitro* in the presence of recombinant RANKL and CSF1<sup>4</sup> and *in vivo* after injection of recombinant RANKL (JMP, unpublished data). Importantly, osteoblast cell lines derived from *ranks*<sup>-/-</sup> mice do not support osteoclast formation indicating that the defect in osteoclastogenesis observed in *ranks*<sup>-/-</sup> mice is attributable to an intrinsic defect in osteoblastic stroma.

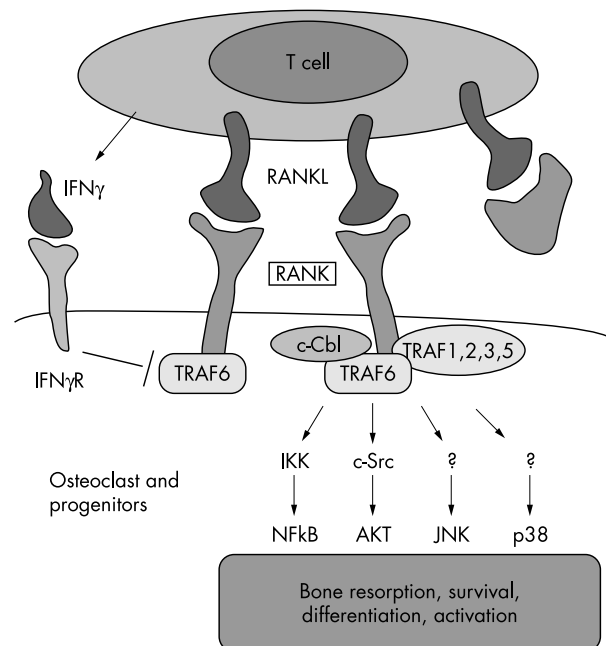
Whereas *csf1* mutant *op/op* mice display a developmental arrest in both monocyte/macrophage and osteoclast lineages, *ranks*<sup>-/-</sup> mice display normal monocyte/macrophage differentiation and normal differentiation of dendritic cells.<sup>1</sup> The osteoclast defect in *csf-1* mutant *op/op* mice is not absolute and older *op/op* mice do have, albeit only few osteoclasts. Moreover, the defect in *op/op* mice can be reversed by transgenic overexpression of Bcl-2 in the osteoclast/monocyte lineage indicating that—in contrast with RANKL—CSF1 expression is not essential for osteoclast development.<sup>14</sup> Whether Bcl-2 overexpression would also rescue the phenotype in *ranks*<sup>-/-</sup> mice needs to be explored. Thus, our genetic data provided the first proof that RANKL is a specific and essential differentiation factor for osteoclast precursors. Moreover RANKL is an essential activation factor for mature osteoclasts (fig 1).

#### RECEPTOR ACTIVATOR OF NF $\kappa$ B LIGAND—RANK

The receptor for RANKL is RANK (receptor activator of NF $\kappa$ B also known as TRANCE-R, ODAR, or TNFRSF11A). RANK is a member of TNF-R superfamily, expressed as a transmembrane heterotrimer on the surface of haematopoietic osteoclasts progenitors, mature osteoclasts, chondrocytes, mammary gland epithelial cells,<sup>10,19</sup> trophoblast cells, and multiple epithelial tumour cell lines (JMP, unpublished data). *In vitro* ligation of RANK with RANKL results in osteoclastogenesis from progenitor cells and the activation of mature osteoclasts.<sup>15-17</sup> Mice with a genetic mutation of RANK are phenocopies of *ranks*<sup>-/-</sup> mice and have a complete block in

osteoclast development that can be restored by reintroduction of RANK into bone marrow progenitor cells.<sup>18,19</sup> The osteopetrosis observed in these mice can be reversed by transplantation of bone marrow from *rankl*<sup>-/-</sup> mice, indicating that *ranks*<sup>-/-</sup> mice have an intrinsic defect in osteoclast function.<sup>19</sup> Thus, the interaction between RANKL expressed by stromal cells/osteoblasts and its receptor RANK expressed on osteoclast precursors are essential for osteoclastogenesis (fig 1). Importantly, in human familial expansile osteolysis, a rare autosomal dominant bone disorder characterised by focal areas of increased bone remodelling,<sup>20</sup> a heterozygous insertion mutation in exon 1 of RANK has been noted that appears to increase RANK mediated NF $\kappa$ B activation and thus might be causal for the disease. All biochemical, functional, and genetic results in humans and mutant mice established the absolute dependency of osteoclast differentiation and activation of mature osteoclasts on the expression of RANKL and RANK.

When RANK on osteoclasts is activated it sends signals into the cells through adapter proteins (fig 2). RANK contains 383 amino acids in its intracellular domain (residues 234–616), which contain three putative binding domains (termed I, II, and III) for tumour necrosis factor receptor associated factors (TRAFs).<sup>21</sup> Mapping of the structural requirements for TRAF/RANK interaction revealed multiple TRAF binding sites clustered in two distinct domains in the RANK cytoplasmic tail. These TRAF binding domains were shown to be functionally important for the RANK dependent induction of NF $\kappa$ B and c-Jun NH2-terminal kinase (JNK) activities. In particular, TRAF6 interacts with membrane proximal determinants distinct from those binding TRAFs 1, 2, 3, and 5. When this membrane proximal TRAF6 interaction domain was deleted, RANK mediated NF $\kappa$ B signalling was completely inhibited while JNK activation was only partially inhibited<sup>21-24</sup> suggesting that interaction with TRAFs is necessary for NF $\kappa$ B



**Figure 2** RANK signalling. After RANKL binding, the TNFR member RANK sends signals into the cells through tumour necrosis factor receptor associated factors (TRAFs) 1, 2, 3, 5, and 6. c-Src and Cbl proteins associate with the cytoplasmic tail of RANK. These RANK associated molecules relay RANK dependent stimulation to downstream pathways such as NF- $\kappa$ B, JNK/SAPK, p38, and Akt/PKB that regulate bone resorption, activation, survival, and differentiation of osteoclasts and dendritic cells. Interferon gamma can inhibit RANKL mediated osteoclastogenesis presumably via induction of TRAF6 ubiquitination and proteolytic TRAF6 degradation. The scheme is based on Arron and Choi.<sup>92</sup>

activation but not essential for activation of the JNK pathway. Indeed mice lacking TRAF6 have a similar bone phenotype as *rankl*<sup>-/-</sup> and *rank*<sup>-/-</sup> mice attributable to a partial block in osteoclastogenesis and defective activation of mature osteoclasts.<sup>25-27</sup> It should be noted that TRAF6 mutant mice still contain a substantial number of TRAP<sup>+</sup> osteoclasts<sup>26</sup> whereas NFκB1/NFκB2 double mutant mice completely lack TRAP<sup>+</sup> osteoclasts.<sup>28-29</sup> Thus, TRAF6 is a critical factor involved in the activation of mature osteoclasts but other TRAFs (and possibly other molecules) seem to be able to partially substitute for the loss of TRAF6 during osteoclast development. In line with these data, osteoclastogenesis can be initiated in *rank*<sup>-/-</sup> mice by transfer of mutant RANK that lacks the TRAF6 binding site.<sup>19</sup>

RANKL also activates the anti-apoptotic serine/threonine kinase Akt/PKB through a signalling complex involving c-Src and TRAF6.<sup>30</sup> c-Src and TRAF6 interact with each other and with RANK after receptor engagement and a deficiency in c-Src or addition of Src family kinase inhibitors blocks TRANCE mediated Akt/PKB activation in osteoclasts. TRAF6, in turn, increases the kinase activity of c-Src leading to tyrosine phosphorylation of downstream signalling molecules such as c-Cbl.<sup>30</sup> Moreover, RANK can recruit TRAF6, Cbl family scaffolding proteins, and the phospholipid kinase PI3-K in a ligand and Src dependent manner. RANKL mediated Akt/PKB activation is defective in *cbl-b*<sup>-/-</sup> dendritic cells.<sup>31</sup> These findings implicate Cbl family proteins as not only negative regulators of signalling, but also as positive modulators of TNFR superfamily signalling. Moreover, these data provided the first evidence of a cross talk between TRAF proteins and Src family kinases. In addition, it should be noted that inhibition of p38 kinases using SB203580 and overexpression of dominant negative p38α or MKK6 can inhibit RANKL induced differentiation of the osteoclast-like cell line RAW264 in in vitro culture systems.<sup>32</sup> Whether JNK/SAPK kinases are also important for osteoclasts development or function, or both, has not been reported yet.

## OSTEOPROTEGERIN

Osteoprotegerin (OPG; "protector of the bone"; also known as osteoclastogenesis inhibitory factor, OCIF) is a secreted protein with homology to members of the TNF receptor family.<sup>5-33</sup> The *opg* gene encodes a 44 kDa protein that is post-translationally modified to a 55 kDa molecule through N-linked glycosylation. Although OPG is a member of the TNFR family, which normally form molecular trimers, OPG is secreted as 110 kDa homodimer. OPG functions as a soluble decoy receptor to RANKL and competes with RANK for RANKL binding. Consequently, OPG is an effective inhibitor of osteoclast maturation and osteoclasts activation in vitro and in vivo.<sup>4-5</sup> High systemic levels of OPG in OPG transgenic mice cause osteopetrosis with normal tooth eruption and bone elongation and inhibit the development and activity of endosteal, but not periosteal, osteoclasts.<sup>5</sup> As expected, OPG deficient mice display severe osteoporosis associated with a high incidence of fractures,<sup>34-35</sup> indicating that the level of bone mass correlates with the levels of OPG in mice.

Expression of OPG in ST2 stromal cell line and human bone marrow stromal cells is downregulated by bone resorbing factors such as vitamin D3 [1,25(OH)2D3], prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), or glucocorticoids and upregulated by Ca<sup>2+</sup> ions and TGFβ.<sup>33-36-37</sup> OPG is also expressed in follicular dendritic cells (identified as FDCR-1) and is upregulated after CD40 stimulation.<sup>38</sup> In addition to osteoporosis, some but not all OPG mutant mice develop calcification of their large arteries<sup>34</sup> and RANKL and RANK transcripts are detected in the calcified arteries of OPG<sup>-/-</sup> mice.<sup>39</sup> Transgenic OPG delivered from mid-gestation through adulthood does prevent the formation of arterial calcification in *opg*<sup>-/-</sup> mice by blocking a process resembling osteoclastogenesis.<sup>39</sup> These data indicate that the

OPG/RANKL/RANK signalling pathway may play an important part in both pathological and physiological calcification processes. Such findings may also explain the observed high clinical incidence of vascular calcification in the osteoporotic patient population.<sup>40</sup> As women with osteoporosis have increased incidence of strokes,<sup>41-42</sup> OPG, RANKL, and RANK might play, similar to the CD40 and CD40L system,<sup>43</sup> a part in the pathogenesis of atherosclerosis, strokes, or heart attacks via a yet unknown effect on blood vessels.

All genetic and functional experiments by many different groups indicate that the balance between RANKL-RANK signalling and the levels of biologically active OPG regulate development and activation of osteoclasts and bone metabolism (fig 1). Intriguingly, all factors that inhibit or increase bone resorption via osteoclasts act via regulation of RANKL-RANK and/or OPG. Thus, it seems that the complex system of osteoclast regulated bone remodelling is critically dependent on these three molecules. However, although RANKL is also expressed in many other tissues than the bone, osteoclast development is restricted to the bone microenvironment suggesting that another tissue specific factor might exist that acts in concert with RANKL/RANK. It has recently been shown in vitro that TNFα and IL1 can apparently induce the development of TRAP<sup>+</sup> osteoclasts in the absence of RANK/RANKL.<sup>44-45</sup> However, in our own genetic experiments using RANK deficient osteoclast progenitors, TNFα as well as IL1 dependent osteoclastogenesis are strictly dependent on RANK expression. Thus, whereas TNFα and IL1 seem to potentiate the development of osteoclasts,<sup>46</sup> presumably via activation of common second messenger systems such as NFκB activation, the effects of both IL1 and TNFα on osteoclasts rely on the expression of RANKL/RANK. It should be also noted that mutations of TNFα, TNFR1, or TNFR2 do not cause any changes in bone metabolisms or osteoclast development/activation in vivo.

In addition to the association between RANKL and OPG, OPG can also bind to the TNF family molecule TRAIL at low stoichiometry (about 10 000 times less binding to TRAIL than to RANKL).<sup>47</sup> OPG-Fc binds TRAIL with an affinity of 3.0 nM, which is slightly weaker than the interaction of TRID-Fc or DR5-Fc with TRAIL. Functionally, high doses of OPG inhibit TRAIL induced apoptosis of Jurkat cells and TRAIL can block the anti-osteoclastogenic activity of OPG.<sup>47</sup> These data suggest potential cross regulatory mechanisms by OPG and TRAIL. However, it is still not known whether OPG-TRAIL interactions have any functional relevance in vivo. Importantly, OPG expression is induced by oestrogen in cell lines and in vivo,<sup>48-49</sup> which might explain postmenopausal osteoporosis in women, that is, reduced ovarian functions leads to reduced oestrogen levels and hence reduced OPG levels, which release RANKL from the inhibition by the decoy receptor. Injection of OPG into ovariectomised female rats blocks bone loss and osteoporosis normally associated with the loss of ovarian function.<sup>5</sup> Thus, OPG and/or modulation of RANKL-RANK function via small molecules are promising avenues to prevent postmenopausal osteoporosis. In essence, OPG seems to function in bone loss similar to insulin in diabetes: injection of OPG prevents osteoclast activation and osteopenia in essentially every model system of osteoclast mediated bone loss.

## ROLE OF RANKL AND RANK IN THE IMMUNE SYSTEM

At around the same time when we had the first evidence that RANKL might play a part in osteoclast development, RANKL (TRANCE) was independently cloned by two other groups as a molecule expressed on the surface of activated T cells.<sup>7-8</sup> Both soluble and membrane-bound RANKL is produced by activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells.<sup>7-9</sup> RANKL is also expressed in lymph nodes, spleen, thymus and intestinal lymphoid patches,<sup>9</sup> and immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes.<sup>8</sup> RANKL

expression in T cells is induced by antigen receptor engagement and is regulated by calcineurin, ERK1/ERK-2, and PKC regulated signalling pathways.<sup>8,9</sup> RANK is expressed on the surface of dendritic cells, mature T cells, and haematopoietic precursors and RANKL-RANK interactions can induce cluster formation, Bcl-XL expression, survival, CD40 expression, and IL12 production in dendritic cells.<sup>7,8,50</sup> In contrast with CD40L/CD40, RANKL/RANK signalling does not change the expression of cell surface molecules such as MHC class II, CD80, CD86, and CD54. Whereas CD40L is primarily expressed on activated CD4<sup>+</sup> T cells, RANKL is expressed on both activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells.<sup>9,51</sup> Moreover, the maximal level of RANKL after the initial T cell activation event is 48 hours and high levels of RANKL expression are sustained until 96 hours, while CD40L is rapidly expressed and downregulated.<sup>52</sup> Thus, CD40L-CD40 interactions might primarily control the initial priming stage whereas RANKL-RANK might act at later time points than CD40L during the immune response.

OPG was found in a screen to identify novel genes expressed in follicular dendritic cells and OPG can be found on the cell surface of DCs probably by capturing of soluble OPG to the cell membrane via binding of a hyalurinic acid binding region present in OPG.<sup>38</sup> Thus, like the interactions between CD40L and CD40, or CD28 and CD80/CD86, it has been suggested that RANKL/RANK might regulate dendritic cell functions, T cell activation, and T cell-dendritic cell communication *in vitro*.<sup>8,53</sup> Based on this studies, various groups are currently trying to control the dendritic cell fate via RANKL-RANK and OPG to modulate *in vivo* dendritic cell survival and to enhance the efficiency of dendritic cell based vaccinations for anti-tumour therapy or the treatment of autoimmune diseases. Moreover, at least in theory it is also possibly that OPG might modulate this interaction.

It has been shown that treatment of antigen pulsed mature dendritic cells with soluble RANKL *in vitro* increases the number and persistence of antigen presenting dendritic cells in the draining LNs *in vivo*.<sup>50</sup> Furthermore, RANKL treatment increased antigen specific primary T cell responses. Interestingly, significant memory responses were observed only in mice injected with RANKL treated dendritic cells.<sup>50</sup> Although the mechanisms are not clear, these increased primary and memory T cells responses after vaccination with RANKL treated dendritic cells could be attributable to increased/ altered cytokine production such as expression IL12 and/or increased number of antigen pulsed dendritic cells. Moreover, it has been suggested that CD40L/CD40 interactions regulate T/B responses whereas RANKL/RANK might have a role in memory T cell responses.<sup>54</sup> However, whereas CD40L is essential for the T cell dependent B cell responses such as germinal centre formation, affinity maturation and class switching,<sup>55,56</sup> germinal centre formation, Ig class switching and the production of neutralising antiviral Abs are not overtly affected in *rankl*<sup>-/-</sup> mice and all the B cell defects of these mutant could be explained by the absence of lymph nodes (Young-Yun Kong and J M Penninger, unpublished data). In the final analyses of all the published genetic and functional studies on RANKL, RANK, and OPG, it seems that although these molecules can influence some aspects of lymphocyte and dendritic cell functions, none of these molecules plays an essential function in T cells, B cells, or dendritic cells that cannot be compensated for by other molecules such as CD40L/CD40. Thus, the essential and true function of RANKL/RANK in the immune system and communication between dendritic cells and T cells needs to be elucidated. For example, as expression of these molecules can be controlled by sex hormones,<sup>10,48</sup> we speculate that this system might control sex specific differences in immunity and could be involved in the higher incidence of autoimmune diseases like arthritis in women and/or the often observed improvement of rheumatoid arthritis during pregnancy.

During the initial analyses of our *rankl*<sup>-/-</sup> and later *rank* mutant mice, a completely unexpected immunological

phenotype became evident: *rankl*<sup>-/-</sup> and *rank*<sup>-/-</sup> mice lack all lymph nodes but display intact splenic architecture and develop Peyer's patches normally, suggesting that RANKL and RANK have a specific and essential role in lymph node organogenesis.<sup>4,18,19</sup> Defective lymph node development in *rankl*<sup>-/-</sup> mice correlates with a significant reduction in lymphotoxin LT $\alpha$  $\beta$ <sup>+</sup> $\alpha$ 4 $\beta$ 7<sup>+</sup>CD45<sup>+</sup>CD4<sup>+</sup>CD3<sup>-</sup> cells and their failure to form clusters in the rudimentary mesenteric lymph node anlage.<sup>57</sup> Transgenic RANKL mediated restoration of lymph node development required LT $\alpha$  $\beta$  expression on CD45<sup>+</sup>CD4<sup>+</sup>CD3<sup>-</sup> cells as lymph node formation could not be induced in LT $\alpha$ <sup>-/-</sup> mice. The authors proposed that both RANKL and LT $\alpha$  $\beta$  regulate the colonisation and cluster formation by CD45<sup>+</sup>CD4<sup>+</sup>CD3<sup>-</sup> cells during lymph node organogenesis.<sup>57</sup> Importantly, RANKL disruption provided the first evidence that development of lymph nodes and Peyer's patches can be genetically uncoupled. The exact cellular and molecular mechanism of RANKL-RANK regulated lymph node morphogenesis needs to be established.

## T CELLS AND BONE: OSTEOIMMUNOLOGY

Bone remodelling and bone loss is controlled by a balance between RANKL/RANK and the RANKL decoy receptor OPG. As RANKL is made by T cells after antigen-receptor stimulation, we asked the question whether T cell derived RANKL can indeed regulate the development and activation of osteoclasts—that is, whether activated T cells can modulate bone turnover via RANKL. In an *in vitro* cell culture system, activated T cells can directly trigger osteoclastogenesis via RANKL.<sup>9</sup> Importantly, systemic activation of T cells *in vivo* leads to a RANKL dependent increase in osteoclastogenesis followed by bone loss. All *in vitro* and *in vivo* effects of T cells on osteoclasts could be blocked by the administration of the decoy receptor OPG.<sup>9</sup> In a recent study it has been confirmed that transgenic overexpression of RANKL in T cells restores osteoclastogenesis in a *rankl*<sup>-/-</sup> background and partially restores normal bone marrow cavities.<sup>58</sup> These data showed that systemic activation of T cells leads to bone loss, indicating that, through their production of RANKL, T cells are crucial mediators of bone loss *in vivo*. The results also provided a novel paradigm for T cells as regulators of bone physiology.

As mutant mice that lack T cells still have normal bone cavities and tooth eruption, T cells are probably not required for normal bone homeostasis. However, chronic systemic T cell activation such as in autoimmune diseases or viral infections or local inflammation within the bone attributable to metastasis, infections, bone implants such as artificial hips, and fractures, or joint inflammation in arthritis probably attracts T cells that then actively participate in bone remodelling via production of RANKL. Moreover, glucocorticoids, which are used to treat autoimmune diseases and allergic disorders, strongly induce RANKL expression and decrease OPG.<sup>6,59</sup> Intriguingly, it has been recently shown that ovariectomy induced bone loss in wild type mice but not in T cell deficient nude mice, which was restored by adoptive transfer of wild type T cells. Moreover, this effect seems to depend on TNF $\alpha$ -TNFRp55 interactions that act presumably upstream of RANKL.<sup>60</sup> If this observation holds true in humans, it is possible that T cells are the principal cells that contribute to bone loss in old age.

These findings provide a molecular explanation for bone loss associated with diseases having immune system involvement, such as adult and childhood leukaemias, cancer metastasis, autoimmunity, and various viral infections. Inhibition of RANKL function via OPG or a related molecule might therefore prevent bone destruction in multiple diseases. Moreover, it has now become clear that many other molecules such as osteopontin that have functions in bone physiology also have important roles in the immune systems.

## RANKL/RANK CONTROL OSTEOCLAST ACTIVATION AND BONE LOSS IN ARTHRITIS

Bone loss represents an important unsolved problem in rheumatoid arthritis (RA). The skeletal complications of RA consist of focal bone erosions and periarticular osteoporosis at sites of active inflammation, and generalised bone loss with reduced bone mass.<sup>61</sup> Local bone loss at the affected joints frequently results in lifelong crippling. Arthritis in humans and animal models is characterised by synovial inflammation, erosion of bone and cartilage, severe joint pain, and ultimately lifelong crippling.<sup>62</sup> For example, in Lewis rats, experimental induction of arthritis by subcutaneous injection of bacterial products in adjuvant leads to severe inflammation in the bone marrow and soft tissues surrounding joints accompanied by extensive local bone and cartilage destruction, loss of bone mineral density, and crippling.<sup>63</sup> This condition in rats, called adjuvant induced arthritis, mimics many of the clinical and pathological features of human RA. Lesions in rat adjuvant induced arthritis are dependent on T cell activation,<sup>64</sup> and T cells in the inflamed joints and draining lymph nodes of affected rats have been shown to produce many proinflammatory cytokines.<sup>62</sup>

In the adjuvant induced arthritis model, RANKL protein is expressed on the surface of synovial effector T cells isolated at the clinical onset of arthritis.<sup>9</sup> Inhibition of RANKL via OPG had no effect on the severity of inflammation. However, OPG treatment completely abolished the loss of mineral bone density in the inflamed joints of these animals in a dose dependent manner. Histologically, OPG treated arthritic rats exhibited minimal loss of cortical and trabecular bone, whereas untreated arthritic animals developed severe bone lesions characterised by partial to complete destruction of cortical and trabecular bone, and erosion of the articular cartilages. Bone destruction in untreated arthritic rats correlated with a dramatic increase in osteoclast numbers whereas OPG treatment prevented the accumulation of osteoclasts.<sup>9</sup> These results showed that RANKL is a key mediator of joint destruction and bone loss in adjuvant arthritis. Importantly, whereas untreated rats experienced severe crippling, rats treated with OPG at the onset of disease—similar to a patient consulting a doctor at the onset of joint swelling—did not show any signs of clinical crippling.

Alteration of cartilage structures leading to cartilage collapse constitutes a critical step in arthritic joint destruction. Controversy exists whether cartilage destruction occurs independently of bone loss, or whether damage to the subchondral bone indirectly causes cartilage deterioration.<sup>65</sup> In untreated arthritic rats, partial or complete erosion of the cartilage in both the central and peripheral regions of joint surfaces is observed. In striking contrast, neither cartilage erosion nor matrix degeneration in the centres of joint surfaces occurred in OPG treated animals.<sup>9</sup> OPG could protect the cartilage by maintaining the underlying subchondral bone therefore insulating the overlying cartilage from the inflammatory cell infiltrates in the bone marrow. As both RANKL and RANK are expressed on chondrocytes,<sup>3, 15</sup> and *rankl*<sup>4</sup> as well as *rank* mutant mice<sup>19</sup> exhibit significant changes in the columnar alignment of chondrocytes at the growth plate, it is possible that RANKL/RANK play a direct part in cartilage growth and cartilage homeostasis. RANK, RANKL, and OPG expression have been recently observed in normal cartilage.<sup>66</sup> However, the functional relevance of RANKL-RANK expression in chondrocytes is still not known. Thus, inhibition of RANKL activity by OPG can prevent cartilage destruction, a critical, irreversible step in the pathogenesis of arthritis.

Arthritis can occur in the absence of T cells.<sup>67</sup> Using *in situ* hybridisation of inflamed rat joints and isolation of different cell populations from these joints, we could show that RANKL is indeed expressed in lymphocytes, macrophages, and especially in synoviocytes.<sup>9</sup> In line with these findings, genetic

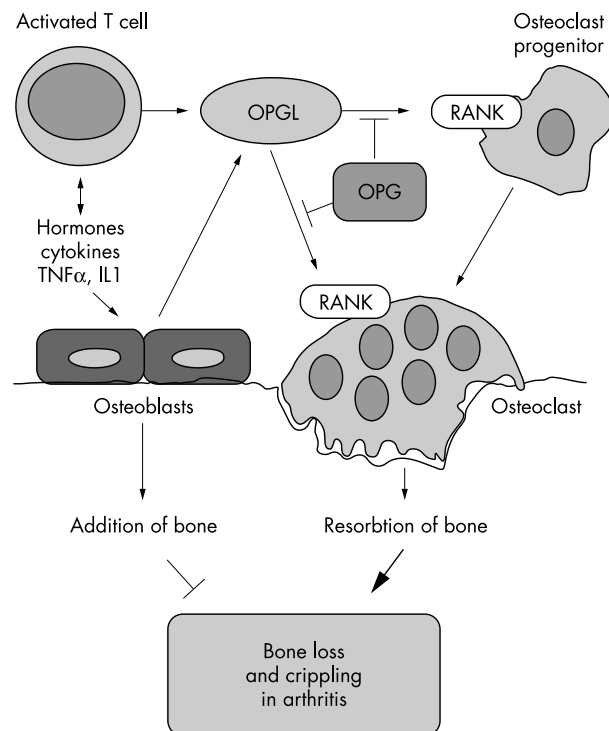
ablation of RANKL does not prevent inflammation in an antibody mediated model of arthritis using the K/BxN serum transfer model.<sup>68</sup> Multinucleated TRAP positive osteoclast-like cells were abundant in resorption lacunae in areas of bone erosion in arthritic control mice, and were completely absent in arthritic RANKL mutant mice, demonstrating the absolute requirement for RANKL in osteoclastogenesis in this serum transfer model of inflammatory arthritis.<sup>69</sup> Cartilage damage was still observed in both arthritic TRANCE/RANKL knockout mice and arthritic control mice but a trend towards milder cartilage damage in the *rankl*<sup>-/-</sup> mice was noted. Thus, it seems that RANKL is not essential for cartilage destruction, but clearly plays a yet unidentified modulatory part.<sup>69</sup>

It has recently been shown that interleukin 1 and to a lesser extent TNF $\alpha$  are critical mediators of antibody induced arthritis in the serum transfer model.<sup>70</sup> Moreover, inhibition of RANKL via OPG prevents bone loss and has a beneficial effect on cartilage destruction without affecting inflammation in a TNF $\alpha$  induced arthritis model<sup>71</sup> indicating that TNF $\alpha$  triggered joint destruction is critically dependent on RANKL expression.<sup>72</sup> It has also been recently suggested that the RANKL-RANK system plays an important part for osteoclastogenesis in both local and systemic osteolytic lesions in autoimmune type II collagen induced arthritis in mice.<sup>73</sup> Thus, in all animals for arthritis studied so far, the RANKL/RANK system is the trigger of bone loss and crippling making OPG the prime drug candidate for therapeutic intervention in different forms of arthritis.

To extend these rodent studies to human patients with arthritis, we collected inflammatory cells from the synovial fluids of patients with adult or juvenile RA and patients with osteoarthritis and evaluated OPG and RANKL expression. All RA patients and patients with advanced osteoarthritis tested ( $n > 40$ ) in our study exhibited RANKL expression in inflammatory cells while OPG expression was not detectable.<sup>9</sup> Thus, the correlation between RANKL expression in inflamed joints and arthritis was absolute. To distinguish which cells were producing RANKL, inflammatory synovial fluids were separated into T cell and non-T cell populations. Consistent with our results in rats, both synovial T cell and non-T cell populations from RA patients expressed RANKL, but not OPG.<sup>9</sup> In subsequent studies, it has been shown that activated synovial T cells, monocytes, and synovial fibroblasts express both membrane bound and soluble forms of RANKL<sup>61, 74</sup> and that RANKL mRNA is present in cells isolated from the pannus and synovial membrane regions of RA patients.<sup>75</sup> Moreover, activated human T cells expressing RANKL can induce osteoclastogenesis from autologous peripheral monocytes, which can be blocked by dose dependent inhibition using OPG.<sup>74</sup> These data confirm our initial findings in rodent adjuvant arthritis models, and suggest that RANKL is the principal mediator of bone destruction in human arthritis. Moreover, these results provide a unifying mechanism between T cell activation, cytokine production, and osteoclast activation and joint destruction via the RANKL/OPG/RANK ligand-receptor system that explains the spectrum of skeletal disorders in RA.<sup>62</sup>

## A MOLECULAR SCENARIO OF T CELL REGULATED BONE LOSS

In inflammatory or autoimmune disease states, activated T cells produce RANKL and proinflammatory cytokines such as TNF $\alpha$ , IL1, IL6, or IL11, all of which can induce RANKL expression in osteoblasts and bone marrow stromal cells.<sup>1, 76</sup> Thus, it seems that T cells promote bone resorption directly via RANKL expression and indirectly via expression of proinflammatory cytokines that mediate RANKL expression in non-T cells (fig 3). These results are consistent with findings that T cells and non-T cell populations express RANKL in arthritic joints. Inhibition of RANKL has no effect on inflammation, but



**Figure 3** Rational drug design to interfere with arthritic disease. Activated T cells produce cytokines such as TNF $\alpha$ , IL1, IL11, and IL17 that lead to RANKL expression on osteoblasts. Moreover, activated T cells directly express and produce RANKL that induces osteoclast formation and activation. The soluble decoy receptor for RANKL, OPG, blocks both pathways. In addition to inhibiting the functions of cytokines to alleviate inflammation, inhibition of RANKL via OPG might be useful to block osteoclast activation and crippling in arthritis irrespective of the disease trigger.

completely prevents bone loss and partially protects cartilage.<sup>7</sup> It has been shown that bone resorption induced by local injection of IL1 $\beta$  or TNF $\alpha$  over the calvaria of mice can be blocked by concurrent systemic injection of OPG, indicating that RANKL is the mediator of the bone damaging effects of TNF $\alpha$  and IL1 $\beta$  in vivo.<sup>77</sup> Although inhibition of TNF $\alpha$  and IL1 using soluble receptor antagonists to some extent prevent inflammation and bone loss in arthritis,<sup>78,79</sup> inhibition of RANKL function via OPG might therefore prevent bone destruction and cartilage damage in arthritis irrespective of the initial trigger.

Reduced bone mineral densities can also be seen in many human diseases such as adult and childhood leukaemia,<sup>80</sup> chronic infections such as hepatitis C or HIV,<sup>81</sup> autoimmune disorders such as diabetes mellitus<sup>82</sup> and lupus erythematosus,<sup>83</sup> allergic diseases such as asthma,<sup>84</sup> lytic bone metastases in multiple cancers such as breast cancer,<sup>85</sup> and of course arthritis.<sup>62</sup> These osteopenic disorders can cause irreversible crippling, thereby severely disrupting the lives of many patients. For example, many patients with lupus require hip replacement surgery and essentially all children that survive leukaemia experience severe bone loss and growth retardation. In North America and Europe 1 in 100 people develop RA and 1 in 10 people develop osteoarthritis. In addition, T cell derived RANKL contributes to alveolar bone resorption and tooth loss in an animal model that mimics human periodontal disease. The alveolar bone resorption around the teeth can be inhibited with OPG.<sup>86</sup> It should be noted that OPG can prevent cancer cell induced bone destruction,<sup>87-89</sup> cancer metastases associated bone pain, and pain related neurochemical reorganisation of the spinal chord.<sup>90</sup> In most of these osteopenic disorders, disease pathogenesis correlates with the activation of T cells (fig 3).

However, short-term activation of T cells does not result in any detectable bone loss and even in some chronic T cell and TNF $\alpha$  mediated diseases such as ankylosis spondylitis,<sup>91</sup> T cell activation does not result in bone loss. Moreover, T cells are working constantly to fight off the universe of foreign particles in which we live, so, at any point in time, some T cells are activated.<sup>92</sup> So what is it that prevents these T cells from causing extensive bone loss every time we have an infection? A crucial counter-regulatory mechanism, by which activated T cells can inhibit the RANKL induced maturation and activation of osteoclasts has been recently discovered (fig 2).<sup>93</sup> It turns out that interferon gamma blocks RANKL induced osteoclast differentiation in vitro. Moreover, interferon gamma receptor knockout mice develop more bone destruction in inflammatory arthritis than normal mice. Mechanistically, interferon gamma can activate the ubiquitin-proteasome pathway within the osteoclasts, resulting in the degradation of TRAF6. Thus, it seems that interferon gamma can prevent uncontrolled bone loss during inflammatory T cell responses. Moreover, T cell derived IL12 alone and IL12 in synergy with IL18 inhibits osteoclast formation in vitro<sup>94</sup> and IL4 can abrogate osteoclastogenesis through STAT6 dependent inhibition of NF $\kappa$ B signalling.<sup>95,96</sup> Thus, multiple T cell derived cytokine might be able to interfere with RANK signalling and therefore osteoclastogenesis and osteoclast functions. In the future it will be interesting to determine the mechanisms that control the balance between T cell mediated bone loss and inhibition of osteoclastogenesis.

## CONCLUSIONS

It has become clear that inhibition of RANKL mediated activation of RANK via OPG or a related molecule ameliorates many osteopenic conditions. RANKL inhibition seems to be the most rational and advisable strategy to prevent bone destruction in multiple diseases and to possibly eradicate major human diseases such as osteoporosis and to curtail crippling in arthritis, diseases that affect millions of people.

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