

## REPORT

IKK $\beta$  as a target for treatment of inflammation induced bone loss

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The transcription factor nuclear factor (NF)- $\kappa$ B is well recognised as a pivotal player in osteoclastogenesis and inflammation induced bone loss. Here, the authors discuss their recent results, obtained using a genetic approach in mice, that indicate the importance of IKK $\beta$ , and not IKK $\alpha$ , as a transducer of signals from receptor activator of NF- $\kappa$ B (RANK) to NF- $\kappa$ B. Ablation of IKK $\beta$  results in lack of osteoclastogenesis and unresponsiveness of IKK $\beta$  deficient mice to inflammation induced bone loss. In the need of a more effective therapy for the treatment of inflammatory diseases causing bone resorption, specific inhibition of IKK $\beta$  represents a logical alternative strategy to the current therapies.

Generally, inflammation is a beneficial host response to foreign challenge or tissue injury that leads ultimately to restoration of tissue structure and function. In fact, inflammation is an integral part of innate immunity. However, prolonged inflammation that is not resolved ceases to be beneficial and contributes to the pathogenesis of many disease states.<sup>1</sup> Excessive bone resorption is a major pathological factor in chronic inflammatory diseases such as periodontitis, osteoporosis, and arthritis, and it is now clear that dysregulation of immune and inflammatory responses is crucial for initiating the bone destruction associated with these conditions.<sup>2</sup> The bone-resorbing osteoclasts are known to play a pivotal role in focal bone erosion in rheumatoid arthritis (RA)<sup>3</sup> and in animal models of arthritis.<sup>4</sup> Study of tissues obtained from the bone-pannus in RA demonstrated the presence of multinucleated osteoclast-like cells,<sup>3,5</sup> and in situ hybridisation revealed that these cells expressed mRNA for definitive osteoclast markers such as tartrate resistant acid phosphatase (TRAP), cathepsin K, and calcitonin receptor. In addition, electron microscopic analysis of subchondral bone from damaged RA metacarpals confirmed the presence of resorption areas typical of osteoclast activity.<sup>6</sup> In normal bone physiology osteoclasts differentiate from their haematopoietic precursors and their differentiation is dramatically dependent on osteoblastic/stromal cells of mesenchymal origin that provide a physical support for nascent osteoclasts and produce several soluble and membrane associated factors that stimulate the proliferation and/or differentiation of haematopoietic osteoclast precursors.<sup>7</sup> Imbalances between osteoclast and osteoblast activities can arise from a variety of hormonal changes or perturbations of inflammatory and growth factors, resulting in skeletal abnormalities characterised by decreased (osteoporosis) or increased (osteopetrosis) bone mass,<sup>7–9</sup> and the focal net loss of bone in sites of inflammation, as found in RA.<sup>3</sup> In animal models in which expression of key proinflammatory mediators has either been abolished through gene knockouts or their activities modulated through genetic and biochemical

blockade, the decrease in the inflammatory response is closely associated with reduction in the degree of bone and cartilage destruction.<sup>10–13</sup> Pettit and colleagues demonstrated that arthritis can be induced in mice lacking osteoclasts (due to the deletion of the key osteoclast differentiation factor, receptor activator of nuclear factor (NF)- $\kappa$ B ligand (RANKL)), although bone erosion does not occur.<sup>14</sup> Similar results were obtained in mice lacking the transcription factor *c-fos*, which is also required for osteoclast maturation.<sup>12</sup> Despite the development and progression of inflammation, these mice were resistant to focal bone erosion as a result of the absence of osteoclasts. Thus, interference with osteoclast formation or maturation represents an attractive strategy for the treatment and prevention of inflammation induced bone loss, suggesting that blockade of RANKL signalling in combination with an anti-inflammatory cytokine may have effects on both bone erosion and inflammation.

## THE RANKL–RANK SYSTEM IN PHYSIOLOGICAL AND PATHOLOGICAL BONE REMODELLING

Two proteins crucial for osteoclast development and activation are RANK (receptor activator of NF- $\kappa$ B) and its ligand, RANKL. RANKL is a member of the tumour necrosis factor (TNF) family of cytokines and its expression is regulated by a number of factors that induce bone resorption including vitamin D<sub>3</sub>, glucocorticoids, interleukin (IL)-1, IL-6, and TNF $\alpha$ .<sup>15–17</sup> RANKL activates mature osteoclasts and directs osteoclast differentiation from monocyte/macrophage precursors together with macrophage-colony stimulating factor (M-CSF).<sup>17,18</sup> The in vivo significance of the RANKL–RANK signalling pathway has been verified by the observations that ablation of either protein in mice results in severe osteopetrosis and a total lack of osteoclasts,<sup>19,20</sup> whereas a deficiency in osteoprotegerin (OPG), which binds to RANK preventing activation of RANK signalling, results in osteoporosis.<sup>21</sup> A crucial target of RANKL signalling is transcription factor NF- $\kappa$ B, a finding that implicates this transcription factor in osteoclast differentiation. A critical role for NF- $\kappa$ B in osteoclastogenesis is supported by the fact that gene specific deletion of both its p50 and p52 subunits causes severe osteopetrosis through the absence of osteoclasts.<sup>22,23</sup> In osteoclasts, RANK induces the activation of Akt, which is blocked by the phosphatidylinositol 3 kinase (PI3K) inhibitor LY294002.<sup>24,25</sup> Furthermore, LY294002 reduces the RANK mediated survival response of osteoclasts.<sup>25</sup> The PI3K inhibitor also displays a potent inhibitory effect on osteoclast differentiation,<sup>24</sup> which may result from a reduced survival of osteoclast precursors during differentiation. Although direct evidence for RANK activation of PI3K remains to be demonstrated, it has been shown in osteoclasts that RANK

**Abbreviations:** IKK, I $\kappa$ B kinase; IL, interleukin; LPS, lipopolysaccharide; OPG, osteoprotegerin; RA, rheumatoid arthritis; RANKL, receptor activator of nuclear factor (NF)- $\kappa$ B ligand; TNF, tumour necrosis factor; TNF-R, TNF receptor

activated the Src tyrosine kinase. The relevance of Src activity to RANK signalling is underscored by the osteopetrotic phenotype of mice deficient in Src.<sup>26</sup> In Src deficient mice osteoclast motility, and therefore bone resorption, are prevented due to lack of association of Src with gelsolin,<sup>27</sup> and thereby inhibiting formation of actin filaments and downregulating the level of Pyk2 and c-Cbl.<sup>28, 29</sup>

The RANKL-RANK system also represents a direct link between synovial T cell infiltration and bone erosion in RA. There is mounting evidence that T lymphocytes regulate osteoclast formation in arthritis. The requirement for RANKL in mediating osteoclast differentiation and function in inflammatory arthritis has been supported by several lines of evidence.<sup>3, 12, 14, 16, 30-32</sup> Initial observations demonstrated that activated T cells provide a source of RANKL for subsequent osteoclast differentiation in rat adjuvant induced arthritis.<sup>3</sup> RANKL production 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>3</sup> In particular, RANKL seems to be a principal pathogenetic factor that causes bone and cartilage destruction in arthritis. Inhibition of RANKL function via its natural decoy receptor OPG prevents bone loss in postmenopausal osteoporosis and cancer metastases and completely blocks bone loss and crippling in various rodent models of arthritis. Perhaps, the most important evidence of the crucial role that osteoclasts play in inflammation induced bone loss comes from the study in which OPG was successfully used to block bone erosion in collagen induced arthritis mice.<sup>3</sup>

## ROLE OF TNF $\alpha$ IN INFLAMMATION INDUCED BONE LOSS

Additional cytokines and growth factors that are also produced by cells of the inflamed synovium, primarily the proinflammatory cytokines TNF $\alpha$  and IL-1, can also stimulate osteoclast development, thus providing a potential link between the inflammatory process and bone destruction.<sup>33-35</sup> Similar to RANKL, TNF $\alpha$  is a potent osteoclastogenic factor that enhances proliferation and differentiation of osteoclast precursors through its type 1 receptor, TNF-R1.<sup>35, 36</sup> However, permissive levels of RANKL are required for optimal TNF $\alpha$  induced osteoclastogenesis, most likely due to the inability of TNF $\alpha$  to support calcium intake. TNF $\alpha$  mediates RANKL stimulation of osteoclast differentiation through an autocrine mechanism.<sup>37</sup>

TNF $\alpha$  exerts its biological functions through two receptors: TNF-R1 (p55) and TNF-R2 (p75). Both receptors are expressed on a wide variety of cell types including bone marrow haematopoietic cells.<sup>38</sup> Amongst the two receptors it is TNF-R1 that mediates most of the biological effects of TNF $\alpha$ , including programmed cell death and the activation of NF- $\kappa$ B.<sup>39</sup> Upon oligomerisation, TNF-R1 binds to and recruits TNFR associated death domain protein (TRADD) molecules and binds indirectly to Fas associated death domain protein (FADD) through an interaction between the death domains of FADD and TRADD. This interaction leads to the activation of a caspase cascade responsible for programmed cell death.<sup>40</sup> In contrast, TNF-R2 lacks a death domain and interacts directly with TRAF (TNF associated factor) 2. Although, TRAF 2 activates both NF- $\kappa$ B and c-Jun-N-terminal kinase (JNK),<sup>41</sup> TNF-R2 make very little contribution to NF- $\kappa$ B activation.<sup>42</sup>

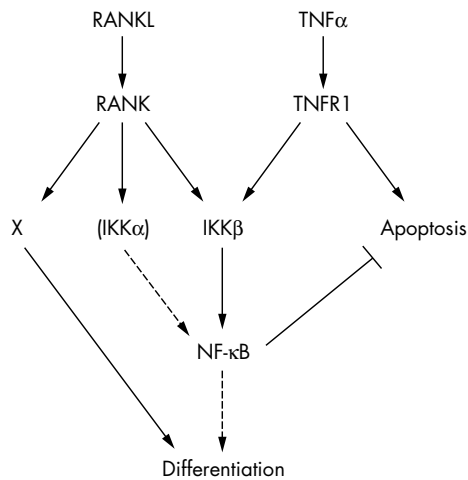
Osteoclast recruitment by TNF $\alpha$  is probably essential to the pathogenesis of inflammatory osteolysis, whereas TNF-R1 promotes osteoclastogenesis, TNF-R2 was shown to inhibit this process.<sup>36</sup> TNF $\alpha$  is produced primarily by activated T cells and activated macrophages within the inflamed synovial tissue in RA.<sup>43, 44</sup> The prominent role of TNF $\alpha$  in driving

inflammation has made it a target for biologically based therapeutics currently used for treatment of RA.<sup>45-49</sup> TNF $\alpha$  alone is sufficient to induce arthritis and joint destruction in a murine model. Mice constitutively expressing human TNF $\alpha$  develop polyarthritis, which is characterised by significant focal bone erosion as well as generalised bone loss.<sup>11, 12, 32</sup> Conversely, mice deficient in TNF $\alpha$  demonstrated a heterogeneous phenotype when challenged with serum transfer arthritis. The absence of TNF $\alpha$  signalling mostly conferred resistance to synovitis and bone erosion, but approximately a third of the animals studied did develop clinical signs of arthritis, albeit at a delayed rate and reduced severity compared with wild-type littermates.<sup>10</sup> Furthermore, focal bone erosion, correlating roughly with the degree of inflammation, was evident within the affected joints in the animals that did develop clinical arthritis (CIA).<sup>10</sup> This study supported previous observations in which mice deficient in TNF $\alpha$  were subject to collagen induced arthritis,<sup>50</sup> suggesting that TNF $\alpha$  independent pathways can compensate for the loss of TNF $\alpha$  signalling in mediating inflammation and subsequent bone erosion.<sup>10, 50</sup> TNF $\alpha$  antagonists, either alone<sup>51-54</sup> or in combination with the immunosuppressant methotrexate<sup>45-49, 55-59</sup> have demonstrated efficacy in reducing signs and symptoms of RA and arresting progression of erosions in a large number of patients with RA. Despite its apparent efficacy, it is not effective in all patients. Some of them, indeed, do not respond to anti-TNF $\alpha$  therapy, and complete disease remission, including the prevention of bone loss, is not always achieved. This suggests that, as in experimental arthritis models, alternative pathways that mediate inflammation and bone erosion may contribute to the heterogeneity of disease, and that in cases in which TNF $\alpha$  blockade is insufficient to control the disease process, alternative therapeutic strategies need to be considered.

## NF- $\kappa$ B AND IKK $\beta$ AS REGULATORS OF INFLAMMATION AND BONE REMODELLING

NF- $\kappa$ B activity is regulated through interaction with specific inhibitors, I $\kappa$ Bs, which trap NF- $\kappa$ B dimers in the cytoplasm.<sup>60</sup> In response to cell stimulation with proinflammatory and innate immune stimuli, such as TNF $\alpha$ , IL-1, or bacterial endotoxin (lipopolysaccharide (LPS)), the I $\kappa$ Bs are phosphorylated at two conserved serines and targeted to rapid ubiquitin dependent proteolysis.<sup>61</sup> I $\kappa$ B phosphorylation is carried out by the I $\kappa$ B kinase (IKK), a complex composed of three subunits: IKK $\alpha$ , IKK $\beta$ , and IKK $\gamma$ /NF- $\kappa$ B essential modulator (NEMO).<sup>62</sup>

IKK $\alpha$  and IKK $\beta$  serve as the catalytic subunits, whereas IKK $\gamma$ /NEMO is the regulatory subunit. IKK $\alpha$  and IKK $\beta$  contain similar kinase domains with essentially identical activation loops.<sup>63, 64</sup> Despite their structural and biochemical similarities, IKK $\alpha$  and IKK $\beta$  are functionally distinct.<sup>62</sup> Whereas IKK $\beta$  is essential for NF- $\kappa$ B activation in response to proinflammatory and innate immune stimuli, IKK $\alpha$  is not required for such responses.<sup>65-68</sup> IKK $\alpha$ , however, plays a unique and critical role in development of the epidermis,<sup>65</sup> but its ability to induce keratinocyte differentiation is independent of its protein kinase activity or NF- $\kappa$ B.<sup>69</sup> Recently, IKK $\alpha$  was found to be required for B cell maturation, another unique function that is not provided by IKK $\beta$ .<sup>70</sup> This function is dependent on IKK $\alpha$  kinase activity, but instead of being mediated through inducible I $\kappa$ B degradation, is exerted via a second NF- $\kappa$ B activation pathway, dependent on processing of the NF- $\kappa$ B2/p100 precursor protein to the mature p52 subunit.<sup>70</sup> This pathway requires the activity of another protein kinase, NIK (NF- $\kappa$ B inducing kinase), which may function as an activator of IKK $\alpha$ .<sup>70</sup> It was observed that NIK deficient osteoclast precursors do not respond to RANKL in an in vitro



**Figure 1** Schematic model of receptor activator of nuclear factor (NF)- $\kappa$ B ligand (RANKL) and tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) signalling during osteoclastogenesis and inflammation induced bone loss. X, a pathway other than I $\kappa$ B kinase (IKK)/NF- $\kappa$ B that is activated by RANKL binding to RANK and is essential for production of functional osteoclasts. IKK $\alpha$  function in RANK signalling is dispensable. TNFR1, TNF receptor 1.

differentiation system devoid of osteoblasts.<sup>71</sup> However, *aly* mice, which carry a point mutation in the *Nik* gene that prevents NIK activation, are not osteopetrotic.<sup>72</sup> Nor was osteopetrosis reported for *Nik*<sup>-/-</sup> mice.<sup>71</sup> In addition, we found that a mutation that prevents IKK $\alpha$  activation has no effect on bone development or inflammation induced bone loss in vivo.<sup>73</sup>

Nonetheless, a pivotal role for transcription factor NF- $\kappa$ B in regulation of inflammation has been well recognised.<sup>74-75</sup> As mentioned above, the relevance of NF- $\kappa$ B pathway to osteoclastogenesis is underscored by the osteopetrotic phenotype of mice lacking the NF- $\kappa$ B1/p50 and NF- $\kappa$ B2/p52 subunits.<sup>22-23</sup> Interestingly, a deficiency in a single subunit has no effect on osteoclast formation or maturation. The question, however, is which catalytic subunit is required for NF- $\kappa$ B activation during osteoclastogenesis and inflammation induced bone loss.

NF- $\kappa$ B controls the expression of the proinflammatory cytokines IL-1 $\beta$  and TNF $\alpha$ , which are important mediators of inflammation in RA. In turn, both TNF $\alpha$  and IL-1 $\beta$  are potent inducers of NF- $\kappa$ B activation, suggesting an interdependence of persistent NF- $\kappa$ B activation and sustained IL-1 $\beta$  and TNF $\alpha$  production. Indeed, expression of a non-phosphorylatable variant of the NF- $\kappa$ B inhibitor I $\kappa$ B $\alpha$  (srI $\kappa$ B $\alpha$ ) abrogated the induction of IL-1 $\beta$  and TNF $\alpha$  in human macrophages and primary fibroblast-like synoviocytes (FLS).<sup>76-77</sup> More importantly, a small synthetic peptide that disrupts the interaction between IKK $\beta$  and the IKK $\gamma$  regulatory subunit and therefore prevents IKK activation was found to inhibit inflammation-induced bone loss in a mouse model of arthritis.<sup>78</sup>

A recent study from our laboratory established that IKK $\beta$ , but not IKK $\alpha$ , is essential for inflammation induced bone loss and is required for osteoclastogenesis in vivo.<sup>73</sup> Bone marrow cells deficient in IKK $\beta$  do not form osteoclasts in vitro when stimulated with RANKL. Furthermore, mice lacking IKK $\beta$  in haematopoietic cells and hepatocytes, *Ikk $\beta$ <sup>d</sup>* mice, are osteopetrotic, due to the lack of osteoclasts, indicating that IKK $\alpha$  function is dispensable in vivo in the RANK signalling pathway (fig 1).

However, the main function of IKK $\beta$  in osteoclastogenesis is to prevent TNF $\alpha$  induced apoptosis of osteoclast precursors (see fig 1). Indeed, bone marrow cells deficient in IKK $\beta$  are extremely sensitive to TNF $\alpha$  induced apoptosis and die in

response to elevated TNF $\alpha$ . Loss of TNF-R1 prevents apoptosis in IKK $\beta$  deficient BM cells and restores the presence of osteoclasts in mice that lack both IKK $\beta$  and TNF-R1 in the relevant cells, *Ikk $\beta$ <sup>d</sup>/Tnfr1<sup>-/-</sup>* mice. Nonetheless, the prevention of TNF $\alpha$  induced death reveals that IKK $\beta$  is also required for maturation of functional osteoclasts, because *Ikk $\beta$ <sup>d</sup>/Tnfr1<sup>-/-</sup>* osteoclasts are defective in bone resorption. Inflammation induced bone loss is prevented in mice lacking IKK $\beta$ , because IKK $\beta$  deficient osteoclasts and preosteoclasts are killed by TNF $\alpha$ . Once the effect of TNF $\alpha$  is eliminated by ablation of its receptor, inflammation induced bone loss is restored in *Ikk $\beta$ <sup>d</sup>/Tnfr1<sup>-/-</sup>* mice.<sup>73</sup> Thus, despite the inability of IKK $\beta$  and TNF-R1 deficient osteoclasts to undergo functional maturation (that is, become active in bone resorption) under non-inflamed condition, in the presence of a strong inflammatory stimulus, such as the one generated by LPS-injection into the joint, these cells undergo maturation after all.<sup>73</sup> The inflammatory cytokines that induce the functional maturation of *Ikk $\beta$ <sup>d</sup>/Tnfr1<sup>-/-</sup>* osteoclast precursors remain to be identified.

## THERAPEUTIC INTERVENTIONS

Knowledge of the pathogenic mechanisms of inflammatory arthritis has led to the design of targeted therapies that are effective in suppression of inflammation and prevention of joint destruction. The current anti-inflammatory and anti-rheumatic drugs used to treat RA include glucocorticoids, aspirin, sodium salicylate, sulfasalazine, and gold compounds, all of which have been shown to block NF- $\kappa$ B activity.<sup>79</sup> The list of therapeutic agents that inhibit NF- $\kappa$ B also includes numerous natural and synthetic antioxidants, immunosuppressants, and natural plant compounds, suggesting that the ability to suppress NF- $\kappa$ B activation at least partially accounts for their therapeutic effects.<sup>79</sup> These compounds are neither potent nor selective for this pathway, however, and may have a range of undesirable side effects as a result of their non-specific nature. Consequently, response to treatment in RA patients is not always complete, and in a subset of patients, focal bone erosion progresses despite therapy. More clear answers were obtained using animals with genetically inactivated NF- $\kappa$ B signalling. Ablation of the *nf- $\kappa$ b1* and *rel* genes rendered the affected animals refractory to development of collagen induced arthritis (CIA).<sup>80</sup> Transgenic mice expressing a super-repressor form of I $\kappa$ B $\alpha$  (srI $\kappa$ B $\alpha$ ) in the T lineage were similarly refractory to CIA.<sup>81</sup> These genetic studies are in a good agreement with the experiments that used highly specific inhibitors of NF- $\kappa$ B. However, the safety of long term use of specific NF- $\kappa$ B inhibitors remains to be elucidated. Genetic studies revealed that NF- $\kappa$ B activity is required for provision of innate immunity and prevention of opportunistic infections.<sup>82</sup> Highly specific inhibitors, local delivery, and short term treatments should alleviate the possible side effects associated with systemic inhibition and minimise the risk of general immunosuppression.

In this context, the results from our knockout experiments<sup>73</sup> and those obtained by the use of IKK peptide inhibitor<sup>78</sup> are exciting. They indicate that specific and selective inhibition of the IKK $\beta$  subunit and the classical NF- $\kappa$ B activation pathway represent an effective approach to the treatment of inflammatory diseases causing bone resorption. Furthermore, during inflammation, proinflammatory cytokines such as TNF $\alpha$  and IL-1 $\beta$  are induced and strongly potentiate RANKL induced osteoclastogenesis, although such factors cannot induce osteoclast differentiation on their own.<sup>73-74</sup> TNF $\alpha$  signalling through TNF-R1 has the potential to induce apoptosis through caspase 8, a process that is prevented by IKK $\beta$  dependent NF- $\kappa$ B activation.<sup>39</sup> Once IKK $\beta$  is inhibited, TNF $\alpha$  induced apoptosis can

eliminate osteoclast progenitors deficient in *Ikk $\beta$* , thereby preventing inflammation induced bone destruction. Thus, IKK $\beta$  inhibition presents a logical strategy for the therapy of numerous bone resorbing inflammatory disorders in which TNF $\alpha$  is elevated, such as RA. However, the efficacy of such an approach would be severely compromised if TNF $\alpha$  signalling, responsible for the elimination of IKK $\beta$  inhibited osteoclast progenitors, is blocked by anti-TNF $\alpha$  drugs, such as infliximab and etanercept. Thus, when IKK $\beta$  inhibitors will become available it is unlikely that they will be useful in conjunction with the currently available anti-TNF $\alpha$  therapeutic agents.

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