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PERSPECTIVES

Effects of RANKL Inhibition on Inflammation and Immunity

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Abstract

Receptor activator of nuclear factor-κB ligand, RANKL, is a member of the TNF superfamily and its actions are not limited to bone, but also affect markedly the maturation and activation of the immune system (1-3). Consequently, the development of denosumab – a fully human monoclonal antibody to RANKL – for use as a novel therapeutic agent in osteoporosis, rheumatoid arthritis (RA) and cancer, may give rise to some questions as to its immunological safety. However, recent reports claim that contrary to anti-TNFs, the bone- and cartilage-sparing effects of denosumab in patients with RA are not coupled with any apparent amelioration on the clinical score of joint inflammation (4). Admittedly, this observation suggests that RANKL is not a major pro-inflammatory cytokine in this setting, and the lack of effect of RANKL inhibition on the immune system is at times taken as a point in fact, but is this the right conclusion? *IBMS BoneKEy*. 2009 March;6(3):116-126.

Introduction

Considering the spatial proximity of immune and bone cells in stem cell niches of the bone marrow, it has been proposed that these cells influence each other not only in terms of their development, maturation and activation (1;5), but also in terms of the renewal of stem cells and progenitor cells. Hence osteoblasts play an important role in the formation of hematopoietic stem cell (HSC) niches, in lymphocyte development, and in the engraftment and maintenance of HSCs in the bone marrow (6). Conversely, the development and activation of mesenchymal stem cells (MSCs) leading to mature osteoblasts, as well as the differentiation of hematopoietic precursors of osteoclasts, are controlled and sustained by growth factors and cytokines produced not only by the bone cells themselves but also by surrounding bone marrow cells, mostly monocytes and T cells (7).

Many cytokines produced by monocytes and/or T cells have actually been pinpointed as stimulators of bone resorption (8-13). Activated $CD4^+$ T cells, through the expression of RANKL, play a major part in osteoclast activation (1), whereas mature B cells, due to the production of osteoprotegerin (OPG), may suppress RANKL-induced osteoclastogenesis (14). Hence, in various inflammatory and autoimmune diseases such as rheumatoid arthritis (RA), lupus erythematosus, inflammatory bowel disease (IBD), and multiple sclerosis, osteoclast-mediated bone loss is a result of the intricate communication between bone and immune cells, particularly T cells and dendritic cells (DCs) (15). This cross-talk between the two systems has also been implicated in post-menopausal bone loss (16). As RANKL is a prominent cytokine produced by T cells and an activator of DCs (1;17), its inhibition, similar to that of TNF, might actually play a dichotomous role in both osteoclastogenesis/bone loss and inflammatory, and perhaps immune, reactions.

Influence of RANKL Inhibition in Inflammatory Disorders

Inflammation is a physiological response and its purpose is to localize and eliminate the causative agents, and to repair the surrounding damaged tissues. While inflammation represents a normal response to tissue injury and contributes to normal healing and repair, it can cause significant tissue damage when inflammatory responses are driven by hypersensitivity reactions and autoimmune injury.

Unlike the effects of agents blocking TNF-α or anti-IL-6 in controlling inflammatory processes in RA (18-21) and other auto-immune diseases (22;23), the inhibition of the RANKL/RANK pathway protects against bone erosion in RA without an apparent reduction of inflammatory parameters (4; 24-26). *RANKL*(-/-) mice remain subject to inflammatory arthritis induced by serum transfer (24), while in TNF-α transgenic (Tg) mice that develop arthritis spontaneously, OPG administration did not modify the inflammatory course (25). In a rat model of adjuvant-induced arthritis, administration of soluble OPG dramatically reduced cortical and trabecular bone loss without tempering the severity of joint inflammation (26). In a mouse model of IBD – the $CD4^{+}CD45RB^{Hi}$ T-cell transfer model – administration of OPG-Fc dramatically improved bone density, but failed to improve inflammatory parameters and infiltration of the gastrointestinal tract by inflammatory cells (27). Hence, in rodents, blockade of TNF-α or RANKL is not equally efficient in the prevention of inflammation (and susceptibility to infection) and bone loss, respectively.

There is one notable exception to the above observations. Indeed, under specific conditions RANKL antagonism may influence the inflammatory reaction. Thus, in IL-2-deficient mice that spontaneously developed an autoimmune disease characterized by hyperactivation of $CD4^{+}$ T cells with multi-organ inflammation and massive T-cell infiltration of the gastrointestinal tract, the administration of Fc-OPG not only led to a significant increase in bone density and improvement of gastrointestinal tissue architecture, but also reduced T-cell infiltration in the colonic mucosa (28). The latter effect was attributed to an inhibitory effect of Fc-OPG on DC-dependent T-cell activation and infiltration in colonic tissues. However, it is noteworthy that in IL-2-deficient mice both DCs and T cells are hyperactivated, and this hyperactivation may be due more specifically to an increased production of RANKL by proliferating T cells. Consequently, while Fc-OPG may control inflammation by modulating the activation state of DCs and T cells in this particular setting, it may still prove ineffective in modulating inflammation in IL-2-competent mice or in inflammatory conditions driven by multiple (and redundant) cytokines. It should be noted, however, that in the absence of a control group receiving an Fc-control molecule, it remains difficult to ascribe all of the immunomodulatory properties in this model to OPG itself.

The Role of RANKL/RANK signaling in the Ontogeny and Development of Lymphoid Tissues

Having established that RANKL inhibition has no major effects on inflammatory reactions (with one exception, see above), the next step is to examine whether the same applies to immune processes.

Contrary to the inflammatory response that is non-specific and lacks memory, the immune response is characterized by a high degree of specificity and memory, involving specialized and highly differentiated cells of the innate and adaptative immune system. In order to provide a powerful, specific response, immune cells undergo numerous stages of development until maturation, consisting of the elimination of self-reactive immune cells on the one hand, and the preservation and expansion of cells reactive to allo-antigens on the other. These maturation steps towards specificity feature, particularly, NK cells and T and B lymphocytes. The development of lymphoid organs is an extremely complex process that depends on the consecutive expression of

adhesion molecules, cytokines and chemokines, eventually leading to lymph node organogenesis. Each secondary lymphoid organ requires specific molecules at distinct stages of its development, some of them being essential and others being unnecessary. Lymph nodes (LNs), Peyer's patches and nasal-associated lymphoid tissue (NALT) seem to follow a common developmental program, whereas spleen organogenesis undergoes a more complex process. Studies in mice have revealed that numerous genes are mandatory for lymphoid tissue development (29). Among them, members of the TNF family, such as surface lymphotoxin LTα1β2 that signals through the lymphotoxin β receptor (LTβR), play a predominant part in LN organogenesis, since mice deficient in LTα, LTβ or LTβR lack LNs (30-32). Due to its binding to RANK, RANKL is also crucial for LN formation (1), since in the absence of RANKL or RANK, LNs do not develop, whereas the formation of Peyer's patches, NALT and splenic microarchitecture is not affected (1;2). Thus *RANK(-/-)* mice present a defect in LN organogenesis (33) due to the absence of RANK-mediated signaling through TNF-receptor-associated factor 6 (TRAF-6), which is also crucial to LN development (34). Analysis of LN development in RANKL-deficient mice revealed that the impaired LN development is due to a defect in colonization and cluster formation of hematolymphoid precursor cells (2), particularly specific T cells, $\alpha4\beta7^{+}CD4^{+}CD3^{-}$ T cells. These T cells express RANKL and are key regulators of stromal cells involved in the development of organized secondary lymphoid structures (35). Since LN organogenesis consists of different stages, RANKL is not required in the early stages of LN formation and development but rather in later stages III or IV when hematopoietic lineage precursor cells colonize LNs. The fact that the transgenic expression of RANKL in *RANKL*(-/-) mice partially rescues LN development and $CD4^{+}$ T cells colonizing the LNs confirms that the RANKL/RANK pathway is essential during later stages, and not at the beginning, of LN organogenesis (2). In addition to a deficiency in LN organogenesis, *RANKL*(-/-) mice have both decreased thymus size and thymic cellularity as RANKL contributes to early thymocyte development (1). However, mature CD4+ and CD8+ T cells appeared normal in *RANKL*(-/-) mice indicating that thymus function is normal and the number of lymphocytes in peripheral blood remains normal in *RANKL*(-/-) mice (matching levels found in wild type animals) due to extramedullary hematopoiesis in the spleen. In addition, *RANK*(-/-) mice boast normal thymic development, a normal percentage of thymocytes and T-cell precursors and adequate markers of thymocyte development (33), suggesting that RANKL may also function through another receptor.

Two observations, however, indicate that the development of immune T cells may resist inhibition of RANK-mediated signaling. First there is evidence that high expression of OPG in OPG-Tg mice does not affect formation of the thymus or spleen, nor does it lead to lymph node agenesis (36), as OPG-Tg rats exhibit normal lymph node development at gestational day 11 when OPG levels are already high (36), suggesting that even residual effects of RANKL may be sufficient in this regard. Second, patients with mutations in the *TNFSF11 (RANKL)* gene or in the *TNFRSF11A (RANK)* gene have normal lymphocyte counts in the peripheral blood and do not have any major abnormalities in T-cell phenotypic and functional properties (37;38).

B Cell Development

RANKL contributes to the development of B cells, with *RANKL*(-/-) mice exhibiting reduced numbers of B cells in the spleen and a marked alteration of B cell development with defective transition of pro-B to pre-B (1). In *RANK*(-/-) mice, the developmental defect in the B cell lineage is massive with a decrease in mature peripheral B cells and a marked reduction in femoral marrow cellularity (33). Interestingly, patients with mutations in the *TNFRSF11A (RANK)* gene failed to produce antibodies and the number of their mature B cells was significantly decreased (38), whereas

patients with mutations in the *TNFSF11 (RANKL)* gene did not present such a defect or any other immunological defects (37). Therefore the RANKL/RANK signaling pathway may be more crucial to murine than human B cell maturation. As far as the role of OPG in B cell development is concerned, the study of pro-B cells in *OPG*(-/-) mice demonstrates an increased proliferation of these cells but a deficit in antibody response to antigen due to an alteration in the isotype switching of IgG immunoglobulins, suggesting that OPG controls B cell maturation and development (39). In contrast, OPG-Tg mice present neither a defect in B cell development and maturation nor an impaired humoral response to immune challenges (36).

In summary, mouse genetic models clearly indicate that the RANKL/RANK/OPG system is involved in the development of lymphoid organs, T and B cells, whereas experiments of nature in humans suggest that loss-of-function mutations in this system only have limited detrimental effects on the development of immunity.

Role of RANKL in Mature Immune Cells and Immune Reactions

Although experiments on transgenic mice illustrate the non-redundant role of RANKL/RANK signaling in the organization of immune tissues, inhibition of RANKL in adults is not expected to affect these developmental processes. In newborn babies, however, immune cells interact with each other in order to initiate an innate and adaptive immune response and to produce pro- and anti-inflammatory cytokines, chemokines, and antibodies. DCs and monocyte/macrophages are key components of the innate immune response and are major antigen-presenting cells priming T cells to proliferate and initiate an adaptive immune response (40). In turn, activation and proliferation of T cells control antigen dispersion and, due to the secretion of cytokines, trigger a feedback amplification loop that further increases DC and monocyte/macrophage functions, as well as B cell proliferation and antibody production. B cells also control the dissemination of antigens by producing a large array of antibodies. All of the cells involved in the processes of innate and adaptive immunity express RANKL, RANK or OPG molecules: hence RANKL is produced not only by bone marrow stromal cells and osteoblasts but also by T cells; monocytes and DCs, like (pre-) osteoclasts, express RANK, while the RANKL antagonist and TRAIL-binding factor OPG is expressed by mature osteoblasts and B cells (26;39;41-43) (Fig. 1).

RANKL and T cells

Although the total number of T lymphocytes, as well as their production of IFN-γ and IL-2, are decreased in *RANKL*(-/-) mice (see above), they do proliferate adequately, and the subsets of $CD4^+$ and $CD8^+$ T cells occur in normal amounts, probably owing to redundant activation pathways (such as CD40-CD40L; CD28-CD80/86) (1). Moreover, when OPG or RANKL is added to wild type murine T-cell cultures, their proliferation and production of cytokines remain unaffected, suggesting that RANKL signaling per se is not crucial to mature T cells (1). Similarly, treatment of mice with RANKL-Fc *in vivo* does not affect T-cell properties according to cytokine production and proliferation assays, nor did it modify T-lymphocyte infiltration of inflammatory tissues (44).

RANKL and B cells

As mentioned above, OPG-Tg mice present no defects in B cell development or maturation, nor an impaired humoral response to immune challenges (36). Indeed, OPG-Tg mice mount a normal B and T cell immune response when challenged with various antigens, confirming that these mice are immunocompetent. Moreover, the defective IgG switch observed in *OPG*(-/-) mice (39) did not occur in mice treated with OPG-Fc: when these mice were injected with a pneumovax vaccine, they produced IgM or IgG subtypes identical to those of mice injected with non-fused-Fc (controls) (45). These results also suggest that RANKL inhibition would be innocuous with regard to the development of auto-

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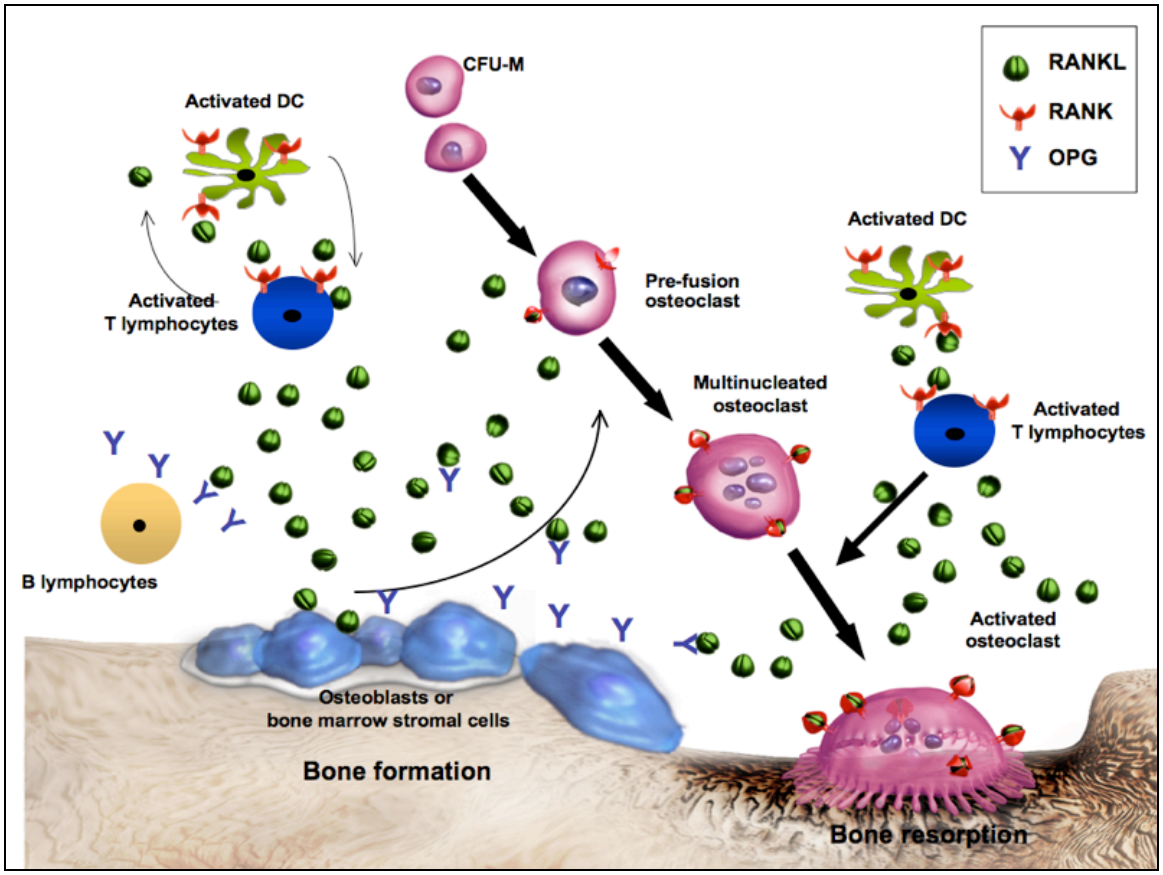

Fig. 1. Expression of RANKL, RANK or OPG molecules by immune cells. Cells involved in the processes of innate and adaptive immunity express RANKL, RANK or OPG molecules. RANKL is an essential mediator of osteoclast formation, function, and survival and is produced not only by bone marrow stromal cells and osteoblasts but also by T cells. RANKL acts by binding to the RANK receptor that is expressed by monocytes and dendritic cells (DCs). The RANKL antagonist and TRAIL-binding factor OPG is expressed by mature osteoblasts and B cells (as well as by other cell types not shown here) and inhibits RANKL-induced osteoclastogenesis. Adapted by permission from Macmillan Publishers Ltd: *Nature*, Boyle WJ, *et al*. 2003 May 15;423(6937):337-42, copyright 2003.

immunity, since autoimmune diseases and some allergic diseases are partly due to a defect in IgG switching (46;47).

RANKL and Dendritic Cells (DCs)

T cells that express RANKL play an agonistic role in DC activation *in vitro* due to co-stimulatory processes involving CD40L/CD40 interaction (43). Hence RANKL enhances DC survival, antigen presentation (48;49) and DC production of cytokines (50). Accordingly, in *OPG*(-/-) mice, DCs induce a more pronounced T cell proliferation (39), probably due to the increased survival of DCs, thus boosting the DC-T cell interaction. However, in *RANKL* (-/-) mice, DC development and function are intact and *RANKL*(-/-) DCs efficiently stimulate alloreactive T cells (1). In *RANK* (-/-) mice, the pattern is similar, with an intact development of DCs, normal expression of DC surface markers and co-stimulatory markers, as well as a preserved capacity to induce proliferation and activation of T cells (33). Once more, patients with mutations in either the *TNFSF11 (RANKL)* or *TNFRSF11A (RANK)* gene present no defect in dendritic cell functions; moreover, phenotypic and functional analyses of DCs did not reveal any major abnormalities in these subjects (37;38). Thus, although RANKL appears to be an effective co-stimulatory factor, it is not essential for DC activation.

RANKL and Monocyte-Macrophages

Monocyte-macrophages are closely related to osteoclasts and express RANK on their cell surfaces. Monocytes from peripheral blood as well as monocytic cell lines both differentiate into osteoclasts when cultured with M-CSF and RANKL (51). As demonstrated *in vitro*, RANKL may also influence monocyte functions (52). Stimulation of monocytes with RANKL induces the expression of Bcl-xl, increases the production of pro-inflammatory cytokines such as IL-1β and TNF-α, chemokines, co-stimulatory molecules (CD80 and CD86) and MHC-II on their cell-surfaces, suggesting that RANKL protects monocytes from apoptosis, increases their phagocytic properties and activates antigen presentation (52). However, both numbers and distribution of monocyte-macrophages in *RANKL*(-/-) mice were normal (1). Differentiation and function of monocyte-macrophages were also preserved in *RANK*(-/-) mice, demonstrating that, contrary to osteoclastogenesis, the RANKL/RANK pathway is not crucial to macrophage development (33). RANKL is closely related to the co-stimulatory molecule CD40L (43), a T-cell surface protein known to be essential for activation of monocyte-macrophages. As demonstrated by Padigel and co-workers, the RANKL/RANK pathway may influence monocyte-macrophage function when other essential co-stimulatory molecules are missing, such as in *CD40L* (-/-) mice (53). Whether RANKL/RANK signaling plays a similar role in patients with hyper-immunoglobulin M syndrome due to CD40 mutation or with CD40L deficiency is currently unknown. In contrast, in the presence of CD40 signaling, the physiological importance of the RANKL/RANK pathway on DCs and monocyte-macrophage cells and their ability to mount an immune response is rather limited.

Most importantly, with regard to the pharmacological effects of denosumab on immunity and the control of infection, blockade of RANKL function *in vivo* by RANK-Fc did not alter the functions of monocytes, nor did it amplify inflammatory processes in mouse models of LPS-endotoxic shock and inflammatory arthritis (52). LPS is a component of cell walls of Gram-negative bacteria and a potent activator of monocyte-macrophage function through its interaction with CD14 and toll-like receptor 4 (TLR4). LPS activation of monocyte-macrophages also induces co-stimulatory proteins such as CD40, CD80 and CD86, which in turn activate T cells. Therefore, even if the RANKL/RANK pathway is inhibited, redundancy of activating pathways and immune cell interaction will sustain the immune response to pathogens. As further proof of this concept, OPG administration to mice infected with mycobacterium bovis did not alter their immune responses (45).

Summary and Perspective

RANKL, OPG and/or RANK are not only expressed by osteoblasts and osteoclasts, but also by T cells, B cells, DCs and monocyte-macrophages. Absence of RANKL or RANK during embryogenesis results in thymus and LN defects in mice, but this phenomenon is not observed in humans. Moreover, blocking the RANKL/RANK pathway in adult animals does not apparently lead to major immune cell dysfunctions. Clinically, inhibition of RANKL binding to RANK has proven to be effective in preventing bone resorption and safe in patients with osteoporosis, RA and likely cancer. As a corollary of its apparent lack of effects on the immune system, RANKL inhibition does not seem to prevent inflammation driven by T cells in RA or particular models of IBD. Whether the inhibition of T cell-mediated, RANKL-dependent activation of DCs and monocyte-macrophages by OPG or denosumab may impact the ability to generate immune reactions (against infection primarily) in some tissues, i.e., the lung and skin, remains to be further evaluated.

Conflict of Interest: Dr. Ferrari reports that he receives research support from Amgen and is an advisory committee member and lectures occasionally at conference symposia for Merck Sharp & Dohme, the Alliance for Better Bone Health (Sanofi Aventis/P&G), Amgen, Eli Lilly (Switzerland), Servier (Switzerland),

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