Osteoporos Int (2011) 22:435–446
DOI 10.1007/s00198-010-1326-y

REVIEW

Do RANKL inhibitors (denosumab) affect inflammation and immunity?

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Received: 1 December 2009 / Accepted: 24 May 2010 / Published online: 23 June 2010

Abstract Receptor activator of nuclear factor kappa B ligand (RANKL) and its natural antagonist, osteoprotegerin (OPG), are, respectively, an indispensable factor and a potent inhibitor for osteoclast differentiation, activity, and survival. The development of a human monoclonal antibody to RANKL, denosumab, constitutes a novel approach to prevent fragility fractures in osteoporosis, skeletal complications of malignancy, and potentially bone erosions in rheumatoid arthritis (RA). In addition to being expressed by osteoblasts, RANKL is abundantly produced by activated T cells, and synoviocytes in RA, whereas its receptor, RANK, is also expressed by monocytes/macrophages and dendritic cells. However, in preclinical and clinical studies of RA—including patients with some degree of immunosuppression—RANKL inhibitors did not significantly alter inflammatory processes. RANKL, RANK, and OPG deficiency in murine models highlights the important role of this pathway in the development and maturation of the immune system in rodents, including functions of T and/or B cells, whereas OPG overexpression in mice and rats seems innocuous with regard to immunity. In contrast, loss-of-function mutations in humans have more limited effects on immune cells. In clinical studies, the overall rate of infections, cancer, and death was similar with denosumab and placebo. Nevertheless, the risk of severe infections and cancer in some specific tissues remains to be carefully scrutinized.

Keywords Denosumab · Immunity · Inflammation · OPG · Osteoporosis · RANKL

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Introduction

Local and systemic bone loss (osteoporosis) that occurs in inflammatory diseases and gonadal steroid deficiency, subchondral bone erosion in rheumatoid arthritis (RA), as well as osteolytic bone metastasis, are all caused by the bone-resorbing effects of osteoclasts. Two factors secreted primarily by bone marrow stromal cells and osteoblasts are necessary and sufficient to induce differentiation of hematopoietic precursors common to the monocyte/macrophage and osteoclast lineages into multinucleated, bone-resorbing cells: colony-stimulating factor-1 (CSF-1 or M-CSF) and the tumor necrosis factor (TNF)-related cytokine receptor activator of nuclear factor kappa B ligand (RANKL) [1, 2]. By engaging specific adaptor molecules, such as the TNF receptor-associated factor TRAF6 and the Grb-2-associated binder-2 Gab2, RANKL binding to its receptor RANK further promotes activation and survival of mature osteoclasts [3–5]. In both mice and humans, deleting or inactivating mutations of RANKL and RANK genes results in the absence of osteoclasts and osteopetrosis [6–9].

Osteoblasts/stromal cells also produce osteoprotegerin (OPG), a decoy receptor which binds RANKL, thus preventing its own binding to RANK. Thereby, OPG exerts a negative regulation on osteoclastogenesis, promotes apoptosis of mature osteoclasts, and ultimately inhibits bone resorption [10]. Hence, OPG-deficient mice exhibit increased bone turnover, a reduction in cortical and trabecular bone volume, and they develop spontaneous fractures [11]. In contrast, overexpression of OPG or RANK-Fc (that functions as a RANKL inhibitor) in transgenic rodents causes high bone mass, but without the typical features of osteopetrosis, as in these models, bone mass correlates with the level of OPG or RANK-Fc expression [10, 12, 13]. Hence, the discovery of the RANKL/RANK/OPG pathway has led first to the development of recombinant OPG (rhOPG) and OPG-Fc, then to that of a fully human IgG2 monoclonal antibody to RANKL named denosumab, as a novel therapeutic agent for potential application in osteoporosis [14, 15], RA [16], and cancer [17].

Besides being produced by osteoblasts, RANKL is also abundantly expressed by activated $CD4^+$ T cells, whereas mature B cells express OPG [18, 19]. Many cytokines produced by monocytes and/or T cells have actually been identified as stimulators of bone resorption, including interleukin (IL)-1, IL-4, IL-6, IL-7, IL-15, IL-17, interferon (IFN)-γ, and TNF-α [20–28]. Hence, T lymphocytes are key to the process of local and systemic bone loss associated with inflammation, autoimmune diseases, and graft rejection [29, 30] and also play a role in bone loss due to estrogen deprivation [31]. The point is that RANKL-producing T cells not only contribute to osteoclast activation [18, 19] but also to the co-stimulation of other RANK-expressing cells, such as dendritic cells (DC) and monocytes/macrophages [32, 33] (Fig. 1).

In addition to inhibiting osteoclastogenesis, RANKL inhibitors could therefore potentially modulate and/or inhibit an array of T-cell-mediated reactions, which has raised some concerns about their specificity and safety. This review focuses on the experimental evidence as to the role of the RANKL/RANK pathway and its inhibitors on inflammation and immunity.

Effects of RANKL inhibitors on inflammatory diseases

Comparing and contrasting the effects of RANKL and TNF inhibitors

Administration of agents blocking TNF-α or IL-6 in both animal disease models and humans has proven efficient in controlling inflammatory processes characteristic of RA [34–37] and other autoimmune diseases [38, 39]. Treatment of RA with TNF-specific antagonists, such as etanercept, infliximab, and adalimumab, also prevents subchondral erosions and cartilage destruction [35, 40–42], whereas their effects on the preservation of systemic bone mass seem limited when withdrawal of corticosteroids is taken into consideration [41, 43, 44]. Treatment of patients suffering from RA or other inflammatory disorders by biological agents that target members of the TNF family has shown an increase in the number of infections and hematologic malignancies (Table 1) [45–47]. Admittedly, TNF inhibitors significantly increase the risk of contracting tuberculosis and of its reactivation [45, 48]. Several investigators demonstrated that transmembrane TNF-α (TmTNF) plays an important role in the inflammatory response to infection, as TmTNF contributes to the induction and regulation of Th1-type cytokines and chemokine expression which is crucial for the development of bactericidal granulomas and resistance to mycobacterium infections [49, 50]. Moreover, TNF-α also appears to fulfill an important regulatory function in controlling repair and regenerative processes after tissue damage [51].

Similar to TNF, RANKL is abundantly produced by infiltrating T cells and synoviocytes in RA [7, 52–54]. In contrast to TNF inhibitors, however, OPG administration in animal models of RA, such as the rat model of adjuvant-induced arthritis and TNF-α transgenic (Tg) mice—which spontaneously develop arthritis—did not alter the severity of joint inflammation, despite a marked reduction of periarticular bone erosions, as well as of cortical and trabecular bone loss at distant skeletal sites [7, 55]. Even $RANKL^{-/-}$ mice remained susceptible to arthritis induced by serum transfer [55]. Recently, Stolina et al. compared the effects of the RANKL inhibitor OPG-Fc, the TNF-α inhibitor pegsunercept, and the IL-1 inhibitor anakinra on rats with established adjuvant- or collagen-induced arthritis [56]. Parameters of local and systemic inflammation (paw swelling and serum levels of pro-inflammatory cytokines) were dramatically decreased by anti-TNF-α or anti-IL-1 therapy, while local, but not systemic, bone loss was partially reduced. In contrast, OPG-Fc therapy inhibited local and systemic bone loss in both arthritis models without modifying local or systemic inflammation.

In a phase II clinical trial, administration of 60 or 180 mg s.c. of denosumab every 6 months to patients with active RA (and receiving methotrexate) halted the progression of subchondral bone erosions and systemic bone loss, although without an apparent reduction of clinical inflammation and joint space narrowing [7, 16, 55, 57]. Altogether, these observations suggest that—contrary to anti-TNFs—RANKL and its inhibitors do not play a major part in T-cell-mediated inflammatory processes in RA, which could be accounted for by the redundant effects of an array of pro-inflammatory cytokines [58]. Moreover, in the

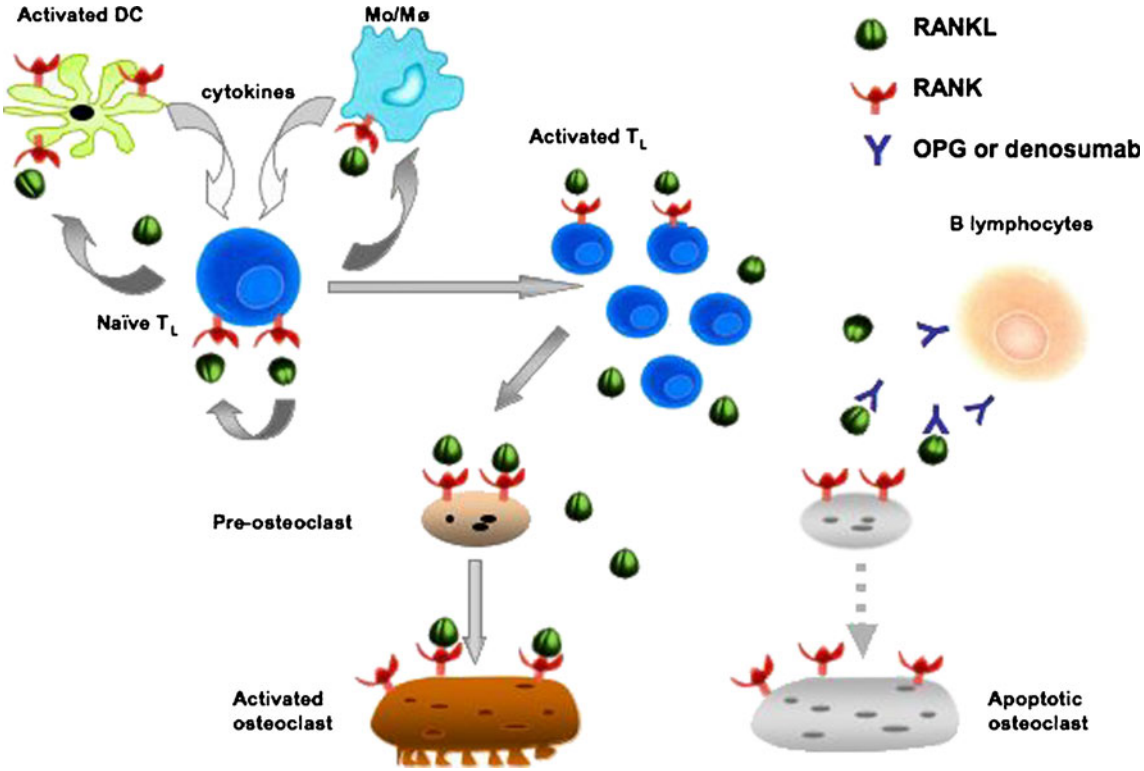

Fig. 1 RANKL-mediated crosstalk between immune cells and osteoclasts. RANK ligand is an essential mediator of osteoclast formation, function, and survival and is produced not only by bone marrow stromal cells and osteoblasts but also by T cells. RANKL binds to the RANK receptor, which is expressed by monocytes/macrophages (Mo/Mø) and osteoclast precursors, dendritic cells (DCs)/antigen-presenting cells (APC), and T cells. 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 inhibit RANKL-induced osteoclastogenesis

above clinical trial, the rate of adverse events and serious infections requiring hospitalization did not differ between patients treated with denosumab and those with placebo, although the episodes of influenza (self-reported), upper respiratory tract, and urinary tract infections were numerically higher in the denosumab group. Hence, in a recent survey, rheumatology experts raised some concerns that the risk of infection with RANKL inhibitors could be worsened if employed concomitantly to anti-TNFs and/or other biological agents [59].

Inflammatory bowel diseases

In the CD4$^+$ CD45RB^{Hi} T-cell transfer mouse model of inflammatory bowel disease (IBD), the effects of OPG-Fc administration were similar to those in RA: While dramatically improving bone density, OPG-Fc did not modify inflammatory parameters nor reduce infiltration of the gastrointestinal tract by inflammatory cells (colitis) [60]. In contrast, in IL-2-deficient mice—that spontaneously develop an autoimmune disease characterized by hyperactivation of CD4$^+$ T cells with multi-organ inflammation and massive T-cell infiltration of the gastrointestinal tract—administration of OPG-Fc not only led to a significant increase in bone density but also improved gastrointestinal tissue architecture [61]. In this particular model, by antagonizing RANKL expressed by T cells, it is possible that OPG-Fc interrupted the amplification loop of T-cell activation and infiltration in colonic tissues mediated by DCs (Fig. 2). Hence, in models where major immunologically active molecules are deleted (e.g., IL-2 KO and CD40 KO [62]), RANK/RANKL signaling may become the main co-stimulatory pathway for crosstalk between T and B cells and DCs [32, 62, 63].

Whether these models are of clinical relevance however remains uncertain, and it is therefore hardly surprising that in most circumstances, RANKL inhibitors did not alter tissular inflammation.

Effects of RANKL/RANK/OPG on T- and B-cell-mediated immunity

Effects on the immune system organogenesis and T cells

While innate immune responses induced by inflammation represent a physiological (and sometimes pathophysiological), non-specific reaction to pathogens (and self-antigens)

Table 1 Properties of anti-tumor necrosis factor and anti-RANKL antibodies in humans

Description	Anti-TNF	Anti-RANKL
Drug structure	Soluble TNFRII-human Fc fusion protein (etanercept)	Human monoclonal IgG2 antibody (denosumab)
Potential indications	Rheumatoid arthritis	Osteoporosis
	Juvenile chronic arthritis Psoriatic arthritis	Skeletal complications of malignancy
	Ankylosing spondylitis	Rheumatoid arthritis
	Psoriasis	
Effect on T cells	Induces apoptosis	NA
	Increases Treg	
Effect on B cells	No direct effect	NA
Effect on mono-mφ	Induces apoptosis Decreases production of inflammatory cytokines	NA
Adverse events		
Infectious	Tuberculosis	Cellulitis/erysipelas[a]
	Bacterial infections	Diverticulitis[a]
	Intracellular pathogens	
	Upper respiratory tract	
Tumoral	Lymphoma, mostly non-Hodgkin's	Breast?[b]
		Reproductive (ovary)?[b]
		Gastrointestinal?[b]
Others		Dermatitis and Eczema[c]
		Hypersensitivity?
Auto-antibodies	Very frequent: ANA, anti-dsDNA, anti-cardiolipin, but their formation is not associated with specific clinical syndrome	None

Concerning RANKL antagonist effects on immune cells in animals and in vitro, see text

NA data not available in humans

[a] In the post-menopausal osteoporosis and HALT trials combined

[b] In the post-menopausal osteoporosis trials only. The "?" indicates that the significance of these observations is still unclear (according to the FDA advisory committee for reproductive health drugs, August 2009)

[c] In the post-menopausal osteoporosis trials

and lack memory, the adaptive immune response is characterized by a high degree of specificity and memory, involving specialized and highly differentiated cells. A prerequisite of specific innate and adaptive immune responses is the stepwise maturation of T and B lymphocytes, and there are distinct stages in the development of lymph node (LN) organogenesis that require the specific expression of adhesion molecules, cytokines, and chemokines. LN, Peyer's patches, and nasal-associated lymphoid tissue (NALT) seem to follow a common developmental program, whereas spleen organogenesis undergoes an even more complex process.

Studies in mice have mapped numerous genes essential for lymphoid tissue development [64], including members of the TNF family such as surface lymphotoxin LTα1β2 and RANKL. LTα1β2 signals through the lymphotoxin β receptor (LTβR), and mice deficient in LTα, LTβ, or LTβR lack LN [65–67]. Similarly, in the absence of RANKL, RANK, or TRAF-6, LN cannot develop, whereas the formation of Peyer's patches, NALT, and splenic microarchitecture is not affected [6, 18, 68, 69]. In RANKL-deficient mice, impaired LN development results from a defect in colonization and cluster formation of hematolymphoid precursor cells [68], particularly $\alpha4\beta7^+$ $CD4^+$ $CD3^-$ T cells. Thymus size and cellularity of these mice are diminished, indicating that RANKL further contributes to early thymocyte development [18]. Although the total number of T lymphocytes as well as their production of IFN-γ and IL-2 is lower in $RANKL^{-/-}$ mice, they do proliferate adequately, and the subsets of $CD4^+$ and $CD8^+$ T cells occur in normal amounts, probably owing to redundant co-stimulatory pathways (such as CD40-CD40L and CD28-CD80/86), indicating that thymus function remains unaffected. Intriguingly, $RANK^{-/-}$ mice boast normal thymic development, a normal percentage of thymocytes and T-cell precursors [6]. Consequently, as suggested by

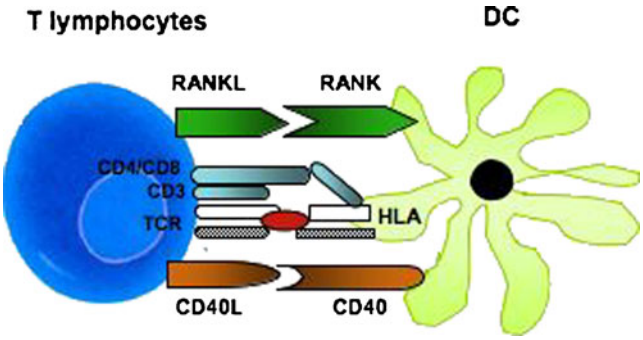

Fig. 2 Role of RANKL/RANK signaling on co-stimulation of innate immune cells. Interaction between T lymphocytes and DC as well as their activation occur (1) through the T-cell receptor (TCR)-HLA complex and (2) through co-stimulatory complexes such as CD28/B7 or CD40L/CD40 molecules. RANKL interaction with RANK promotes DC survival [62] and enhances monocyte functions [33]. This interaction is similar to that of the co-stimulatory CD40L/CD40 interaction [98]. CD40L is rapidly expressed by activated T cells, whereas RANKL expression occurs within 48 h. Consequently, CD40L/CD40 interaction is key to controlling the priming stage, whereas RANKL/RANK interaction may take place later and is of a more accessory nature [99]

these authors, RANKL signaling in the thymus might also be mediated through a receptor other than RANK [6].

Contrasting with RANKL- and RANK-deficient mice, highly expressing OPG-transgenic (OPG-Tg) mice present no alterations of LN, thymus, or spleen [70]. OPG-Tg rats present normal LN development at gestational day 11 when OPG levels are already high [70], suggesting that even residual effects of RANKL may be sufficient in this regard. Similarly, the administration of RANKL-Fc in mice did not affect cytokine production and proliferation of T cells, nor did it modify T-lymphocyte infiltration of inflammatory tissues [71]. Moreover, when OPG or RANKL are added to wild-type murine T-cell cultures, their proliferation and production of cytokines remain unaffected, suggesting that RANKL signaling is not crucial for mature T-cell functions [18].

Finally, patients with mutations in the *TNFSF11* (*RANKL*) gene or in the *TNFRSF11A* (*RANK*) gene have normal lymphocyte counts of the peripheral blood and do not present major abnormalities in T-cell phenotypic and functional properties [8, 9].

Effects on B cells

RANKL$^{-/-}$ mice exhibit a marked alteration of B-cell development with defective transition of pro-B to pre-B and reduced numbers of B cells in the spleen [18]. In RANK$^{-/-}$ mice, the developmental defect in the B-cell lineage is massive with a decrease in mature B cells in the bone marrow and peripherally [6]. In three out of the eight patients with osteoclast-poor osteopetrosis due to mutations in the *TNFRSF11A* (*RANK*) gene, the number of mature B cells was significantly decreased, and their serum immunoglobulin levels (particularly IgG) were low. Moreover, two of these patients failed to produce antibodies to tetanus toxoid vaccination [9]. Of note, their Ig level—although reduced—was not as low as that observed in patients with common variable immune deficiency. Furthermore, the reasons for the hypogammaglobulinemia in patients with mutations in the *TNFRSF11A* (*RANK*) gene may be as follows: (1) in response to RANKL, osteoclasts are unable to produce high levels of APRIL, a player of B-cell activation and isotype switching, and (2) the marked osteopetrosis limits anatomic niches for B-cell development and survival in bone marrow [72]. In addition, patients with osteoclast-poor osteopetrosis due to mutations in the *TNFSF11* (*RANKL*) gene affecting its cell surface presentation were not reported to suffer from immunological deficiencies nor to be particularly prone to infection [8]. To explain the discordant observations concerning B-cell functions in these patients with RANKL and RANK mutations, it has been hypothesized that the mutant RANKL protein might still retain some selective activity on immune cells [8] which, to our knowledge, has not yet been substantiated.

In OPG$^{-/-}$ mice, proliferation of pro-B cells is increased, but there is a deficit in antibody response to antigen due to an alteration in the isotype switch of IgGs, suggesting that OPG is essential for B-cell maturation and development through its inhibitory effect on TRAIL [73, 74]. In contrast, OPG-Tg mice and rats present neither a defect in B-cell development and maturation nor impaired humoral response to immune challenges [70], confirming that these rodents are immunocompetent. In addition, in mice treated with OPG-Fc and injected with a pneumovax vaccine, production of IgM and IgG subtypes was identical to that of mice injected with control-Fc (Table 2) [75]. Moreover, administration of denosumab to adult ovariectomized cynomolgus monkeys over a period of 15 months had no apparent effect on basal immune parameters and did not elicit any effect on T, B, or NK cells at any time point [76].

In summary, mouse genetic models clearly indicate that the RANKL/RANK/OPG pathway is essential to the development of lymphoid organs and T and B cells, whereas experiments of nature in humans show that loss-of-function mutations in this system have only moderate and selective effects on B-cell immunity. On another side, overexpression of OPG even from the embryonic stage, as well as administration of RANKL antagonists, do not appear to impair the development and/or activation of T and B cells (Table 3). These results also suggest that RANKL inhibitors do not impair autoimmunity, since autoimmune diseases and some allergic diseases are partly due to a defect in IgG switching [77, 78].

Table 2 Comparative phenotypes of $RANK^{-/-}$, $RANKL^{-/-}$, and $OPG^{-/-}$ mice

	$RANK^{-/-}$ mice	$RANKL^{-/-}$ mice	$OPG^{-/-}$ mice
Bone	Osteopetrotic; no osteoclast present	Osteopetrotic; no osteoclast present	Osteoporotic; osteoclast activation
Thymus	Normal size; thymocyte dvp normal	Reduced size; thymocyte dvp impaired	Normal size
LN	LN agenesis; Peyer's patches smaller	LN agenesis; Peyer's patches smaller	Normal LN dvp; Peyer's patches normal
Spleen	Normal; extramedullary hematopoiesis	Normal; extramedullary hematopoiesis	Normal
L_T	Normal T-cell dvp; CD4/CD8 ratio N	Deficit in early intrathymic T-cell dvp CD4/CD8 ratio N	Normal T-cell dvp; CD4/CD8 ratio N
L_B	B-cell dvp ND; reduced number of B-cells	B-cell dvp impaired; reduced number of B-cells	Increased number of B-cells; altered Ig isotype switching
DC	Normal dvp and function/activation	Normal dvp and function/activation	Increased survival and function/activation

Adapted from Dougall et al. [6]

LN lymph nodes, *dvp* development, L_B lymphocytes B, L_T lymphocytes T; *Ig* immunoglobulin, *DC* dendritic cells, *ND* not determined

Effects of RANKL/RANK/OPG on other immune cells

Immune cells need to 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 to pathogens. DCs and monocyte/macrophages are key components of the innate immune response and are major antigen-presenting cells (APC) priming T cells to proliferate and initiate an adaptive immune response [79]. 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 enhances functions of DC and monocyte/macrophages, as well as B-cell proliferation and antibody production in order to control the dissemination of antigens.

Effects on monocyte–macrophages

Monocyte–macrophages are closely related to osteoclasts and express RANK on their cell surface. Monocytes from peripheral blood as well as monocytic cell lines both differentiate into osteoclasts when cultured with M-CSF and RANKL [1]. Stimulation of monocytes with RANKL induces the expression of Bcl-xl, pro-inflammatory cytokines such as IL-1β and TNF-α, chemokines, co-stimulatory molecules (CD80 and CD86), and MHC-II on

Table 3 Evaluation of RANKL inhibition on immune parameters in preclinical models

		Length of RANKL inhibition		
		Short-term (<3 weeks, OPG- or RANK-Fc)	Long-term (>1 year, dmab)	Life-long (OPG-Tg)
Baseline immune profile	Cellular composition of blood [70]		Cyno	Mouse, rat
	Systemic cytokine levels [70]			Mouse, rat
	Systemic immunoglobulin levels [70, 75]	Mouse		Mouse, rat
	T and B cell proliferation in vitro to specific antigens [70, 75]	Mouse		Mouse, rat
Immune challenge	Delayed contact hypersensitivity to oxazolone in skin [70, 75]	Mouse		Mouse
	Innate response to LPS [70]			Mouse, rat
	T-cell-dependent immune response to KLH [70, 75]	Mouse	Cyno	Mouse
	T-cell-independent immune response to Pneumovax [70, 75]	Muse		Mouse
Infectious disease	BCG bacterial infection [70]	Mouse		
	Influenza viral infection [97]	Mouse		
Autoimmune disease	Immune-mediated arthritis [56]	Rat		
	Inflammatory bowel disease [60]	Mouse		

their cell surfaces, which protects these cells from apoptosis, increases their phagocytic properties, and activates antigen presentation [33]. However, in studies on RANKL$^{-/-}$ mice, both the number and distribution of monocyte–macrophages were normal [18]. 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 [6]. However, the role of RANKL/RANK in monocyte–macrophage functions becomes more prominent when other essential co-stimulatory molecules are missing, such as in mice lacking CD40L [80], a T-cell surface protein that is essential for activation of monocyte–macrophages and a close relative to RANKL [32, 81]. Whether RANKL antagonists would similarly worsen the deficient monocyte–macrophage functions in patients with the hyper-immunoglobulin M syndrome due to CD40 mutation or with CD40L deficiency is currently unknown.

Most important in terms of the pharmacological effects of denosumab on both immunity and 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 mice models of lipopolysaccharide (LPS)–endotoxic shock and inflammatory arthritis [33]. LPS is a component of cell walls of Gram-negative bacteria and a potent activator of monocyte/macrophage function due to 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 interactions 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 response [75].

Effects on antigen-presenting cells (APC)

APC play an essential role in the presentation of foreign peptides or altered self-peptides to T lymphocytes and thus in the induction of antigen-specific T-cell activation. APC are mainly composed of DCs and Langerhans cells (LC), a subset of DCs found in the skin epithelium and in mucosae. T cells that express RANKL play an agonistic role on DC activation in vitro due to co-stimulatory processes involving CD40L/CD40 interaction (see above) [32]. Hence, RANKL enhances DC survival, antigen presentation [62, 63], and production of cytokines [82]. Accordingly, in OPG$^{-/-}$ mice, due to the increased survival of DCs, the DC–T-cell interaction may be prolonged and T-cell proliferation more pronounced [73]. However, in RANKL$^{-/-}$ mice and RANK$^{-/-}$ mice, DC development and function are intact, with normal expression of DC surface markers and co-stimulatory markers, and the appropriate stimulation of alloreactive T cells [6, 18]. Phenotypic and functional analyses of DCs in subjects with either *TNFSF11* (*RANKL*) or *TNFRSF11A* (*RANK*) gene mutations also did not reveal any major abnormalities [8, 9]. Thus, although RANKL appears to be an effective co-stimulatory factor, it is not essential for DC activation.

Like DCs, LCs also express RANK, and LC proliferation and survival is impaired in RANKL$^{-/-}$ mice [83, 84]. Ultraviolet exposure or inflammation upregulated the expression of RANKL by inflamed keratinocytes leading to an increase of regulatory CD4^{+} CD25^{+} T cells [83], which in turn helps to control cutaneous inflammation and hyperallergic responses. Loser et al. [83] demonstrated that in transgenic mice expressing RANKL under the transcriptional control of the keratin-14 (K14) promoter (K14-RANKL Tg mice), the number of regulatory CD4^{+} CD25^{+} T cells was increased by activation of DCs, and contact hypersensitivity responses were decreased.

Effects of RANKL inhibition on immune surveillance of cancer

Both innate and adaptive immune mechanisms are implicated in the surveillance of cancer. Thus, antigen-presenting DCs, CD8 cytotoxic T lymphocytes, and CD4 T helper cells and, more specifically, natural killer (NK), regulatory T cells (Treg), and Tγδ cells interact in controlling and eventually eliminating tumor cells. A large number of cytokines and chemokines have been implicated in the immune response to cancer, since they amplify the inflammatory reaction—as in the case of IL-2—and/or cause lysis (by apoptosis) of tumor cells—as in the case of IFN-γ. It follows that although it is not essential, the immunomodulatory effect of RANK signaling on the various effectors of this system described above could affect immune surveillance of cancer. CD4^{+} T cells in particular can generate direct cytotoxicity via TRAIL. When added together with TRAIL to osteoblast-like cell cultures, OPG prevents TRAIL-induced apoptosis [85]. OPG secreted by prostate cancer cells may also protect them from TRAIL-induced apoptosis [86]. Therefore, the binding of OPG to TRAIL and interference with the natural defense mechanism against tumorigenesis is a potential concern related to OPG treatment. In this regard, denosumab has the advantage over OPG in that it does not bind to TRAIL [87]. On the other hand, inhibiting the excess RANKL commonly produced in bone infiltrated by tumor cells interrupts the

vicious circle of bone lysis stimulation of metastatic growth, which can prevent the skeletal progression of cancer, at least in preclinical models [88].

Risk of infection and cancer associated with denosumab in clinical trials

In a combined cohort of more than 8,000 post-menopausal women with osteoporosis from fracture (FREEDOM) and bone loss prevention [89, 90], the incidence of serious adverse events was similar in the denosumab and placebo (PBO) groups (25.3% and 24.3%, respectively), but denosumab tended to decrease the risk of death (HR, 0.76; CI, 0.55–1.03; p=0.08). In a combined cohort of about 1,700 subjects with non-metastatic breast cancer, i.e., including patients who faced a higher risk of infections and death, the incidence of serious adverse events and death was also similar in denosumab and PBO groups (31.6% vs 27.6% and 5.2% vs 5.6%, respectively) [91, 92]. Combining the results of these four studies (~10,000 subjects), infections were reported in 50.6% of subjects in the PBO group and 50.1% in the denosumab group, while serious infections were found in 4.3% and 3.4% of subjects treated with denosumab and PBO, respectively (NS). Sepsis (≤0.2%) and opportunistic infections (≤2%) including tuberculosis and fungal infections were rare, and their incidence was similar in the two groups. Severe pneumonia (1%), bronchitis (0.2%), and urinary tract infections (0.3%) also occurred in similar proportions in the two groups, while rare cases of severe skin infections (mostly at lower extremities) and diverticulitis were observed more frequently with denosumab than with PBO (erysipelas/cellulitis, 0.2% PBO, 0.4% denosumab; diverticulitis, 0.1% PBO, 0.3% denosumab). Considering the role of RANKL/RANK in the modulation of the immune/inflammatory responses in the skin (see above) and the small but significantly higher number of patients with eczema among post-menopausal women treated with denosumab as compared to placebo (FREEDOM), we suggest that RANKL inhibitors could amplify cutaneous allergic and inflammatory responses rather than increase susceptibility to infection itself. A recent meta-analysis including the FREEDOM trial and using a model of fixed rather than random effects revealed a significantly higher risk of serious infections in women with osteoporosis or osteopenia treated with denosumab than in controls (RR=1.26, CI=1.01–1.57, p=0.04). When patients with non-metastatic breast cancer (HALT trial) were excluded, the risk increase was of borderline significance (RR=1.25, CI=1.00–1.59; p=0.05).

The overall incidence of cancer in post-menopausal women from the FREEDOM trial and the bone loss prevention trial did not differ between denosumab (4.8%) and placebo (4.2%), nor did the number of malignancy-related deaths in both the FREEDOM and HALT prostate cancer [91] trials together (denosumab n=26, PBO n=35). In the combined sample of 8,091 post-menopausal women, there were no differences between denosumab and PBO in the number of skin, lung/mediastinum, or hematopoietic neoplasms including lymphomas, whereas the denosumab group showed a greater number of cases of breast cancer (35 vs 28 in PBO), cancer of the reproductive organs (21 vs 9 in PBO, mostly the ovaries), and of the GI tract (35 vs 24 in PBO). The latter was observed more specifically in the colon, pancreas, and stomach. With regard to the incidence of breast cancer in the two groups, there were only five more cases during year 3 in the group of nearly 4,000 women receiving denosumab than in those receiving PBO. It should also be noted that in the cancer trials (breast and other) involving thousands of patients treated monthly with double-dose (120 mg) denosumab for the prevention of skeletal-related events, overall disease progression and survival did not differ between denosumab and the comparator group (zoledronic acid) [93, 94].

Summary and perspective

RANKL, RANK, and OPG are not only expressed by bone cells but also by T cells, B cells, DCs, and monocyte–macrophages amongst others. Absence of RANKL or RANK during embryogenesis in mice results in thymus and LN defects, but this phenomenon has not been reported in humans with mutations in these genes. Hence, mutations in the *TNFSF11* (*RANKL*) gene do not cause severe immune deficiencies nor have they been reported to result in an increased risk of infection, cancer, or immune disorders [8]. Nevertheless, mutations in the *TNFRSF11A* (*RANK*) gene may cause hypogammaglobulinemia in some patients, with a potentially increased risk of infection [9]. As underscored by Sobacchi et al. [8] "the contrast in the effects of mutations in the RANKL/RANK/OPG system between the knockout mouse phenotype and the human phenotype could be due to species-specific differences."

Blocking the RANKL/RANK pathway in adult rodents and monkeys apparently does not lead to major immune cell dysfunctions, in contrast to TNF-α or IL-1 inhibitors. As a corollary of its apparent lack of major effects on the immune system, RANKL inhibition does not prevent inflammation driven by T cells in RA or IBD. Inhibition by denosumab of RANKL binding to RANK has proven effective in preventing bone resorption and/or fractures and, to be generally safe, in patients with osteoporosis, RA, and non-metastatic (prostate and breast) cancer [16, 89, 91, 92]. Whether inhibition of T cell-mediated, RANKL-dependent

activation of DCs and monocyte–macrophages would affect the capacity to generate and/or control immune reactions (primarily to infection and cancer) in some tissues, like in the skin and colon, however, remains to be scrutinized as a large number of possibly older and thereby more immunocompromised patients might be treated with denosumab in the future. On another side, RANK signaling also plays a role in the development of breast tissue [95], as well as in angiogenesis and endothelial permeability [96], which could be modified by RANKL inhibitors.

As denosumab progresses from investigation to clinical application, and as biological agents targeting cytokines are becoming more common in clinical practice, the efficacy and safety of association of biological agents, such as anti-TNF and denosumab for treatment of RA and other inflammatory diseases, will also need to be specifically investigated.

Acknowledgments We are grateful to Professor Jean-Michel Dayer and to Roswitha Rehm for helpful and critical reading of this manuscript and to Dr. Marina Stolina (AMGEN Co., Thousand Oaks, CA, USA) for her contribution to this manuscript. This work was supported by grant numbers 310000–108453 and PMPDA-110347 from the Swiss National Science Foundation and from AETAS, Swiss Foundation for Ageing Research (SFL). Serge Ferrari has received consultant and speaker fees and research grants from AMGEN.

Conflicts of interest None.

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