3 The Non-Bone-Related Role of RANK/RANKL Signaling in Cancer

Peter A. van Dam, Yannick Verhoeven, and Xuan B. Trinh

Abstract

RANK ligand (RANKL) is a member of the tumor necrosis factor alpha superfamily of cytokines. It is the only known ligand binding to a membrane receptor named receptor activator of nuclear factor-kappa B (RANK), thereby triggering recruitment of TNF receptor-associated factor (TRAF) adaptor proteins and activation of downstream pathways. RANK/RANKL signaling is controlled by a decoy receptor, osteoprotegerin (OPG), but also has additional more complex levels of regulation. It is crucial for the differentiation of bone-resorbing osteoclasts and is deregulated in disease processes such as osteoporosis and cancer bone metastasis. Cells expressing RANK and RANKL are commonly found in the tumor environment. In many tumor types, the RANK/RANKL pathway is overexpressed, and this is in most cases correlated with poor prognosis. RANK signaling plays an important role in the innate and adaptive immune response, generates regulatory T (Treg) cells, and increases the production of cytokines. It is also involved in chemo resistance in vitro. Recent evidence suggests that RANKL blockade improves the efficacy of anti-CTLA-4 antibodies against solid tumors and experimental metastasis. Therefore, there is increasing interest to use RANKL inhibition as an immunomodulatory strategy in an attempt to make immune-resistant tumor responsive to immune therapy.

Keywords

RANK · RANKL · Osteoprotegerin · Microenvironment · Cancer · Bone health · Immunomodulation · PFD-L1 · CTLA-4 · Immune response · Inflammation · Immune tolerance · Angiogenesis

3.1 Background

In most cancer types, only a minority of patients have an improved survival after immune therapy. Mutational burden, neoantigen load, quality and clonality of neoantigens, expression of antigen presenting molecules and immune checkpoints, interferon gamma responsiveness, and composition of the microenvironment (hot versus cold tumors), all influence the beneficial effects of

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immune therapies [1]. Although combinations of immune therapy (e.g., CTLA-4 and PD-L1 blocking) can be synergistic, they do not resolve their diminutive effectiveness in cancer treatment and often induce significant additional toxicity [2–5]. Hence, there is an increasing interest in combining immune therapy with less toxic immune modulating drugs to sensitize immune unresponsive tumors to immune therapies [6]. Recent data suggest that RANK/RANKL inhibition may be an attractive approach to increase the effectiveness of immunotherapy. Signaling between the receptor activator of nuclear factor-kappa B (RANK) and its ligand (RANKL) is essential for the differentiation of bone-resorbing osteoclasts and is deregulated in pathological processes such as postmenopausal osteoporosis or cancer-induced bone destruction [2]. However, cells expressing RANK and RANKL are also commonly found in the tumor microenvironment. RANK signaling plays an important role in the innate and adaptive immune response as it generates regulatory T (Treg) cells and increases production of cytokines [7, 8]. In this chapter, the effects of RANK/RANKL signaling inhibition on the microenvironment of malignant tumors are reviewed. It is hypothesized that this approach may be used to improve the response to immunotherapy (Fig. 3.1).

3.2 The RANK/RANKL Signaling Pathway

The receptor activator of nuclear factor-kappa B ligand (RANKL) was originally identified in T cells and dendritic cells (DC) [2]. It is a type II homotrimeric transmembrane protein that has three known isoforms. RANKL1 and RANKL2 are expressed as membrane bound proteins. RANKL3 is a soluble secreted protein that is formed by cleavage of the membranous counterparts or by alternative splicing [9]. The RANKL has a large cytoplasmic domain containing four cystein-rich repeat motifs and two N-glycosylation sites. The full length RANKL is called RANKL1, in RANKL2 a part of the intracellular domain is deleted, while in RANKL3 the N-terminal part misses [7, 10]. The RANKL is encoded by the TNFS11 gene in humans and is also named osteoclast differentiation factor (ODF), osteoprotegerin ligand (OPGL), or TNF-related activation induced cytokine (TRANCE) [9, 11]. It is the only known ligand binding to a membrane receptor named receptor activator of nuclear factor-kappa B (RANK), which is a type I transmembrane protein belonging to the TNF receptor superfamily (TNFRSF11A) [11, 12]. Binding between RANKL and RANK induces

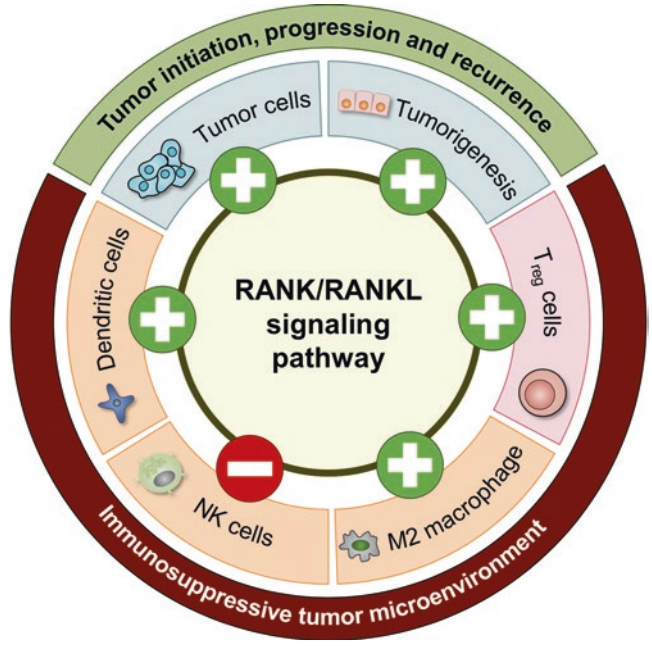

Fig. 3.1 Main effects of RANK/RANKL signaling pathway on tumor growth, immune cells, and microenvironment

trimerization of the receptor. This triggers recruitment of TNF receptor-associated factors (TRAF), adaptor proteins, and activation of downstream signaling pathways (such as NF-kB, AKT/PKB, JNK, and the MAP kinase cascade) [7, 13, 14]. A regulatory system is built into the RANK/RANKL signaling pathway by means of a decoy receptor called osteoprotegerin (OPG, TBFRSF11B) interacting with RANKL [2]. OPG is a soluble glycoprotein that can exist either as a 60-kDa monomer or as a 120-kD dimer but lacks transmembrane or cytoplasmatic domains. The dimerization of OPG increases the affinity of OPG to RANKL dramatically and is essential for RANK/RANKL signal inhibition [15]. Several factors can upregulate OPG expression such as estrogen (which is important for bone metabolism), TRAIL, Wnt, and TNFalpha [7]. On the other hand, it can be downregulated by PTH and TGF-beta [9]. The overall inhibitory effect of OPG on RANKL depends on the balance of its binding to these various ligands [11, 12]. The RANK/RANKL signaling network is further complexed by a second, more recently discovered, decoy receptor for RANKL, LGR4 [14]. LGR4 suppresses canonical RANK signaling by competing with RANK to bind RANKL. The binding of RANKL to LGR4 activates the Gαq and GSK3-β signaling pathway. This suppresses the expression and activity of nuclear factor of activated T cells and calcineurin-dependent 1 (NFATC1) during osteoclastogenesis. Furthermore, functional RANK splicing variants have also been identified, implicating several sophisticated levels of the pathway [16].

3.3 The Functional Role of RANK/RANKL Signaling in Humans

RANK and RANKL can be detected in many different tissues, such as the bone, prostate, thymus, mammary glands, and liver, implicating a functional role in these organs [17]. Studies in mice indicate that RANK/RANKL signaling is required for mammary gland development and lymph node formation [7, 17, 18, 19]. The signaling pathway's crucial role in healthy bone remodeling and bone homeostasis is, however, much better documented [8]. The RANK/RANKL pathway regulates the formation of multinucleated osteoclasts from their monocyte-macrophage precursor cells and subsequently also their activation and survival [8]. By binding RANKL, OPG prevents it to connect to and activate RANK, thereby protecting the skeleton from excessive bone resorption [19, 20]. When deregulated, this pathway may lead to pathological processes such as cancer-induced bone destruction and osteoporosis but also chronic inflammatory processes such as inflammatory bowel diseases and arthritis [14, 20, 21].

Another well-known functional role for RANK/RANKL signaling is that of modulating the immune response. RANK/RANKL and OPG knockout mice showed a disrupted immune phenotype (e.g., impaired T or B cell development) [7, 22]. RANKL can be found in tumor-infiltrating lymphocytes (TILs), immature dendritic cells, B cells, macrophages, and monocytes [2]. RANK activation induces lymphocyte differentiation, T-cell activation, and dendritic cell (DC) survival, triggering intracellular signaling pathways (e.g., MAPK, NFkB, p38, and c-JNK) and even extracellular kinases (ERK) [17, 23, 24, 25]. RANKL can induce the expression of multiple activating cytokines by DCs, including IL-1, IL-6, IL-12, and IL-15, and can enhance DC survival via the induction of the antiapoptotic protein Bcl-xL (B-cell lymphoma-extra large) [2]. Dendritic cells prime and activate T cells during the immune response by processing and presenting antigens to them. The RANKL signal can alter the function of dendritic cells, which may lead to an increase of Foxp3-positive Tregs [7]. Recent evidence suggests that in response to injury, pericytes are also able to modulate local tissue immune responses via several independent pathways including RANKL signaling. In this area, the OPG/RANK/RANKL axis in association with the functions of pericytes may be involved in vasculogenesis, the process of atherosclerosis by altering lipid metabolism, vascular signaling, and angiogenesis [26, 27].

3.4 RANK/RANKL Signaling in Cancer

Several studies documented RANK signaling to be important in a variety of cancers [23–43]. This was recently nicely reviewed by Renema et al. and de Groot et al. [17]. Tregs are a $CD4^+$ helper T-cell subset that can suppress autoimmune responses in the body and are critical to create an immune suppressive environment in cancers [2]. Together with other partners, such as TAMs, they can create a status of local immunosuppression surrounding the tumor [7]. TAMs express immune checkpoint modulators (such as PD-L1) that directly inhibit activated T cells and produce various chemokines that attract other immunosuppressive cells, such as Tregs and myeloid-derived suppressor cells (MDSCs) [11]. In many situations, the RANK network is an important driver to create an immunosuppressive microenvironment, thereby promoting tumor progression. The central role of RANK/RANKL signaling in bone metastasis has been well studied [9]. The RANK signal network has been shown to drive epithelial to mesenchymal transition (EMT), induce stem cell-like phenotypes, promote osteomimicry, and give cancer cells the ability to home to bone [11, 39]. In a large population of breast cancer patients, strikingly high levels of RANK expression in the primary tumor were predictive for the frequency of the later occurrence of bone metastasis [37]. Recently, it seems that RANK signaling is important in the biology of many tumor types beyond bone metastasis [2, 7, 8, 11, 17, 23–43]. RANK and RANKL-expressing cells are commonly found in the tumor microenvironment [2]. The RANKL/RANK pathway is often overexpressed in cancers of the prostate, endometrium, stomach, breast, cervix, stomach, bladder, oesophagus, and thyroid, which is correlated with poor prognosis [23–43]. RANKL has been detected in endothelial cells and implicated in angiogenesis [7].

There is some circumstantial evidence suggesting that paracrine signaling through RANK/RANKL is responsible for the expansion of mammary stem cells observed during pregnancy and luteal cycles [13, 38]. MMTV-RANK transgenic mice are prone to develop mammary tumors, which may be related to activated RANK signaling [35]. Pharmacologic inhibition of RANKL or genetic ablation of RANK reduces (particularly estrogen and progesterone receptor negative) mammary tumor and metastasis development in animal models [32]. Breast cancer cells are able to produce RANKL and stimulate osteoclast differentiation [16, 38, 39]. In humans, high RANK expression is associated with altered mammary differentiation, which suggests that increased RANK signaling may contribute to breast carcinogenesis [13, 40]. High RANK expression was particularly detected in human primary breast adenocarcinomas that lack expression of the hormone receptors, in tumors with high pathologic grade and proliferation index. It is associated with the presence of metastases and poor prognosis [37]. It has been shown in vitro that HIF-1 alpha-induced expression of RANKL initiates increased migration of breast cancer cells via PI3K/AKT signaling, illustrating that the RANK/RANKL pathway also plays an important role in breast cancer progression [41, 42].

Mouse models and randomized studies in humans have shown that combination of antibodies blocking OPG or RANK with chemotherapy, hormone therapy, or targeted drugs resulted in stronger decrease of tumor burden in the bone [8, 17]. However, inhibition of RANK signaling also has a direct effect on tumor cells at other locations [28]. The RANK/RANKL pathway was variably expressed in tumors of the thyroid, and increased serum OPG was also correlated with poor prognosis in gastric, cervical, esophageal, and bladder carcinoma [23–27, 29–32]. Song et al. found that RANK expression was significantly higher in hepatocellular carcinoma (HCC) than in peritumoral hepatic tissue [33]. HCC cell lines express RANK constitutively, and activation of the RANK-RANKL axis significantly promoted migration and invasion ability of HCC cells in vitro. Recently, it has been demonstrated that RANK/RANKL expression is also significantly elevated in endometrial and prostate cancer tissue, particularly in tumors of higher stage [20, 34, 35, 44]. Therefore, there may be a role for RANKL inhibitors as a therapeutic strategy.

3.5 Effects of the RANK/RANKL Signaling Pathway on the Tumor Microenvironment

RANK and RANKL expressing cells are commonly found in the tumor microenvironment [20]. RANKL modulates the immune response by inducing T-cell proliferation and dendritic cell survival [45]. In human breast carcinomas, RANKL is found in tumor-infiltrating lymphocytes (TILs), and RANK is strongly expressed in tumor-associated macrophages (TAMs) [18]. TAMs accumulate in the microenvironment and, depending on their M2 or M1 phenotype, are involved in tumor growth, angiogenesis, and metastasis. RANKL acts as a chemoattractant for these cells [2]. RANK/RANKL signaling in M2 macrophages modulates production of chemokines, promoting the proliferation of Tregs and thereby creating an immunosuppressive environment. As RANKL is mainly produced by Tregs, a vicious circle is established in conjunction with the TAMs mainly expressing RANK [7, 46]. Tumor-infiltrating Tregs have been shown to stimulate mammary cancer metastasis through RANKL-RANK signaling [47]. RANKL treatment enhances survival of mature dendritic cells (DCs) and triggers generation of proinflammatory cytokines (IL-1, IL-6, and IL-12) that can promote differentiation of CD4+ T cells into Th1 cells, providing a major costimulatory factor for CD4+ T-cell responses [47]. RANK is also expressed on NK cells, playing an important role in immunosurveillance. RANKL/RANK is involved in crosstalk between the bone and the immune system. It stimulates osteoclasts to function as antigen-presenting cells, thereby activating CD4+ and CD8+ T cells. A similar phenomenon might also be present in the microenvironment of solid tumors [7]. The crosstalk of tumor cells with the immune system is not completely understood, but the impact of RANK-RANKL signaling on the tumor immune response is likely to be context specific [4]. Due to sequestering OPG by tumor cells or entrapment of OPG by the proteoglycans and glycosaminoglycans of the extracellular matrix, a microenvironment is created that facilitates the expansion of the tumor cells [48]. In addition, OPG can block TRAIL activity, thereby acting as an antiapoptotic and pro-proliferative stimulus for cancer cells [11, 21]. It has been shown that RANK/RANKL signaling can promote the initial stages of cancer development by inducing stemness and epithelial-mesenchymal transition [19]. RANKL (e.g., produced by osteoblasts or bone marrow stromal cells) attracts RANK-expressing cells and induces their migration by activation of specific signaling pathways, such as the MAP kinase pathway [44]. RANKL was also detected in endothelial cells and has been implicated in angiogenesis through Src and phospholipase C-dependent mechanisms [2, 49].

3.6 RANKL Signaling Inhibition

The only commercially available inhibitor of RANKL is denosumab. This drug is a fully human monoclonal antibody that binds RANKL, thereby blocking its interaction with RANK [2, 8]. Denosumab is approved by the Food and Drug Administration for the treatment of osteoporosis and giant cell tumor of the bone and for the prevention and treatment of skeletal complications caused by bone metastases and lytic bone lesions in multiple myeloma [8]. The drug has a well-known and acceptable toxicity profile [8]. It remains unclear whether RANK/RANKL inhibition with denosumab in patients with cancer has any effect beyond the bone. In a post hoc analysis of patients with non-small-cell carcinoma of the lung (NSCLC) that were included in a phase III randomized trial comparing zoledronic acid versus denosumab, a survival benefit was observed (HR 0.80; 95% CI 0.67–0.95, $p = 0.01$) for the patients treated in the denosumab arm [50]. There was no difference in the delay of bone events in both groups, and the beneficial effect of denosumab could be observed in patients with visceral metastasis, as well as in patients with bone metastasis only. However, the recent prospective SPLENDOUR trial could not show any improvement in OS or PFS by adding denosumab to standard first-line therapy in patients with metastatic NSCLC [51].

The effect of adjuvant denosumab in women with early breast cancer was recently studied in two large, multicenter, prospective, randomized trials [52, 53]. In the ABCSG-18 study, it was shown that disease-free survival was significantly better in the denosumab group [52]. This study compared placebo or denosumab 60 mg subcutaneously every 6 months for 5 years in 3425 postmenopausal patients with hormone-sensitive early breast cancer treated with an aromatase inhibitor. In the DCARE study, which assessed 4509 high-risk early breast cancer patients treated with standard therapy either with or without denosumab 120 mg SC every month (for 6 months, then 3 monthly up to 5 years), no improvement in bone metastasis-free, disease-free, or overall survival was reported, even though there was an improvement in time to bone metastasis at site of first recurrence in the denosumab group [53]. It is important to mention that most (95.9%) of these patients had received taxane or anthracycline-based chemotherapy. This raises the hypothesis that chemotherapy may reduce some of the tumor suppressive effects of RANK/RANKL inhibition in the cancer microenvironment. Other explanations may be the differences in molecular characteristics of the tumors of these patient populations, or effects of the menopause and endocrine treatment on the tumor behavior. It is clear that more research is necessary to unravel the effect of denosumab on tumor behavior. In the D-BEYOND trial, the biological effects of two neoadjuvant injections of 120 mg denosumab (1 week apart) in 27 patients with premenopausal primary breast cancer were evaluated [54]. The authors concluded that 2 weeks of RANKL inhibition did not have an effect on the tumor proliferation rate, but significantly increased the number of TILS in the tumor environment, making them theoretically more susceptible for immune therapy. Recently, some additional evidence emerged that RANK/RANKL inhibition may have a role as immune modulator. In preclinical studies, RANKL blockade improves the efficacy of anti-CTLA-4-targeted antibodies in solid tumor models of metastasis [53]. Bakhru et al. showed that antibodies blocking RANKL and CTLA-4 cooperate to increase the frequency of tumor-infiltrating CD4+ T cells expressing cytolytic markers, thereby improving antimelanoma immunity [55]. Addition of RANKL blockade to anti-PD-1 and anti CTLA-4 resulted in superior tumor responses and was most effective if RANKL inhibition was given concurrent or following checkpoint blockade [54]. This triple combination therapy improved T-cell effector function in tumor bearing mice by increasing the proportion of tumor-infiltrating CD4+ and CD8+ T cells that can produce both interferon gamma and TNF. In 2014, Smyth et al. described a case of a rapidly advancing metastatic melanoma with aggressive and symptomatic bone metastases requiring treatment with the anti-RANKL antibody denosumab for palliation in a patient who was concomitantly treated with ipilimumab (an anti-CTLA-4 antibody) [56]. She had a spectacular partial response and was alive at 62 weeks. In a melanoma preclinical model, these authors could demonstrate that monoclonal antibodies (mAbs) directed to CTLA-4 or RANKL have modest antimetastatic activities in monotherapy, but when these drugs were combined at the time of intravenous melanoma inoculation, the development of metastases was significantly reduced. Mechanistically, the combined effect of anti-CTLA-4 and anti-RANKL depends on lymphocytes or natural killer cells. In a retrospective study, Afzal and Shirai evaluated the synergistic effect of immune checkpoint inhibitors and denosumab in metastatic melanoma patients [57]. Eleven (29.72%) out of 37 patients were treated with immune checkpoint inhibitors and denosumab, and the others only immune checkpoint inhibitors. The median progression-free and overall survival in the cohort having the combination treatment, respectively, was 11.6 and 57 months compared with 4.15 and 22.8 months in the control group. Although there are potential confounders, this suggests that adding denosumab to immune checkpoint inhibitors may have a beneficial effect on outcome. In a subsequent study, Ahern et al. assessed the efficacy of a combination of RANKL and CTLA-4 blockade by analysis of tumor-infiltrating lymphocytes, tumor growth, and metastasis in a model using a

variety of neutralizing antibodies and gene-targeted mice [58]. RANKL blockade improved the efficacy of anti-CTLA-4 mAbs against solid tumors and experimental metastases. Treg-depleting anti-CTLA-4 mAbs of the mouse IgG2a isotype showed the highest combinatorial activity. The optimal combination depended on the presence of activating Fc receptors and lymphocytes (particularly natural killer and $CD8^+$ T cells), whereas anti-RANKL alone did not require Fc receptors. T-cell infiltration into solid tumors post anti-RANKL and anti-CTLA-4 was significantly higher, and this was accompanied by increased T-cell effector function. Several studies are currently ongoing, studying the effect of denosumab monotherapy and the combination of RANKL inhibition and immunotherapy [2, 7].

3.7 RANK/RANKL Signaling and Chemo- or Radiotherapy

The role of the RANK/RANKL signaling in drug resistance remains unclear. There is some in vitro evidence suggesting that RANK/RANKL signaling can induce chemoresistance through the activation of multiple signal transduction pathways [59, 60]. However, in a mouse model, RANKL blockade increases the efficacy of cisplatin chemotherapy [60]. At the moment, there are no objective data that RANK/RANKL signaling inhibition has an influence on the effectivity of chemotherapy or radiotherapy in humans [7].

3.8 Conclusion

The role of RANK/RANKL inhibition as an immunomodulatory strategy in combination with other treatment modalities should be further investigated. As denosumab has clear immune-stimulating effects and an interesting toxicity profile, the drug has an attractive potential to be coadministered with immunotherapies for cancer treatment, thereby reinforcing the antitumor immune response. Optimal dosage and sequencing of treatment with other drug combinations warrants further investigation.

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