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Fibroblast-recruited, tumor-infiltrating CD4+ T cells stimulate mammary cancer metastasis through RANKL-RANK signaling

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Abstract

Inflammatory mechanisms influence tumor development and metastatic progression[1]. Of interest is the role of such mechanisms in metastatic spread of tumors whose etiology does not involve pre-existing inflammation or infection, such as breast and prostate cancers. We found that prostate cancer metastasis is associated with lymphocyte infiltration into advanced tumors and elevated expression of the tumor necrosis factor (TNF) family members receptor activator of NF-κB (RANK) ligand (RANKL) and lymphotoxin (LT)[2]. But the source of RANKL and its role in metastasis were not established. RANKL and its receptor RANK control proliferation of mammary lobuloalveolar cells during pregnancy[3] through activation of IκB kinase α (IKKα)[4], a protein kinase that is required for self-renewal of mammary cancer progenitors[5] and prostate cancer metastasis[2]. We therefore examined whether RANKL, RANK and IKKα are also involved in mammary/breast cancer metastasis. Indeed, RANK signaling in mammary carcinoma cells that overexpress the *ErbB2* (*c-Neu*) proto-oncogene[6], which is frequently amplified in metastatic human breast cancers[7,8], was important for pulmonary metastasis. Metastatic spread of *ErbB2*-transformed carcinoma cells was also dependent on $CD4^+CD25^+$ T cells, whose major pro-metastatic function appeared to be RANKL production. RANKL-producing T cells were mainly $FoxP3^+$ and found in close proximity to smooth muscle actin (SMA)-positive stromal cells in mouse and human breast cancers. The T cell-dependence of pulmonary metastasis was replaced by administration of exogenous RANKL, a procedure that also stimulated pulmonary metastasis of RANK-positive human breast carcinoma cells. These results are consistent with the adverse prognostic impact of tumor-infiltrating $CD4^+$ or $FoxP3^+$ T cells on human breast cancer[9,10] and suggest that targeting of RANKL-RANK signaling can be used in conjunction with other therapies to prevent subsequent metastatic disease.

RANK signaling controls osteoclast maturation and bone resorption, and was therefore targeted to prevent bone metastasis in breast and prostate cancers[11]. A humanized RANKL antibody that reduces vertebral fractures in postmenopausal women and androgen-ablated prostate cancer patients was found to be a more potent inhibitor of bone metastasis than other osteoclast-targeting drugs[12,13]. To examine whether RANK signaling may control additional aspects of breast cancer metastasis, we used *MMTV-ErbB2* transgenic mice of the

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FVB/N background[6], in which mammary carcinomas are induced by a gene that is over-expressed in about 30% of human breast cancer[7]. *Rank*$^{-/-}$ mice are osteopetrotic, defective in tooth eruption, and exhibit retarded growth[11]. We therefore generated *MMTV-ErbB2*/*Rank*$^{+/-}$/*Fvb* mice that were indistinguishable from *MMTV-ErbB2*/*Rank*$^{+/+}$/*Fvb* mice in appearance, weight and general health. Although primary tumor development was not affected by reducing *Rank* gene dosage, mammary carcinomas from *MMTV-ErbB2*/*Rank*$^{+/-}$ mice expressed less RANK than *MMTV-ErbB2*/*Rank*$^{+/+}$ tumors and the multiplicity of lung metastases was reduced by 50% (Fig. 1a). The role of RANK-signaling was further examined using an orthotopic tumor model in which primary mammary cancer cells (PCaM) from an *MMTV-ErbB2* tumor were injected into the #2 mammary gland of FVB/N mice. Injection of recombinant RANKL, after inoculation, significantly increased the incidence and multiplicity of pulmonary metastases (Fig. 1b).

To facilitate mechanistic analysis, we generated an immortalized mammary carcinoma cell line, MT2, from an *ErbB2*-induced mammary tumor (Supplementary Fig. 1a). MT2 cells double every 24 hrs (Supplementary Fig. 1b), exhibit epithelial morphology in culture, form colonies in soft agar and mammospheres when grown in Petri dishes without serum (Supplementary Fig. 1c). MT2 cells expressed as much RANK as PCaM cells, but RANK expression was very low in non-transformed mammary epithelial cells (MEC) from tumor-free virgins (Supplementary Fig. 1d). Neither PCaM nor MT2 cells expressed *Rankl* mRNA (Supplementary Fig. 1e). MT2 cells underwent pulmonary metastasis after transplantation into the #2 mammary gland that was further enhanced by RANKL injection (Fig. 1c). To ascertain that the results of these experiments are not affected by a putative immune response elicited by the *ErbB2*-expressing cells, we transplanted MT2 cells into *MMTV-ErbB2/Fvb* mice that are tolerized to the *ErbB2* oncogene[14]. The results were identical to those obtained in normal FVB/N mice (Fig. 1c). Silencing of RANK expression (Supplementary Fig. 1f) reduced pulmonary metastasis of MT2 cells (Fig. 1d) and blocked the response to RANKL(Supplementary Fig. 2a). Neither RANKL administration nor RANK silencing had a significant effect on primary tumorigenic growth of transplanted MT2 cells, which was identical in FVB/N and *MMTV-ErbB2/Fvb* mice (Supplementary Fig. 2b, c). Similar results were obtained by administration of a RANK-Fc fusion protein, which blocks RANK signaling[15], into mice bearing MT2 mammary tumors. Whereas RANK-Fc marginally inhibited tumor growth at the primary site, it greatly reduced the incidence and multiplicity of pulmonary metastasis (Supplementary Fig. 3).

RANK signaling in MT2 cells induced nuclear translocation and phosphorylation of IKKα that was not associated with IKKβ and IKKγ/NEMO, the two other IKK complex components (Supplementary Fig. 4a–c), indicating that RANK signaling targets free IKKα or one that is part of a different complex. As in prostate cancer[2], RANK-mediated IKKα activation repressed expression of the metastasis inhibitor maspin (Supplementary Fig. 5a–c). Expression of ectopic maspin in MT2 cells (Supplementary Fig. 5d) marginally inhibited tumorigenic growth (Supplementary Fig. 5e), while strongly reducing pulmonary metastasis (Supplementary Fig. 5f). Maspin-MT2 tumors expressed nearly 4-fold more maspin than green fluorescent protein (EGFP)-marked MT2 tumors (Supplementary Fig. 5g). Exogenous RANKL treatment, which stimulated EGFP-MT2 metastatic activity and did not influence primary tumor growth by either maspin-MT2 or EGFP-MT2 cells, did not enhance the weak metastatic activity of maspin-MT2 cells (Supplementary Fig. 5h). As expected[5], silencing of IKKα in MT2 cells slowed down tumorigenic growth (Supplementary Fig. 6a, b) and completely inhibited pulmonary metastasis, even in mice given exogenous RANKL (Supplementary Fig. 6c), while up-regulating maspin expression (Supplementary Fig. 6d).

RANKL treatment of freshly isolated primary tumor cells or cells with reduced *Rank* gene dosage did not affect primary mammosphere formation (Supplementary Fig. 7a, b).

Although RANKL was reported to enhance metastatic activity of diverse cancer cell lines by enhancing their motility[16], no significant effect of RANKL on motility or invasiveness of MT2 cells was observed (Supplementary Fig. 7c). *In vivo*, RANKL treatment decreased the number of apoptotic cells in late-stage tumors (Supplementary Fig. 7d). Conversely, IKKα silencing increased the frequency of apoptotic cells in late-stage tumors by 2.2-fold and this was not reversed by RANKL treatment (Supplementary Fig. 7e, f). RANKL treatment of mice injected with RANKL pre-treated MT2 cells via the tail vein enhanced their extravasation into the lung and this effect was RANK dependent (Supplementary Fig. 7g). Correspondingly, *in vitro* RANKL strongly inhibited the apoptotic death of suspended MT2 cells (Supplementary Fig. 7h). These results suggest that the pro-metastatic effects of RANKL and IKKα may be due to increased survival of circulating metastasis-initiating cells.

RANKL expression was detected in tumor stroma but not in the carcinoma portion of *ErbB2*-induced mammary tumors (Fig. 2a; Supplementary Fig. 8b) or within metastatic lung nodules, which were surrounded by a few $CD5^+$ lymphocytes (Supplementary Fig. 8a). As activated lymphocytes are a primary source of RANKL during inflammation[17] and are present in stroma of breast tumors[18], we examined their role in tumor RANKL expression. Staining of parallel tumor sections revealed an almost identical distribution of $RANKL^+$ and $CD5^+$ cells (Fig. 2a), which could be either B1 or T lymphocytes. Correspondingly, tumors grown in *Rag1*$^{-/-}$ mice, which lack mature T and B cells (Supplementary Fig. 9), contained lower amounts of *Rankl* mRNA than tumors grown in WT mice (Fig. 2b) and were completely devoid of $RANKL^+$ cells (Fig. 2a). A similar decrease in *Rankl* mRNA was seen in tumors grown in *Cd4*$^{-/-}$ mice (Fig. 2c), which lack only $CD4^+$ T cells (Supplementary Fig. 9). Tumors raised in *Cd4*$^{-/-}$ mice were almost completely devoid of $RANKL^+$ cells, although they contained a few adjacent $CD5^+$ cells (Fig. 2a). To further identify the major RANKL-expressing cell type in metastatic mammary tumors, we grew MT2 cells in mammary glands of *Rag1*$^{-/-}$ mice that were reconstituted with splenic B cells, $CD4^+$ T cells or $CD8^+$ T cells from WT FVB/N mice. Reconstitution efficiency was confirmed by flow cytometry at the experiment's end (Supplementary Fig. 10). Only $CD4^+$ T cell reconstitution resulted in a substantial increase in tumor *Rankl* mRNA above the low basal level in mock-reconstituted *Rag1*$^{-/-}$ mice (Fig. 2d). We also isolated B cells, $CD4^+$ and $CD8^+$ T cells from spontaneous *MMTV-ErbB2* tumors and analyzed them for *Rankl* mRNA, which was highest in $CD4^+$ T cells (Fig. 2e). Likewise, $CD4^+$ T cells from MT2 mammary tumors grown in WT FVB/N mice contained much more *Rankl* mRNA than B cells from the same tumors (Fig. 2f). Despite their lower contribution to *Rankl* mRNA expression, $CD8^+$ T cells were more numerous within mammary tumors than $CD4^+$ T cells and both T cell subsets were far more numerous than tumor-infiltrating B cells (Supplementary Fig. 11a). The absence of lymphocytes in *Rag1*$^{-/-}$ mice did not affect the amount of tumor-associated macrophages (Supplementary Fig. 11b) and no differences in composition of tumor stroma were found between tumors grown in FVB/N or *MMTV-ErbB2/Fvb* mice (Supplementary Fig. 2d, e). Amongst different types of $CD4^+$ T cells, $FoxP3^+$ Treg cells exhibited the most similar distribution to $RANKL^+$ cells in parallel mammary tumor sections (Fig. 2g). Notably, Treg cells in breast cancer are associated with an invasive phenotype and poor prognosis[9,10]. The majority of $FoxP3^+$ and $RANKL^+$ cells were in contact with SMA^+ cells within the tumor stroma (Fig. 2g). Congruently, $CD4^+CD25^+$ tumor T cells, which are $FoxP3^+$ enriched[19], expressed 4-fold more *Rankl* mRNA than $CD4^+CD25^-$ T cells (Fig. 2h). Co-staining of tumor sections revealed that FoxP3 and RANKL were co-localized (Supplementary Fig. 12a). These results suggest that tumor-infiltrating $CD4^+FoxP3^+$ Treg cells are the most critical cells for maintaining high RANKL expression within the microenvironment of metastatic mammary tumors.

The proximity of RANKL$^+$ T cells to SMA$^+$ cells, which expressed fibroblast markers (Supplementary Fig. 13), prompted us to examine whether the latter express T cell-attracting chemokines. CCL5/RANTES, a T cell chemokine involved in breast cancer metastasis[20,21] was expressed by, but not PCaM from mammary tumors (Fig. 2i). Expression of CCR1, one of the cognate CCL5 receptors[22], was strongly elevated in tumor CD4$^+$ T cells relative to splenic CD4$^+$ T cells of tumor-bearing or naïve mice (Supplementary Fig. 12b, left panel). Notably, CD4$^+$CD25$^+$ tumor T cells expressed more *Ccr1* mRNA than CD4$^+$CD25$^-$ tumor T cells (Supplementary Fig. 12b, right panel). Furthermore, tumor-infiltrating FoxP3$^+$ T cells were in close proximity to CCL5$^+$ stromal cells (Fig. 2j) and preferentially attracted CD4$^+$CD25$^+$ T cells relative to CD4$^+$CD25$^-$ T cells from spleens of tumor-bearing mice, although the latter were 6-times more abundant in unselected splenocytes (Fig. 2k, left panel). This recruitment was partially dependent on CCL5/RANTES (Fig. 2k, right panel). CCL5 neutralization also inhibited recruitment of tumor-associated CD25$^+$ cells *in vivo* (Fig. 2l).

To examine whether CD4$^+$ T cells were required for stimulation of pulmonary metastasis, we transplanted freshly isolated PCaM cells from *MMTV-ErbB2*-induced tumors into mammary glands of WT, *Rag1*$^{-/-}$, *Cd4*$^{-/-}$ and *Cd8*$^{-/-}$ mice, all in the FVB/N background. Although primary tumor volume was a bit larger in *Cd4*$^{-/-}$ and *Rag1*$^{-/-}$ mice than in WT or *Cd8*$^{-/-}$ mice (Supplementary Fig. 14a), pulmonary metastasis was greatly diminished in *Cd4*$^{-/-}$and *Rag1*$^{-/-}$ mice, but unaltered in *Cd8*$^{-/-}$mice (Fig. 3a, Supplementary Fig. 14b). PCR-mediated *ErbB2* mRNA quantitation confirmed a 3-fold decrease in pulmonary metastasis in *Cd4*$^{-/-}$ mice (Fig. 3b). A dramatic decrease in pulmonary metastasis was exhibited by *Rag1*$^{-/-}$ mice inoculated with MT2 cells (Fig. 3c) but this was restored by transplantation of CD4$^+$ T cells and to a much lesser extent by CD8$^+$ T cells, whereas B cell transplantation had no effect (Fig. 3d). Administration of exogenous RANKL to MT2-inoculated *Rag1*$^{-/-}$ mice restored pulmonary metastasis in the complete absence of T cells (Fig. 3e). CD4$^+$CD25$^+$ were far more effective in restoring pulmonary metastasis in MT2-inoculated *Rag1*$^{-/-}$ mice (Fig. 3f) and this effect was almost completely blocked by administration of RANK-Fc (Fig. 3g).

We examined whether RANK signaling stimulates metastasis of human breast carcinoma cells. Of 19 cell lines, Au565, SKBR-3, ZR-75-1 and SUM-1315 were the highest RANK expressors (Fig. 4a; Supplementary Fig. 15a). None of the examined cell lines expressed more RANKL than normal human mammary epithelial cells with progesterone (Supplementary Fig. 15b). ZR-75-1 and Au565 cells were inoculated into the #2 mammary glands of nude mice. Whereas Au565 cells failed to form tumors, ZR-75-1 cells formed slow growing primary tumors (data not shown) that gave rise to pulmonary metastases in 20% of mice with an average multiplicity of less than 1 nodule per lung (Fig. 4b). Human RANKL administration increased metastasis incidence and multiplicity by 3-fold and 10-fold, respectively (Fig. 4b). Furthermore, RANKL administration decreased *maspin* expression in ZR-75-1 tumors by 2.5-fold (Fig. 4c). RANK-negative T47D cells did not respond to RANKL administration (Supplementary Fig. 16). RANKL$^+$ and FoxP3$^+$ cells were also present in the tumor-associated stroma of human breast cancer (Fig. 4d), in close proximity to SMA$^+$ cells (Fig. 4e). RANKL expression was higher in invasive ductal carcinomas (IDC) than in ductal carcinomas in situ (DCIS) or lymph node-positive tumors (Fig. 4f).

Our results provide a new mechanistic explanation for the association of CD4$^+$ and Treg markers with a more aggressive behavior in advanced breast cancers[9,10,18,23], by demonstrating that tumor-infiltrating CD4$^+$CD25$^+$FoxP3$^+$ T cells are a major source of RANKL production. RANKL expressed by these cells stimulates metastatic progression through RANK, expressed by metastasizing breast/mammary carcinoma cells

(Supplementary Fig. 17). These findings and the demonstration that the pro-metastatic function of T cells can be replaced by exogenous RANKL are different from those obtained in the *MMTV-PyMT* model in which $CD4^+$ Th2 cells stimulate tumor progression through IL-4 which caused the M2 polarization of tumor-associated macrophages[18]. Our findings in *MMTV-ErbB2* mice correlate well with human breast cancer. In both cases, $RANKL^+$ and $FoxP3^+$ Treg cells were concentrated in the tumor stroma and were not in contact with carcinoma cells. Furthermore, in both cases, tumoral $FoxP3^+$ cells correlated with invasion, metastasis and poor prognosis[9,10,23,24]. Recruitment of $CD4^+CD25^+$ T cells to CAFs is partially dependent on CCL5/RANTES, a chemokine associated with human breast cancer grade and metastasis[20,21]. Our findings, which suggest that CAFs and CCL5/RANTES act through T cells in addition to direct effects on carcinoma cells[20], are consistent with the observation that CAF depletion in mammary tumors decreased Treg infiltration and pulmonary metastasis[25]. We suggest that the potent pro-metastatic effect of tumor-infiltrating Treg cells can be dismantled through the use of RANKL-RANK antagonists, which should leave the potential anti-tumorigenic activity of Th1 cells intact. Therefore, RANK signaling inhibitors may be used in conjunction with surgical resection of primary breast tumors and anti-tumor immunotherapy to prevent recurrence of metastatic disease. It should also be noted that RANKL can mediate the breast cancer promoting activity of progestins, which induce its expression by pre-malignant cells[26,27].

METHODS SUMMARY

A detailed Methods section is available in Supplementary Information. C57BL6 $Rank^{+/-}$mice[11] were backcrossed to FVB/N *MMTV-ErbB2* mice for at least 6 generations. FVB/N *MMTV-ErbB2*/$Rank^{+/-}$ mice were intercrossed to generate FVB/N *MMTV-ErbB2*/$Rank^{+/+}$ and FVB/N *MMTV-ErbB2*/$Rank^{+/-}$ mice. $Cd4^{-/-}$, $Cd8^{-/-}$ and $Rag1^{-/-}$ mice were maintained in the FVB/N background. Single PCaM or MT2 cell suspensions were prepared and injected into the #2 mammary gland. Tumor size was measured weekly with a caliper. At the end of the study, mice were sacrificed and primary tumors and lungs were analyzed.

Supplementary Material

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Acknowledgments

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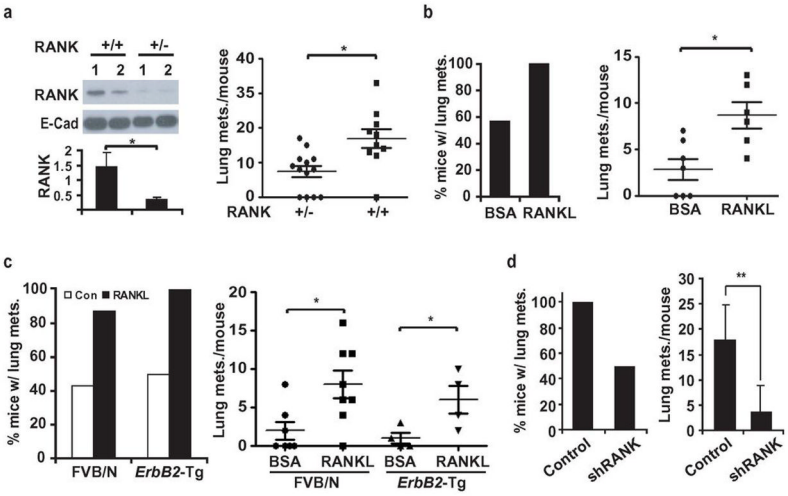

Figure 1. RANK-signaling in mammary carcinoma cells enhances metastasis

a, *MMTV-ErbB2*/*Rank*$^{+/+}$*/Fvb* and *MMTV-ErbB2*/*Rank*$^{+/-}$*/Fvb* mice were sacrificed 8 weeks after tumor onset. RANK expression in primary tumors was analyzed by immunoblotting (each lane a tumor from a different mouse, upper left) and quantitated by densitometry of relative to E-cadherin (bottom left; means +/− s.e.m.; n=6). Lung metastasis multiplicity was analyzed by H&E staining of lungs from *MMTV-ErbB2/Rank*$^{+/+}$*/Fvb* (n=10) and *MMTV-ErbB2/Rank*$^{+/-}$*/Fvb* (n=13) mice. Means +/− s.e.m. and data for individual mice are shown (right panel). **b,** PCaM cells from *MMTV-ErbB2/Fvb* mice were isolated and grafted (1×10^6 cells) into the #2 mammary glands of FVB/N mice subjected to twice weekly intra-tumoral injections of BSA or recombinant RANKL (80 μg/kg), starting one week post-inoculation. At day 35, lungs were isolated, sectioned, H&E stained, and metastatic lesions counted. Shown are means +/− s.e.m. (n=6) as well as values for individual mice. **c**, MT2 cells (1×10^6) were grafted into #2 mammary glands of FVB/N or *MMTV-ErbB2*/*Fvb* mice treated with BSA or RANKL and analyzed for pulmonary metastases as in **a**. Data are presented as incidence and multiplicity of pulmonary metastases (mean +/− s.e.m.; n=7–8). **d**, Mice were inoculated with 1×10^6 shRANK-transduced or control MT2 cells and lung metastases were measured 56 days later. Data are presented as incidence and multiplicity of pulmonary metastases (means +/− s.e.m.; n=6). *: P<0.05; **: P<0.01.

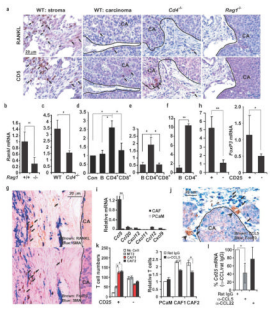

Figure 2. Expression of RANKL in mammary tumors depends on CD4$^+$ T cells

a, Mammary glands of indicated mouse strains were inoculated with 1×10^6 *MMTV-ErbB2* PCaM cells. After 56 days, tumors were isolated, fixed, paraffin embedded and sectioned. Parallel sections were stained with RANKL- and CD5-specific antibodies and counterstained with hematoxylin. Panels show the stroma- and carcinoma-containing regions separated by a line, when relevant. Arrows indicate CD5$^+$ and RANKL$^+$ cells. **b–f,** *Rankl* mRNA expression in tumors. **b**, MT2 cells were transplanted into the #2 mammary glands of indicated mice. After 56 days, tumors were excised and *Rankl* mRNA was quantitated by q-RT-PCR and normalized to *cyclophilin* mRNA. Results are means +/− s.e.m. (n=3). **c**, Freshly-isolated *MMTV-ErbB2* PCaM cells were transplanted as above into indicated mice. After 56 days, tumors were isolated and *Rankl* mRNA was quantified. Results are means +/−s.e.m. (n=3). **d**, MT2 cells were transplanted into mock-, B cell-, CD4$^+$ T cell- or CD8$^+$ T cell-reconstituted *Rag1*$^{-/-}$ mice, 3 days after reconstitution. *Rankl* mRNA in tumors was analyzed as above. Means +/− s.e.m. (n=3). **e**, Spontaneous *MMTV-ErbB2* tumors were dissociated into single cell suspensions. Tumor-infiltrating lymphocytes were enriched by positive selection and *Rankl* mRNA was quantified as above. Means +/− s.e.m. (n=3). **f**, Tumor-infiltrating B cells and CD4$^+$ T cells were purified from MT2 formed tumors and analyzed for *Rankl* mRNA. Means +/− s.e.m. (n=3). *: P<0.05; **: P<0.01. **g,** Parallel sections of tumors raised in WT mice as in **a.** were stained with FoxP3 (brown)-, RANKL (brown)- and SMA (blue)-specific antibodies without counterstaining. Arrows: FoxP3$^+$ and RANKL$^+$ cells. The stroma and carcinoma regions are separated by a line. **h,** Tumor-infiltrating CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells were purified and analyzed for *Rankl* and *FoxP3* mRNA expression as above. Means +/− s.e.m. (n=3). *: P<0.05; **: P<0.01. **i**, Cancer associated fibroblasts (CAF) and PCaM cells were purified from *MMTV-ErbB2* tumors. Chemokine mRNAs were quantified by qRT-PCR as above. Means +/− s.e.m. (n=3). **: P<0.01. **j**, Sectioned *MMTV-ErbB2* tumors were stained with CCL5- (brown) and FoxP3-(blue) specific antibodies. **k**, The indicated cell types were plated onto multiwell plates at 2X10^5 cells per well with 3 μg/ml rat IgG or CCL5 antibody. Splenocytes from tumor-bearing *MMTV-ErbB2* mice were added to the upper compartment of Boyden chambers. After 24 hrs, T cells in the bottom compartment were quantified by flow cytometry. CAF1 and CAF2, two independent preparations. Means +/− s.e.m. (n=3). *, P<0.05. **l,** Rat IgG, CCL5, or CCL22 antibodies (2 mg/kg) were i.p. injected into FVB/N females bearing MT2 tumors twice weekly. After one week, tumors were excised and *Cd25* mRNA was quantified by q-RT-PCR and normalized to *cyclophilin* mRNA. Results are means +/− s.e.m. (n=4). *, P<0.05. CA, carcinoma region.

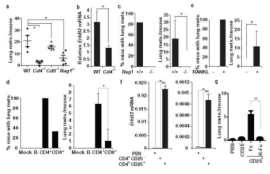

Figure 3. Tumor infiltrating CD4$^+$ T cells stimulate pulmonary metastasis

a–b, Freshly-isolated *MMTV-ErbB2* PCaM cells (1×10^6) were transplanted into the #2 mammary glands of indicated mice. **a**, Lung metastasis incidence and multiplicity were determined 56 days later. Means +/− s.e.m. (n=4–6). Each point represents a value from a single mouse. **b,** Pulmonary metastasis in indicated mice inoculated with PCaM cells was quantified by qRT-PCR analyses of lung RNA with *MMTV-ErbB2* specific primers. Means +/− s.e.m. (n=4). **c**, MT2 cells were transplanted as above. After 56 days, pulmonary metastasis incidence and multiplicity were quantified. Means +/− s.e.m. (n=6). **d**, MT2 cells were transplanted into *Rag1*$^{-/-}$ mice reconstituted with indicated cell types, 3 days after reconstitution. After 35 days, lung metastasis incidence and multiplicity were quantified. Means +/− s.e.m. (n=3). **e,** MT2-inoculated *Rag1*$^{-/-}$ mice were treated with BSA or RANKL (80 μg/kg) twice weekly, starting one week post-inoculation. After 35 days, lung metastasis incidence and multiplicity were determined. Means +/− s.e.m. (n=6–7). **f**, MT2 cells were transplanted into *Rag1*$^{-/-}$ mice reconstituted with indicated cell types as in **d.** Lung metastases were measured by Q-RT-PCR of *ErbB2* mRNA on day 56 post-inoculation. Each plot represents *ErbB2* mRNA expression from an independent littermate group. Means +/− s.e.m. (n=3). **g**, MT2 cells were transplanted into *Rag1*$^{-/-}$ littermate females reconstituted with PBS, CD4$^+$CD25$^-$ or CD4$^+$CD25$^+$ cell, as above. After one week, the CD4$^+$CD25$^+$ reconstituted mice were treated with control Fc or RANK-Fc (2.5 mg/kg) twice weekly. Pulmonary metastasis multiplicity was calculated on day 35 post-inoculation. Means +/− s.e.m. (n=4–5). *: P<0.05; **: P<0.01.

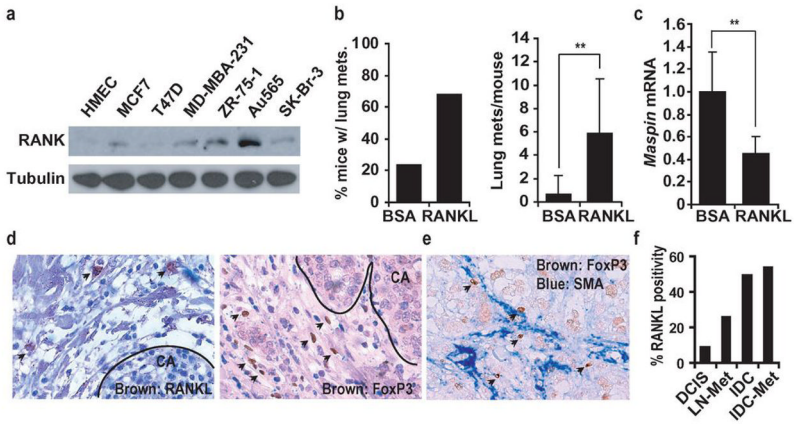

Figure 4. RANKL in human breast cancer

a, RANK expression in human breast cancer cell lines was analyzed by immunoblotting. **b**, ZR-75-1 cells were transplanted into the #2 mammary glands of *Nude* mice. Mice were treated with BSA or RANKL (80 μg/kg) twice weekly, starting one week post-inoculation. After 84 days, lung metastasis incidence and multiplicity were determined. Means +/− s.e.m. (n=6). **c**, *Maspin* mRNA in ZR-75-1-transplanted tumors treated with BSA or RANKL was analyzed by qRT-PCR. Means +/− s.e.m. (n=6). **: P<0.01. **d,** Human breast cancer sections were stained with FoxP3- and RANKL-specific antibodies and counterstained with hematoxylin. Arrows: $FoxP3^+$ and $RANKL^+$ cells. CA, carcinoma region. **e,** Human breast cancer sections were stained with FoxP3- (brown) and SMA- (blue) specific antibodies without counterstaining. **f**, Tissue arrays containing 50 samples each of ductal carcinoma in situ (DCIS), invasive ductal carcinoma without metastasis(IDC), IDC with metastasis (IDC-Met), and lymph node macro-metastasis (LN-Met) were analyzed for RANKL-expressing cells. Samples were considered positive when at least two cells/field (200x) were $RANKL^+$.