

Co-administration of RANKL and CTLA4 Antibodies Enhances Lymphocyte-Mediated Antitumor Immunity in Mice

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Abstract

Purpose: Novel partners for established immune checkpoint inhibitors in the treatment of cancer are needed to address the problems of primary and acquired resistance. The efficacy of combination RANKL and CTLA4 blockade in antitumor immunity has been suggested by recent case reports in melanoma. Here, we provide a rationale for this combination in mouse models of cancer.

Experimental Design: The efficacy and mechanism of a combination of RANKL and CTLA4 blockade was examined by tumor-infiltrating lymphocyte analysis, tumor growth, and metastasis using a variety of neutralizing antibodies and gene-targeted mice.

Results: RANKL blockade improved the efficacy of anti-CTLA4 mAbs against solid tumors and experimental metastases, with regulatory T-cell (Treg)-depleting anti-CTLA4 mAbs of the mouse IgG2a isotype showing greatest combinatorial activity. The opti-

mal combination depended on the presence of activating Fc receptors and lymphocytes (NK cells for metastatic disease and predominantly CD8⁺ T cells for subcutaneous tumor control), whereas anti-RANKL alone did not require FcR. The significantly higher T-cell infiltration into solid tumors post anti-RANKL and anti-CTLA4 was accompanied by increased T-cell effector function (cytokine polyfunctionality), and anti-RANKL activity occurred independently of Treg depletion. The majority of RANKL expression in tumors was on T cells whereas RANK-expressing cells were mostly tumor-associated macrophages (TAM), with some expression also observed on dendritic cells (DC) and myeloid-derived suppressor cells (MDSC).

Conclusions: These results provide a rationale for the further investigation of RANKL-RANK interactions in tumor immunity and a basis for development of translational markers of interest in human clinical trials. *Clin Cancer Res*; 23(19); 5789–801. ©2017 AACR.

Introduction

Immunotherapy has shown great promise in the treatment of certain advanced solid organ malignancies, most prominently in melanoma. Breakthrough trials using ipilimumab (a monoclonal antibody targeting CTLA4, the archetypal T-cell immune checkpoint) demonstrated, for the first time, improved survival in advanced melanoma; recent pooled analysis of long-term survival

data for ipilimumab monotherapy showed a plateau of 21% in the overall survival (OS) curve commencing at about 3 years (1). More recently, adjuvant ipilimumab improved survival in stage 3 resected melanoma (2). Combining anti-CTLA4 with an mAb-targeting another checkpoint, PD-1, produced superior tumor responses and survival benefit in advanced melanoma, demonstrating the importance of combination immunotherapy targeting non-redundant mechanisms of immune evasion by tumors (3–5). However, one challenge in the immunotherapy of solid cancers is the discovery of new targets for patients who display primary resistance to current immunotherapy combinations.

The possible utility of receptor activator of NF-κB ligand (RANKL) pathway blockade as a feasible therapeutic partner for anti-CTLA4 mAbs was suggested by a case report of a patient with rapidly progressive metastatic melanoma with skeletal and extra-skeletal metastasis, who received denosumab (anti-RANKL mAb) for palliation of bony pain, but then experienced an unexpected and rapid near-complete response to single agent ipilimumab (6). This report was accompanied by a preclinical experiment that showed a combination of anti-RANKL and anti-CTLA4 mAbs significantly reduced experimental mouse melanoma lung metastases via natural killer (NK) cells (6). A second case report of complete response following ipilimumab and denosumab in metastatic melanoma followed (7). However, to date the general utility and precise mechanisms of action of anti-RANKL and anti-CTLA4 have remained unclear.

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Translational Relevance

This article illustrates the combinatorial efficacy of co-targeting RANKL and CTLA4 in mouse models of subcutaneous and metastatic cancer, predominantly in melanoma. The results provide insight into potential mechanisms of anti-melanoma efficacy as described in case reports of patients receiving combination ipilimumab and denosumab in advanced melanoma, and suggest translational markers for consideration in the design of future clinical trials of similar combinations.

RANK and RANKL are members of the tumor necrosis factor receptor and ligand superfamilies, respectively, with closest homology to CD40 and CD40L. RANK (TNFRSF11a) and RANKL (TNFSF11) are currently best known in clinical practice for their role in bone homeostasis, as the differentiation of osteoclasts from the monocyte-macrophage lineage requires RANKL interaction with RANK expressed on the myeloid osteoclast precursors (8, 9). However, RANKL was initially identified as a dendritic cell (DC)-specific survival factor, which was upregulated by activated T cells and interacted with RANK on the surface of mature DCs to prevent apoptosis (10, 11). The fully human IgG2 anti-RANKL antibody (denosumab) is widely used in clinical practice as a potent and well-tolerated anti-resorptive agent for the prevention of skeletal-related events arising from bone metastases, and the management of giant cell tumor of bone and osteoporosis (12, 13). Intriguingly, denosumab increased OS in a *post-hoc* exploratory analysis of a phase III trial in patients with non-small cell lung cancer and bone metastases, compared with zoledronic acid (14).

Taken together, the investigation of RANK and RANKL as possible novel immunotherapy targets in cancer is a rational approach. Here we have defined the mechanism of action of RANKL-RANK blockade in combination with anti-CTLA4, and provide insight into the combination efficacy observed in the case reports.

Materials and Methods

Cell culture

Mouse melanoma cell lines B16F10 (ATCC) and LWT1, colon cancer cell line CT26, and prostate carcinoma cell line RM-1 were maintained, injected, and monitored as previously described (15–18). The mouse prostate cancer cell line Tramp-C1 was maintained as described previously (19) but without dehydro-solandrosterone. The mouse fibrosarcoma cell line MCA1956 (derived from a MCA-inoculated C57BL/6 mouse) was kindly provided by Robert Schreiber (Washington University School of Medicine, St Louis, MO). All cell lines were routinely tested negative for *Mycoplasma*, but cell line authentication was not routinely performed.

Mice

C57BL/6 wild-type (WT) mice were bred-in-house or purchased from the Walter and Eliza Hall Institute for Medical Research. C57BL/6 perforin-deficient (*pf^{-/-}*), interferon-deficient (*IFN γ ^{-/-}*), Fc receptor deficient (*Fc γ RIII*, *Fc γ RIV*, and

Fc ϵ R γ), *Batf3* transcription factor-deficient (*Batf3^{-/-}*; ref. 20) and FoxP3-DTR (21) mice were bred in-house at the QIMR Berghofer Medical Research Institute (QIMRB). All mice were used between the ages of 6 to 16 weeks. Groups of 5 to 13 mice per experiment were used for experimental tumor metastasis assays and subcutaneous tumor growth. All experiments were approved by the QIMRB Animal Ethics Committee.

Antibodies

Purified anti-mouse anti-RANKL (IK22-5; rat IgG2a; ref. 22), anti-CTLA4 (UC10-4F10; hamster IgG) and control antibodies (1-1 or 2A3; rat IgG2a) were produced in house or purchased from BioXcell (West Lebanon, NH). Anti-CTLA4 clone 9D9 (various isotypes as indicated), and control antibody (1D12; mouse IgG2a), were supplied by Bristol-Myers Squibb. Recombinant human OPG-Fc (consisting of the ligand binding residues of OPG (residues 22–194) fused to the Fc portion of human IgG1; ref. 23) was supplied by Amgen Inc. Antibodies to deplete NK cells (anti-asialoGM1, Wako) or anti-CD8 β (53.5.8, BioXcell) were administered as indicated.

Subcutaneous tumor models

For B16F10 (1×10^5), RM-1 (5×10^4), CT26 (1×10^5), MCA1956 (1×10^6) and Tramp-C1 (1×10^6) tumor formation, cells were inoculated subcutaneously into the abdominal flank of female (B16F10, MCA1956) or male (RM-1, CT26, Tramp-C1) mice. Therapeutic antibody treatment commenced as indicated on day 3 to 12 after tumor inoculation and was given every 2 to 4 days up to a maximum of 4 doses. Digital callipers were used to measure the perpendicular diameters of the tumors. The tumor size was calculated as the product of the two measurements and is presented as mean \pm SEM.

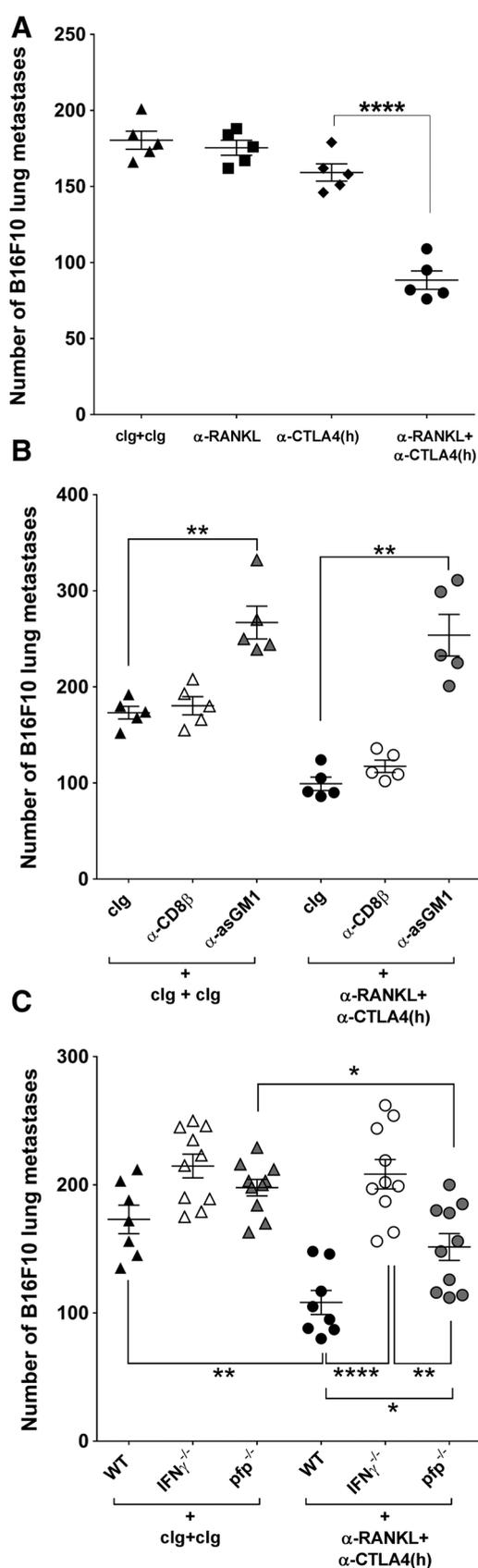
Experimental lung metastasis models

Single-cell suspensions of B16F10 (2×10^5), RM-1 (1×10^5), or LWT1 (7.5×10^5) were injected intravenously into the tail vein of the indicated strains of mice. Lungs were harvested on day 14, and surface tumor nodules were counted under a dissection microscope. Antibody treatments were as indicated, with anti-CTLA4 and/or anti-RANKL mAbs administered on days -1, 0, and 2 relative to tumor inoculation. Antibodies to deplete CD8⁺ T cells or NK cells were administered where indicated on days -1, 0, and 7 relative to tumor inoculation.

Flow cytometry

Tumor-bearing mice were sacrificed at two time points: day 9, or at end-point (when the experiment was terminated due to tumors reaching ethical endpoint size). Tumor and spleen were collected and wet weight was recorded. Single-cell suspensions were generated from indicated organs as previously described (21).

The following antibodies (from Biolegend, eBioscience, BD Biosciences) were used: CD4-BV605 (RM4-5), CD8-BV711 (53-6.7), CD11b-BV650 (M1/70), CD11b-PE (M1/70), CD11c-PE (N418), purified CD16.2 (9E9) followed by goat-anti-hamster FITC, CD206-AF647 and CD206-PECy7 (C068C2), and Zombie Aqua live/dead dye; TCR β -PerCP-Cy5.5 (H57-597), CD45.2-A780 (104), Ly6C/Ly6G (GR-1)-EF450 (RB6-8C5), MHCII-APC (M5/114.15.2), CD265 (RANK)-PE (R12-31); RANKL-AF647 (IK22-5). For intracellular cytokine staining (ICS), cells were stimulated for 4 hours with Cell Stimulation Cocktail (1/1,000;



eBioscience). Cells were then surface stained as described above before being fixed/permeabilized with Cytofix/Cytoperm (BD Biosciences) and stained with IFN γ -AF488 (Biolegend), TNF α -PE (BD Biosciences), and IL-2-Pacific Blue (Biolegend).

For intracellular transcription factor staining, cells were surface stained as described above before being fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience), according to the manufacturer's protocol and stained with FoxP3-EF450 or FoxP3-AF488 (FJK-16s) and Ki67-EF450 (Sol185; eBioscience). All immune cell analysis was first gated on live, single CD45.2⁺. T cells were defined as TCR β ⁺NK1.1⁻. NK cells were defined as TCR β ⁻NK1.1⁺. DCs were defined as CD11c⁺MHCII^{high} cells. Tumor-associated macrophages (TAM) were defined as CD11b⁺F4/80⁺ non-DC cells. MDSCs were defined as CD11b⁺, Ly6C/Ly6G (GR-1)^{hi}, non-TAM, non-DC cells. To determine absolute counts in samples, liquid-counting beads (BD Biosciences) were added immediately before samples were run on a flow cytometer. All data were collected on a Fortessa 4 (BD Biosciences) flow cytometer and analyzed with FlowJo v10 software (Tree Star, Inc.).

Statistical analysis

GraphPad Prism and R software was used for statistical analysis. For column analyses, the Brown-Forsythe test was used to assess equal variances. If non-significant, one-way ANOVA with multiple comparisons was used. In the event of unequal variances between groups, Kruskal-Wallis analysis with Sidak's or Dunnett's multiple comparisons were employed as appropriate. For longitudinal tumor growth analysis, treatment group random effects models were used, for within-experiment mice only. Data were considered to be statistically significant where the *P* value was less than 0.05.

Results

Suppression of lung metastasis by CTLA4 and RANKL co-blockade depends upon NK cells and IFN γ

In mice bearing experimental B16F10 melanoma lung metastases, wild-type (WT) mice treated with the combination of hamster anti-CTLA4 (UC10-4F10) and rat anti-RANKL (IK22-5) mAbs showed superior resistance to metastases compared with mice treated with either antibody alone or control immunoglobulin (cIg; Fig. 1A). The mechanism of action of anti-CTLA4 and anti-RANKL combination therapy was determined in WT mice depleted of CD8⁺ or NK cells or mice deficient for

Figure 1. Suppression of experimental lung metastases by combination anti-CTLA4 and anti-RANKL is NK cell and IFN γ dependent. **A-C**, C57BL/6 wild-type (WT) or gene-targeted mice (as indicated) were injected intravenously with B16F10 melanoma cells (2×10^5), and metastatic burden was quantified in the lungs after 14 days by counting colonies on the lung surface. Mice were treated on day -1, 0, and 2 (relative to tumor inoculation) with cIg, anti-CTLA4 (UC10-4F10, hamster IgG, 200 μ g i.p.) and/or anti-RANKL (IK22-5, 200 μ g i.p.). Some groups of mice were additionally treated on days -1, 0, and 7 with anti-CD8 β or anti-asGM1 (100 μ g/mouse i.p.) to deplete CD8⁺ T cells or NK cells where indicated. Groups of 5 to 10 mice with mean \pm SEM of each group are shown. Experiments were performed once. Statistical significance as indicated was determined by one way ANOVA and Tukey's multiple comparisons (*, *P* < 0.05; **, *P* < 0.01; ****, *P* < 0.0001).

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perforin or IFN γ . As shown in Fig. 1B, the efficacy of the combination relied on the presence of NK cells but not CD8⁺T cells; and IFN γ was critical and to a lesser extent perforin (Fig. 1C). A similar dependence on NK cells was demonstrated for the effective control of prostate carcinoma RM-1 experimental lung metastases following treatment the same with anti-CTLA4 and anti-RANKL combination therapy (Supplementary Fig. S1A).

Anti-RANKL optimally synergises with anti-CTLA4 antibodies of the IgG2a isotype

Given that the immunoglobulin constant region of anti-CTLA4 has been reported to influence antitumor activity (24), we next assessed the impact of how different anti-CTLA4 antibody isotypes synergized with anti-RANKL in suppressing experimental B16F10 lung metastases (Fig. 2). The anti-CTLA4 clone 9D9 has been produced as a number of isotypes, including mouse IgG1, IgG2a and IgG2b; whereas another isotype (IgG1-D265A) contained a mutation that eliminated binding to all Fc γ receptors (Fc γ R; ref. 24). As shown in Fig. 2A, the IgG2a isotype of the anti-CTLA4 (anti-CTLA4; mIgG2a) alone resulted in greater suppression of lung metastases compared with the hamster clone of anti-CTLA4 (anti-CTLA4; h), and this suppression was further increased with the addition of anti-RANKL to either anti-CTLA4 clones. Similarly, significant suppression of RM-1 and LWT1 lung metastases were also seen with anti-CTLA4 (mIgG2a) and anti-RANKL combination therapy (Supplementary Fig. S1B and S1C).

Interestingly, the other three anti-CTLA4 isotypes (IgG2b, IgG1, or IgG1-D265A) were not as effective in suppressing lung metastases as monotherapy compared with the anti-CTLA4 (mIgG2a) isotype as they did not result in significant suppression of metastases compared with the clg treated group (Fig. 2B). However, the addition of anti-RANKL to anti-CTLA4 (h) or anti-CTLA4 (mIgG2a and mIgG2b) resulted in significant suppression of lung metastases compared with clg. Nevertheless, the group treated with anti-RANKL and the anti-CTLA4 (mIgG2a) clone was significantly superior to the combination anti-RANKL with anti-CTLA4 (mIgG2b; Fig. 2B). Overall, anti-RANKL treatment alone did not significantly suppress metastasis, although it significantly improved the control of metastases when used in combination with specific anti-CTLA4 isotypes, most notably mIgG2a (Fig. 2A and B).

Anti-RANKL and anti-CTLA4 suppress subcutaneous tumor growth

Given these results in experimental metastasis models, the efficacy of co-administration of anti-RANKL and anti-CTLA4 mAbs in mice bearing subcutaneous tumors was next assessed (Fig. 3). Similar to the lung metastasis models, optimal B16F10 subcutaneous growth suppression by combination anti-CTLA4 and anti-RANKL was again dependent on antibody isotype, with significant suppression of growth observed with anti-CTLA4 (mIgG2a; Fig. 3A), rather than the hamster isotype (Supplementary Fig. S2A). Antitumor efficacy was also confirmed in additional subcutaneous tumor models, mouse colon cancer CT26 (Fig. 3B) and mouse prostate carcinoma Tramp-C1 (Supplementary Fig. S2B). We next performed a longitudinal analysis of seven independent pooled experiments using B16F10 comparing anti-CTLA4 (mIgG2a) or the Fc γ R-non-engaging clone of anti-CTLA4 (IgG1-D265A) and/or anti-RANKL with control Ig (Supplementary Fig. S2C). Overall our

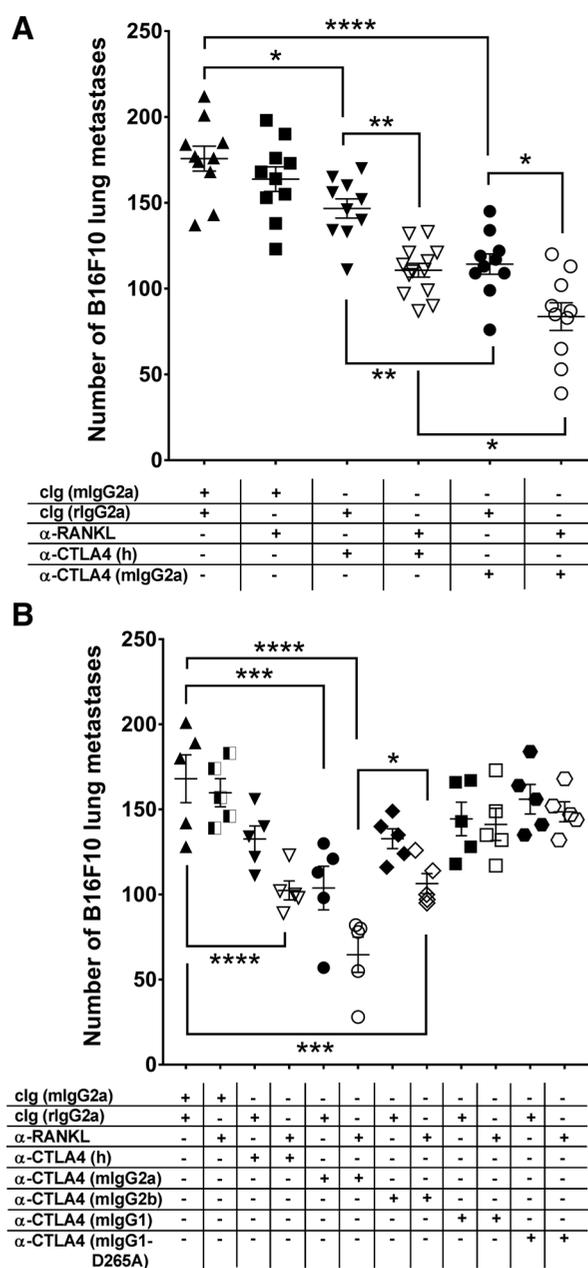


Figure 2. Isotype of anti-CTLA4 affects combinatorial efficacy with anti-RANKL to suppress experimental lung metastases. Groups of C57BL/6 wild-type (WT) mice were injected intravenously with B16F10 melanoma cells (2×10^5) as indicated, and metastatic burden was quantified in the lungs after 14 days by counting colonies on the lung surface (A and B). Mice were treated on day -1, 0, and 2 (relative to tumor inoculation) with clg (1D12, mouse IgG2a), various isotypes of anti-CTLA4 [UC10-4F10 (hamster IgG) or 9D9 (mouse IgG2a, IgG2b, IgG1 or IgG1 D265A), 200 μ g i.p.] and/or clg (2A3, rat IgG2a) or anti-RANKL (IK22-5, 200 μ g/mouse i.p.) as indicated. Means \pm SEM of 5 to 8 mice per group are shown. (A) is a pooled result from two independent experiments, whereas in (B) experiments were performed once. Statistical significance was determined by one way ANOVA, with (A) Tukey's multiple comparisons or (B) Sidak's multiple comparisons where monotherapy anti-CTLA4 is compared with clg, and with combination with anti-RANKL, or combination with clg (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

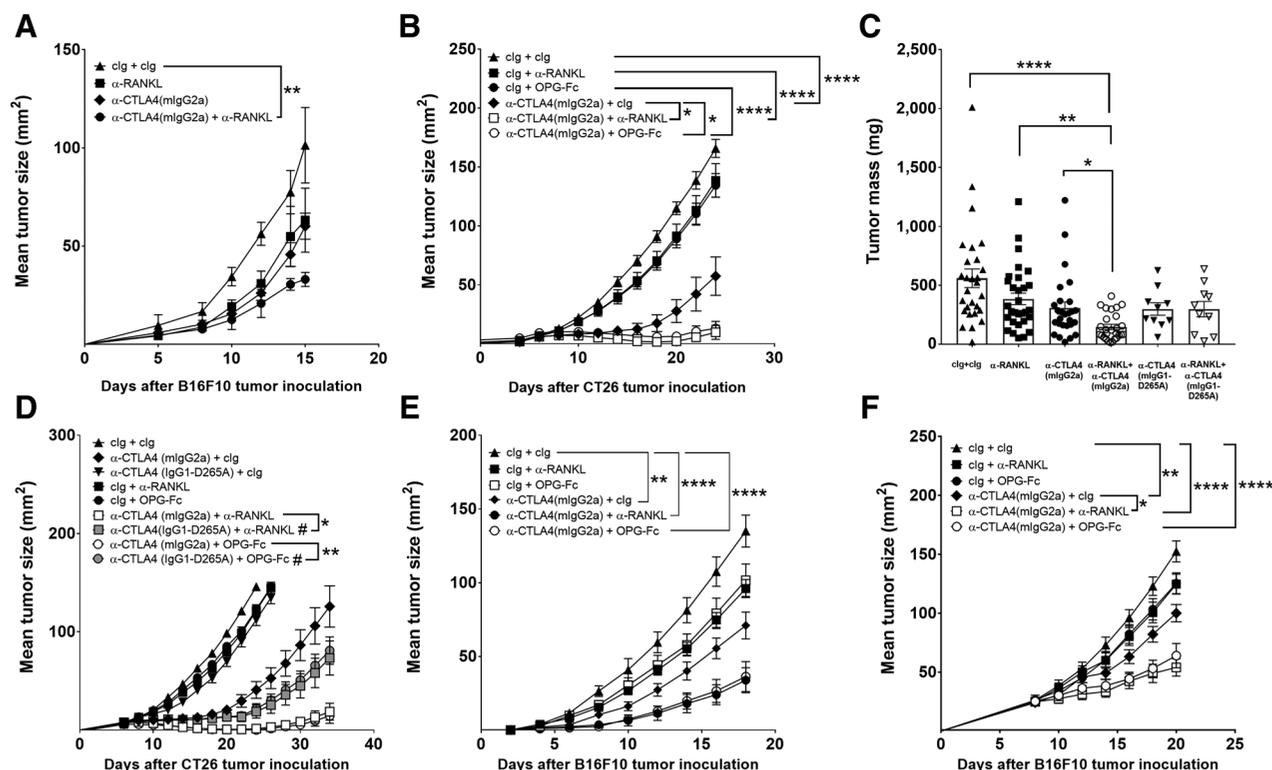


Figure 3.

The IgG2a isotype of anti-CTLA4 combines most effectively with anti-RANKL to suppress subcutaneous tumor growth. Groups of C57BL/6 wild-type (WT) mice were injected subcutaneously with (A, C, E, and F) B16F10 melanoma cells (1×10^5) or (B and D) CT26 colon cancer cells (1×10^5) and tumor growth was measured for different treatment groups. Mice were treated on (A, C, and E) days 3, 7, 11, and 15 (B and D) days 6, 9, 12, and 15, or (F) days 9, 13, 17 (relative to tumor inoculation) with clg (1-1, rat IgG2a, 200 μ g i.p.); 1D12, mouse IgG2a, 50 μ g i.p.), anti-CTLA4 (9D9, mouse IgG2a or IgG1-D265A, 50 μ g i.p.) and/or anti-RANKL (IK22-5, 200 μ g i.p.) as indicated. Some additional groups of mice were treated i.p. with OPG-Fc (60 μ g/mouse) on (B and D) days 6, 9, 12, and 15 (E) days 3, 6, 10, 13, and 16, or (F) days 9, 12, 16, and 19. B16F10 subcutaneous tumor growth (A, E, and F) or CT26 (B and D) subcutaneous tumor growth (displayed as means \pm SEM) of 5–9 mice per group are shown. (A) is a representative growth curve from seven independent experiments, otherwise experiments were performed once. Statistical significance was demonstrated by one way ANOVA, Tukey's multiple comparisons, of tumor size on final day of measurement (including D, for combination arms only remaining at day 34; *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$). In D, # indicates significance for the following comparisons: anti-CTLA4 (mIgG1-D265A) + anti-RANKL compared with either monotherapy, and anti-CTLA4 (mIgG1-D265A) + OPG-Fc compared with either monotherapy, at day 26. C, For six pooled experiments conducted as in (A), but not including the experiment shown in (A), wet tumor masses were recorded after resection from flank. Differences between combination anti-CTLA4 (mIgG2a) and anti-RANKL, either as monotherapy, or clg were significant as indicated (Kruskal-Wallis test, Dunn's multiple comparisons, in which monotherapy arms or clg were compared with combination anti-CTLA4 (mIgG2a) and anti-RANKL; *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$).

data demonstrated the combination of anti-CTLA4 (mIgG2a) with anti-RANKL significantly suppressed tumor growth and resulted in superior efficacy compared with either as monotherapy or with clg (Supplementary Fig. S2C). By contrast, the combination of anti-CTLA4 (IgG1-D265A) and anti-RANKL was superior to clg-treated groups, but combinatorial therapy was not superior to either treatment as a monotherapy (Supplementary Fig. S2C). Similarly, although B16F10 tumor mass at endpoint of mice treated with combination anti-RANKL with anti-CTLA4 (mIgG2a) was also significantly decreased compared with the respective monotherapy-treated groups, there was no additive benefit of adding anti-RANKL to anti-CTLA4 (IgG1-D265A; Fig. 3C).

To confirm these findings, anti-CTLA4 mIgG2a and IgG1-D265A isotypes were assessed in the treatment of subcutaneous CT26 tumors in combination with two different therapies targeting RANKL: anti-RANKL mAb (IK22-5) or the well-validated RANKL blocker OPG-Fc (23). Anti-CTLA4 (mIgG2a) was the

significantly more effective isotype in suppressing tumor growth in combination with either RANKL-directed therapy (Fig. 3D), suggesting that FcR engagement by the anti-CTLA4 mAb was required for optimal combination therapy. Similar results were found in the treatment of fibrosarcoma MCA1956 (Supplementary Fig. S2D). OPG-Fc demonstrated similar efficacy as anti-RANKL mAb when used in combination with anti-CTLA4 (mIgG2a) in CT26 (Fig. 3D) as well as B16F10 subcutaneous tumors (Fig. 3E), confirming efficacy of different RANKL-directed therapies. When treatment was commenced at a later timepoint (with established B16F10 tumors), the combinatorial efficacy of anti-CTLA4 (mIgG2a) with anti-RANKL or OPG-Fc was still observed (Fig. 3F). Despite the optimal effect of anti-CTLA4 (mIgG2a) and anti-RANKL, in the more sensitive CT26 and MCA1956 tumor models, it was obvious that co-blockade using anti-CTLA4 (IgG1-D265A) and anti-RANKL was also more effective than either monotherapy (Fig. 3D, Supplementary Fig. S2D). These data suggested that anti-RANKL provides additional

antitumor benefit to either Treg-depleting anti-CTLA-4 (IgG2a) or blocking only anti-CTLA-4 (IgG1-D265A) in the CT26 and MCA1956 models.

RANKL and RANK expression in the tumor microenvironment

Expression of RANKL and RANK in the B16F10 tumor microenvironment (TME) was next defined (Fig. 4A and B). The majority of intratumor RANKL was expressed by a small fraction of T cells, with a higher proportion expressing RANKL at an earlier timepoint (day 9) and higher in tumor than in spleen, with more CD8⁺ T cells compared to CD4⁺ T cells expressing RANKL in the tumor (Fig. 4A, Supplementary Fig. S3A). Overall, about 20% of tumor-infiltrating leukocytes (TIL) expressed RANK with greater than 90% of those also staining positive for CD11b, suggesting intratumor RANK was expressed almost exclusively by tumor-infiltrating myeloid cells (Supplementary Fig. S3B). Approximately 40% of tumor-infiltrating macrophages (TAM), 60% of MDSCs, and a low proportion of DCs (7%), expressed RANK (Fig. 4B, Supplementary Fig. S3C). Treatment with anti-RANKL did not significantly alter myeloid RANK expression on these cell types (Fig. 4B). Recently, it was reported in a B16 melanoma model that Ly6C^{low}MHCII^{high} intratumor macrophages had an RNA expression profile consistent with an inflammatory M1 subtype, whereas those with MHCII^{low/negative} expression were more consistent with an immunosuppressive M2 phenotype (25). Interestingly, in our B16F10 model, a higher proportion of Ly6C/Ly6G (GR-1)^{low} TAMs expressing RANK had negative or low MHCII expression compared with those not expressing RANK, suggesting that the RANK-expressing TAM population may be more suppressive than those TAMs not expressing RANK (Supplementary Fig. S3D and S3E). Less than 1% of RANKL- or RANK-expressing cells are CD45.2 negative (indicating a negligible level of intratumor expression of either *in vivo*), and additionally all tumor cell lines used in this study when assessed by flow cytometry were negative for RANKL or RANK expression. Thus, RANKL and RANK expression was largely restricted to tumor-infiltrating lymphocytes and myeloid cells in the TME.

Antitumor efficacy of anti-RANKL and anti-CTLA4 (mIgG2a) combination therapy is FcγRIV receptor, IFNγ, and CD8⁺ T-cell dependent

Next, to continue dissecting mechanism of action of the combination, we assessed the reliance of the combinatorial efficacy of anti-RANKL with anti-CTLA4 (mIgG2a) on Fc receptors and the presence and function of effector lymphocytes in the subcutaneous B16F10 tumor model (Fig. 4C). Combination activity of anti-CTLA4 (mIgG2a) and anti-RANKL against B16F10 was abrogated in mice lacking FcγRIV or FcεRγ, but not FcγRIII (Fig. 4C). This is consistent with the described mechanism of action of anti-CTLA4 (mIgG2a), which requires FcγRIV (24, 26). To determine whether Fc receptor engagement is necessary for anti-RANKL treatment alone, and because an Fc-mutant of anti-RANKL was not available, we assessed treatment effect of anti-RANKL in the immunogenic fibrosarcoma MCA1956, where anti-RANKL monotherapy resulted in significant subcutaneous growth suppression. This efficacy was preserved in mice lacking activating FcR (Supplementary Fig. S4A), demonstrating that FcR engagement was not essential for the antitumor activity of RANKL blockade. Next, the role of CD8⁺ T cells and NK cells in the control of B16F10 tumor growth

by anti-CTLA4 (mIgG2a) and anti-RANKL was assessed by the selective depletion of each subset (Fig. 4D). When CD8⁺ T cells were depleted, the antitumor efficacy of the combination therapy was almost completely abrogated. By contrast NK-cell depletion was without effect, demonstrating the reliance of this combination therapy on CD8⁺ T cells (Fig. 4D). Similar to results observed in the metastatic setting, this combination therapy was IFNγ dependent, but does not require perforin (Fig. 4E). The essential role for cross-presenting CD8α⁺ conventional DCs in this combination therapy was also revealed by the use of mice deficient in the transcription factor *Batf3*; the efficacy of the combination therapy was abrogated in these mice compared with WT-treated mice (Fig. 4F). Similarly, combination efficacy against subcutaneous Tramp-C1 was abrogated in *Batf3*^{-/-} mice (Supplementary Fig. S4B).

CD8⁺ T-cell influx into tumor post co-administration of anti-CTLA4 and anti-RANKL

To further understand the mechanism of the combination therapy and the role of CD8⁺ T cells, the composition of TILs was assessed in subcutaneous B16F10 tumors that had been treated with the optimal combination therapy of anti-CTLA4 (IgG2a) and anti-RANKL (Fig. 5). When assessed at tumor endpoint, the proportion of CD45.2⁺ TILs was significantly increased in the combination therapy compared with cIg- or monotherapy-treated groups (Fig. 5A). The increase in CD45.2⁺ TILs in the combination therapy-treated group was largely accounted for by a marked increase in CD8⁺ T cells, both in proportion (Fig. 5B) and absolute numbers (Fig. 5C).

Given the superiority of anti-CTLA4 (mIgG2a) as a partner to RANKL blockade, we next assessed the level of Tregs in the TME, as anti-CTLA4 (mIgG2a) reportedly depletes tumor-infiltrating Tregs via ADCC (24). Interestingly, although the proportion of Tregs (CD4⁺Foxp3⁺) as a percentage of CD4⁺ T cells in the tumor was indeed reduced with anti-CTLA4 (mIgG2a) monotherapy, it was not further reduced with the addition of anti-RANKL antibody (Fig. 5D). In addition, the FcγR-IV expression on CD11b⁺ cells was not further increased with the combination therapy in the tumor (Supplementary Fig. S5A), suggesting that enhanced Treg depletion in the TME does not explain the mechanism of action of this combination. In the spleen, no significant changes in Treg proportion or number were detected between treatment groups, indicating that Treg levels were not altered systemically with the combination (Supplementary Fig. S5B).

To further assess any essential role for Tregs in combinatorial efficacy of anti-RANKL in immunotherapy, the FoxP3-DTR mouse model was employed. In these mice, the diphtheria toxin receptor (DTR) is expressed under the control of the *foxp3* locus, allowing for the conditional and near-complete depletion of Tregs through administration of diphtheria toxin (DT), resulting in enhanced anti-tumor immunity (21). A trend to greater B16F10 subcutaneous tumor growth suppression was seen (Supplementary Fig. S5C and S5D) and a higher proportion of mice were cured when anti-RANKL therapy was given in combination with DT compared with DT alone (Supplementary Fig. S5E). In addition, a similar trend of enhanced growth suppression of subcutaneous RM-1 was also seen in FoxP3-DTR mice treated with DT and anti-RANKL (Supplementary Fig. S5F). FACS analysis of RM-1 tumors at endpoint revealed near-complete Treg depletion by DT alone, with no additional depletion of Tregs noted when anti-RANKL was

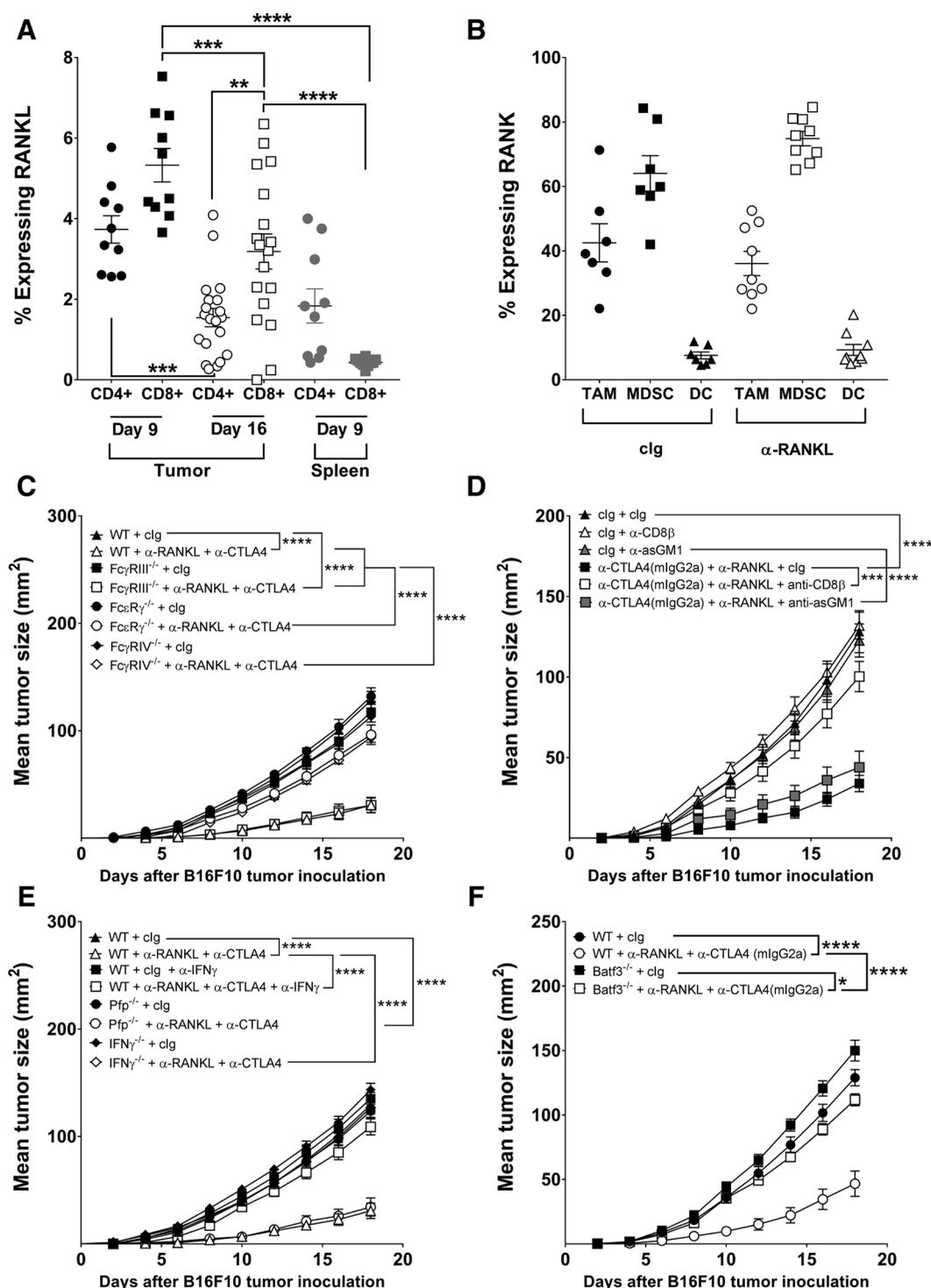
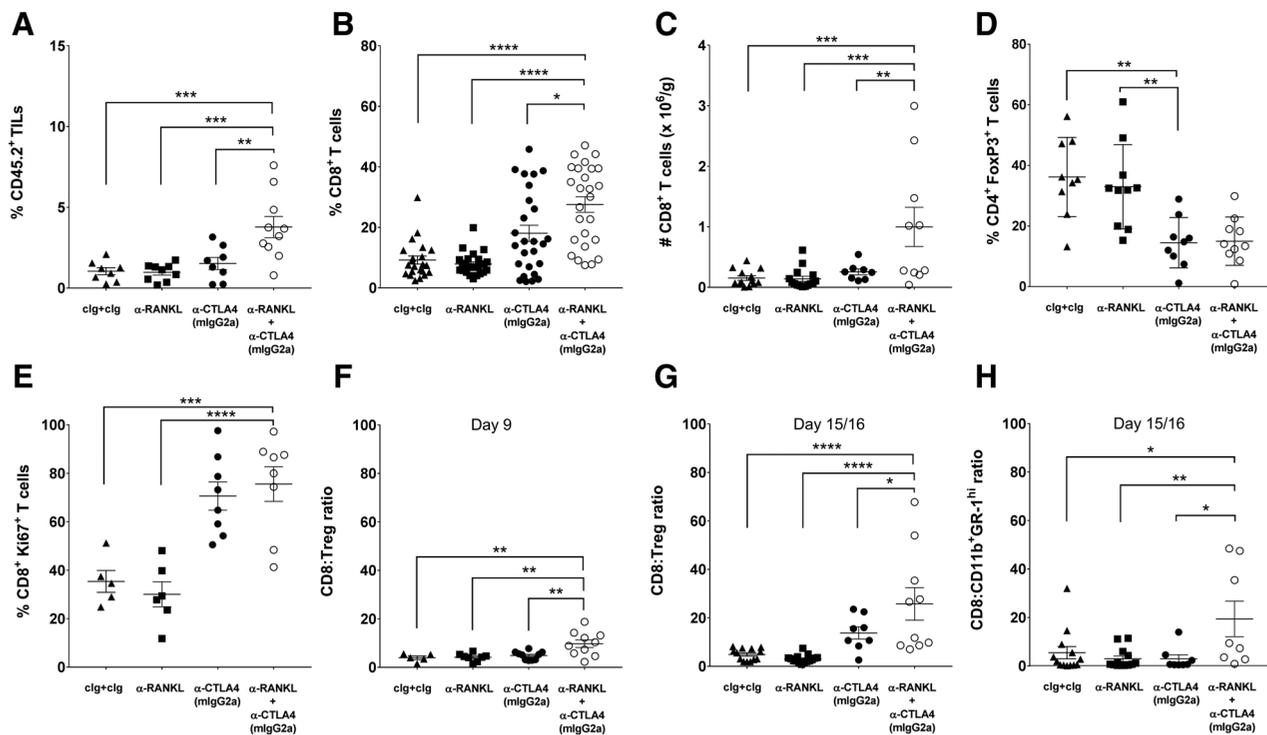


Figure 4.

Efficacy of anti-CTLA4 (mIgG2a) and anti-RANKL combination therapy is FcR-, IFN γ -, and CD8 $^{+}$ T-cell-dependent. Groups of C57BL/6 wild-type (WT) or gene-targeted mice as indicated were injected subcutaneously with B16F10 melanoma cells (1×10^5), and tumors were harvested for FACS at (A) indicated time points or (B) day 16 after tumor inoculation, or (C-F) tumor growth was monitored under different treatment conditions. Mice were treated on day 3, 7, and if experiment ongoing also day 11 and 15 relative to tumor inoculation with clg (1-1; rat IgG2a, 200 μ g i.p. + 1D12; mouse IgG2a, 50 μ g i.p.), anti-CTLA4 (9D9; mouse IgG2a or IgG1-D265A as indicated, 50 μ g i.p.) and/or anti-RANKL (IK22-5, 200 μ g i.p.) as indicated. Some mice were additionally treated intraperitoneally on days -1, 0, and 7 with anti-CD8 β or anti-asGM1 (100 μ g/mouse i.p.) as indicated. (A) Expression of RANKL by T cells, (B) expression of RANK by myeloid subsets indicated, and (C-F) B16F10 subcutaneous tumor growth (displayed as mean \pm SEM) are shown for 3 to 9 mice per group. Two independent experiments, each with 3 to 10 mice per group, are combined in each of (A-B); otherwise experiments were performed once. Differences between groups is significant where indicated as determined by one way ANOVA, Tukey's multiple comparisons (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$). The WT groups treated with clg and combination α -CTLA4 with α -RANKL are the same in (C) and (E) but are displayed in different graphs for ease of interpretation.

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**Figure 5.**

Combined anti-RANKL and anti-CTLA4 therapy results in increased recruitment of CD8⁺ T cells into tumors. Groups of C57BL/6 wild-type (WT) mice were injected subcutaneously with B16F10 melanoma cells (1×10^5) and tumors were harvested for FACS after various treatments. Mice were treated on (A–E, G–H) days 3, 7, and 11 and 15 or (F) days 3 and 7 relative to tumor inoculation with cIg (1-1; rat IgG2a, 200 μg i.p. + 1D12; mouse IgG2a, 50 μg i.p.), anti-CTLA4 (9D9; mouse IgG2a, 50 μg i.p.) and/or anti-RANKL (IK22-5; rat IgG2a, 200 μg i.p.) as indicated. Mice were sacrificed at (A–E, G–H) end-stage relative to ethical end-point for size (day 15 or 16) or (F) day 9, and tumors processed for FACS analysis. (A) CD45⁺ TILs proportion of total live cells, (B) CD8⁺ T cells as a proportion of total CD45⁺ TILs, (C) number of intratumor CD8⁺ T cells, (D) Tregs (defined as TCRβ⁺CD4⁺FoxP3⁺) as a proportion of CD4⁺ T cells, (E) proportion of CD8⁺ T cells expressing Ki-67⁺, ratio of CD8⁺ T cells to Tregs at (F) day 9 and (G) day 15 to 16, and (H) ratio of CD8⁺ T cells to MDSCs for 4 to 8 mice per group are shown. Data are pooled from 2 to 5 independent experiments (A–H). Statistical significance where indicated was determined by (A–C, E–H) one-way ANOVA, Dunnett's multiple comparisons, or (B) the Kruskal-Wallis test, Dunn's multiple comparisons, where each group was compared with anti-CTLA4 (mIgG2a) + anti-RANKL combination therapy (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

combined with DT (Supplementary Fig. S5G). Taken together, the mechanism of action of the combination therapies in these models does not appear to be due to more efficient Treg depletion, and is intact in settings of near-complete intratumoral Treg depletion.

Another potential mechanism of action of anti-RANKL could be the enhancement of T-cell proliferation. However, we did not observe any further increase in Ki-67 expressing CD8⁺ T cells in the combination treated groups compared to anti-CTLA4 monotherapy (Fig. 5E). This suggests that the additional CD8⁺ T cells observed in the tumor post combination treatment might be a result of selective CD8⁺ T cell recruitment. No significant changes were noted in proportions or numbers of myeloid subsets apart from a mild decrease in the proportion of CD11b⁺ cells as a fraction of total TILs with combination therapy, which likely is compensatory for the increased proportion of CD8⁺ T cells (Supplementary Fig. S6A–S6G). In addition, the proportion of TAMs expressing CD206 (an M2 marker) was not altered in either B16F10 or RM-1 subcutaneous tumors (Supplementary Fig. S6H and S6I). Influx of CD8⁺ T cells with the combination therapy, combined with a lack of increase in suppressive immune cells such as Tregs or myeloid cells, may change the TME to favor antitumor

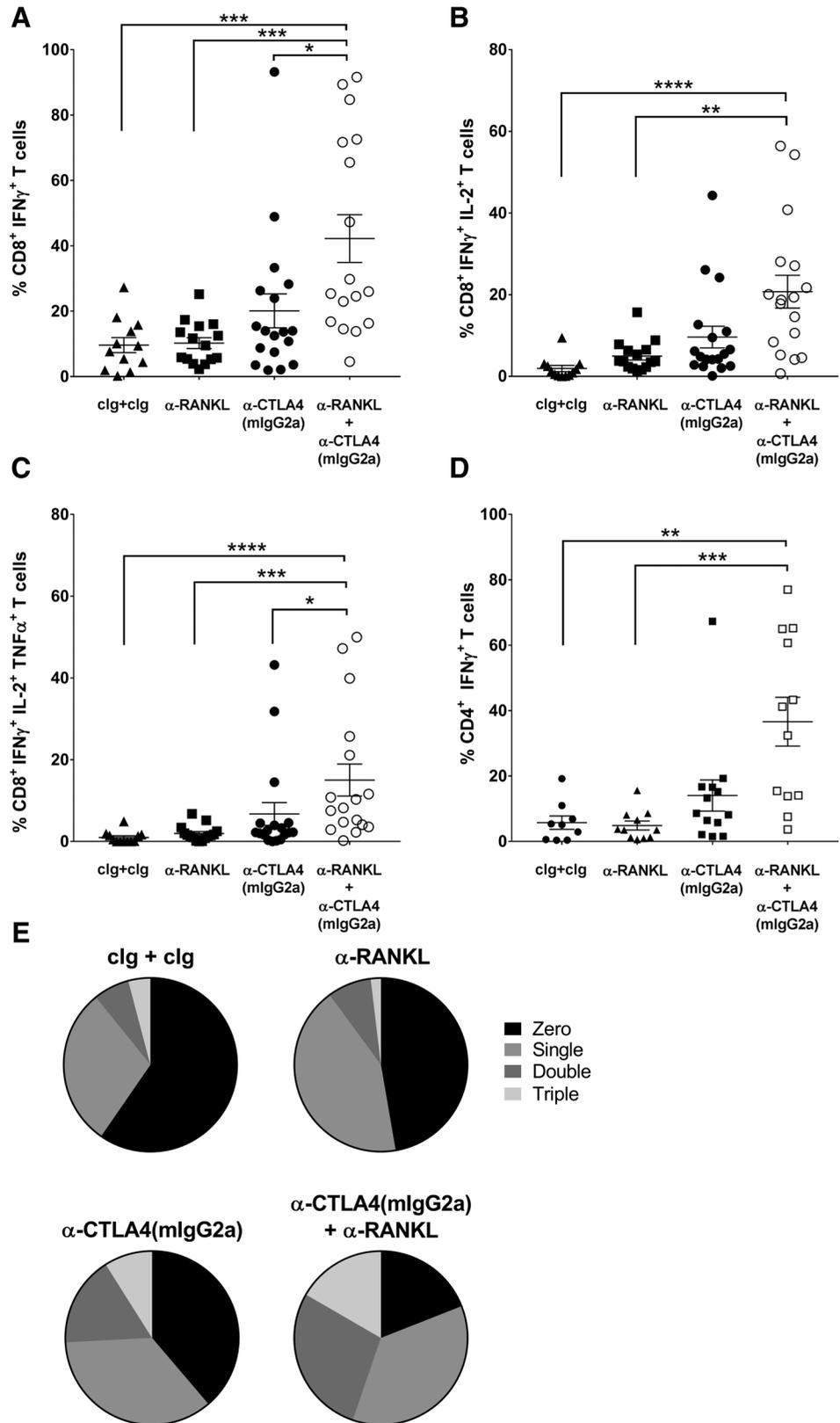
activity. Indeed, significant increase in CD8⁺-to-Treg ratio were noted when measured at an early time-point (day 9; Fig. 5F) or at tumor end-point (Fig. 5G). In addition, the ratio of CD8⁺ T cells to MDSCs was also significantly increased with the combination therapy (Fig. 5H). Importantly, these observed changes were specific for the TME as no significant changes in the proportions of leukocyte subsets were observed in the spleen of these tumor-bearing mice, such as T cells (Supplementary Fig. S6J and S6K).

Anti-RANKL and anti-CTLA4 therapy increases T-cell cytokine production and polyfunctionality

Finally, given that the main changes in TME composition related to the T-cell compartment, we also assessed how this combination affected Th1 cytokine production (IFNγ, TNFα, and IL-2) from CD8⁺ and CD4⁺ T cells in B16F10 tumor at experimental endpoint (day 16; Fig. 6). TNFα was the most commonly produced cytokine *ex vivo* after stimulation, but significant differences were noted in the production of IFNγ by CD8⁺ T cells (Fig. 6A) following combination therapy compared to cIg or monotherapy alone. Furthermore, CD8⁺ T cells co-expressing IFNγ and IL-2 (Fig. 6B) or IFNγ, IL-2 and TNFα (Fig. 6C) was also increased with the combination therapy.

Figure 6.

Anti-RANKL improves the efficacy of anti-CTLA4 by increasing T-cell cytokine polyfunctionality. Groups of C57BL/6 wild-type (WT) mice were injected subcutaneously with B16F10 melanoma cells (1×10^5); mice were sacrificed on day 16 relative to tumor inoculation, and tumors were processed and stimulated *ex vivo* before ICS was performed for expression of cytokines. Mice were treated on days 3, 7, 11, and 15 relative to tumor inoculation with clg (1-1; rat IgG2a, 200 μ g i.p. + 1D12; mouse IgG2a, 50 μ g i.p.), anti-CTLA4 (9D9; mouse IgG2a, 50 μ g i.p.) and/or anti-RANKL (IK22-5; rat IgG2a, 200 μ g i.p.) as indicated. Proportion of CD8⁺ T cells with positive cytoplasmic staining for (A) IFN γ , (B) IFN γ and IL-2 or (C) IFN γ , IL-2 and TNF α co-expression; and (D) proportion of CD4⁺ T cells expressing IFN γ following the indicated therapy is shown for 4–8 mice per group. Two to three pooled independent experiments are displayed in (A–D). **E**, Mean proportion of CD8⁺ T-cell-expressing zero, one, two, or three cytokines (of IFN γ , IL-2, and TNF α) from two pooled experiments is shown for each of four treatment groups as indicated. Statistical significance where indicated was determined by the Kruskal–Wallis test, Dunn’s multiple comparisons where each group is compared with combination anti-CTLA4 (mIgG2a) + anti-RANKL treatment (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).



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Similar findings were also seen with CD4⁺ T cells, particularly in the proportion that produced IFN γ (Fig. 6D). The majority of CD8⁺ T-cell from the clg-treated group produced no cytokines after stimulation, whereas the combination therapy generated T cells with the most polyfunctionality, with monotherapy treatment groups displaying intermediate phenotypes (Fig. 6E and Supplementary Fig. S7). The effect of the combination therapy on cytokine polyfunctionality was TME-specific, as these differences were not observed in the splenic T cells of tumor-bearing mice (Supplementary Fig. S8).

Discussion

Longer-term OS data for key immunotherapy trials in advanced melanoma are now maturing, showing encouraging results but with significant scope for improvement. Subsequent trial experience and pre-clinical studies have underlined the importance of a combinatorial approach, with superior tumor responses and survival (at 2 years) reported with combination nivolumab and ipilimumab compared with ipilimumab alone which is likely to translate into longer-term survival benefit although this data is awaited (27). A priority now is to improve combination immunotherapy to overcome primary and acquired resistance to existing therapies; experimental strategies using novel partners in combination with either ipilimumab or mAbs targeting the PD-1/PD-L1 axis have reached the clinical trial stage (28–30). In this study, we have presented data indicating the combinatorial efficacy of anti-CTLA4 with molecules blocking RANKL–RANK interactions in metastatic and subcutaneous models, and demonstrated its mechanism of action is due to increased tumor-localized CD8⁺ T cells and enhanced effector function.

The full efficacy of CTLA4 blockade, at least in mice, relies on depletion of Tregs in addition to blocking the interaction of CTLA4 on effector T cells (Teff) with B7 ligands, which results in negative regulation of their function (31). We have shown that the optimal combinatorial efficacy of the anti-RANKL and anti-CTLA4 combination is achieved when using the anti-CTLA4 (mIgG2a) isotype. This isotype was previously demonstrated to selectively deplete intratumoral Tregs via antibody-dependent cell-mediated cytotoxicity (ADCC) involving upregulation of Fc γ RIV expression on CD11b⁺ TILs, thereby enhancing antitumor immunity (24, 26). The requirement for activating Fc γ Rs has previously been established in mice for another anti-CTLA4 isotype (9D9 mIgG2b; ref. 32), as has the requirement of Fc γ RIIIA in the human setting in the ex-vivo lysis of Tregs by monocytes from peripheral blood when cultured with ipilimumab (33). However, in our models, neither increased depletion of Tregs, nor further upregulation of Fc γ RIV on myeloid cells was seen with the combination of this IgG2a mAb with anti-RANKL. Indeed, additional efficacy of combination anti-RANKL with DT-induced Treg depletion was seen in FoxP3-DTR mice compared with DT alone, where both DT-containing arms showed >95% Treg depletion compared with clg at endpoint. Thus, the combinatorial efficacy with anti-RANKL remained intact even when more Tregs were depleted than could be achieved through the use of anti-CTLA4 (mIgG2a), indicating that the mechanism of action of anti-RANKL was not directly on Tregs. These findings are in contrast with the previously reported roles for RANKL-expressing Tregs in the promotion of metastasis in a mouse model of RANK-expressing mammary carcinoma (34), or the role of systemic Treg

control via cutaneous RANKL–RANK interactions in the restraint of UV-induced cutaneous inflammation (35). Furthermore, we have shown that monotherapy efficacy of anti-RANKL is independent of activating FcR.

Overcoming T-cell exhaustion is a hallmark of current clinically available cancer immunotherapy, with restoration of effector T-cell function (such as IFN γ production; ref. 36). In our study, blockade of RANKL when added to anti-CTLA4 further improved effector function of intratumor T cells as measured by production of Th1-type cytokines such as IFN γ and IL-2, and increased the proportion of polyfunctional T cells. Such polyfunctional T cells have been shown to be important in the control of cancer and certain chronic viral infections and vaccination (37–40). Our data demonstrated the combination therapy led to marked increase of tumor-infiltrating CD8⁺ T cells most likely through recruitment rather than increased proliferation. In a study of human extra-mammary Paget's disease, the majority of RANK expression was associated with M2 macrophages, which produced the chemokine CCL17 when stimulated with RANKL, resulting in recruitment of T cells (41, 42). Although not addressed in this study, future analysis of chemokine production in the TME under the conditions of RANKL/RANK blockade may give further insights into this mechanism in the setting of cancer immunotherapy.

It is unclear whether the activity of anti-RANKL in combination with specific anti-CTLA4 isotypes resulted from the disruption of an immunosuppressive or tolerogenic axis between RANK-expressing myeloid cells and RANKL-expressing lymphocytes in the TME, although an essential role for DC-mediated cross-presentation was suggested through the use of *Batf3*-deficient mice. In our models, the most abundant RANK-expressing cells were TAMs, with expression also detected by DCs and MDSCs. The conditions under which myeloid cells express RANK in the TME and its downstream functions after RANK ligation remain unknown. Interactions between RANKL-expressing T cells and RANK-expressing myeloid cells can result in differential outcomes depending on factors such as anatomical site and pathologic setting. In one study, ligation of RANK on mucosal DCs by RANKL (but not splenic, mesenteric or peripheral lymph node DCs) resulted in increased IL-10 production, and oral RANKL administration at the time of antigen challenge increased oral tolerance, suggestive of a role for RANKL/RANK in tolerance induction (43). In a separate study, DCs that were co-cultured with genital squamous cell carcinoma cell lines showed an IL-10^{high} IL-12^{low} phenotype and functional deficits in a mixed lymphocyte reaction, but this phenotype could be reversed with RANKL blockade, which also resulted in increase in DC expression of co-stimulatory proteins (44). It is currently unknown whether the interaction between RANK-expressing DCs in the TME and RANKL-expressing lymphocytes results in a tolerogenic signal, although this supposition would be supported by our finding that treatment efficacy is abrogated in mice lacking *Batf3*.

RANK and RANKL are also implicated in the development of select lymphoid organs: gene-targeted mice deficient for RANK or RANKL lack peripheral lymph nodes, display thymic abnormalities, and are osteopetrotic with resultant reduction in B-cell development; in addition, a role for RANK and RANKL in the embryological development of *Aire* positive medullary thymic epithelial cells (mTEC) has been described (8, 9, 45–47). RANKL blockade as a mechanism of altering

central tolerance has been demonstrated to increase tumor antigen-specific T-cell immunity in young (3–5-week-old) mice (48). The mouse thymus reaches peak stromal cellularity at 4 weeks, and thereafter into adulthood the turnover of certain thymic epithelial cells is 10 to 14 days (49). In the current study, all mice were aged at least 6 weeks, and time from treatment commencement to experimental completion was typically 12 to 13 (but up to 20) days, meaning that alteration of thymic negative selection through depletion of mTEC subsets is unlikely to account for the antitumor efficacy of the combination studied.

The combination of RANKL blockade with alternative immune checkpoint inhibitors, such as those targeting PD-1, should now be assessed. Although the published case reports suggest unusually deep and brisk antitumor responses with combination ipilimumab and denosumab, antibodies targeting PD-1 have demonstrated superior antitumor responses compared with ipilimumab as monotherapy with a more acceptable toxicity profile (50). These results suggest that exploration of the combination of anti-RANKL and anti-PD1/PD-L1 are warranted, to assess whether the improvement of effector T-cell responses seen with the combination studied here could be recapitulated. In humans, the currently available anti-RANKL mAb is well tolerated; in cancer trials, adverse events suggestive of either immune suppression or autoimmunity were not higher than with bisphosphonate therapy (13, 51).

Overall, these results suggest a potential role for combining antibodies targeting RANKL with immune checkpoint blockade. In clinical trials of such combinations, translational markers of interest would include those of T-cell function and proliferation, and functional markers of investigational RANK⁺ myeloid subsets. Identification of the essential RANK-expressing suppressive myeloid population(s) for combinatorial efficacy would be a key goal, as this may provide a biomarker to identify patients suitable for this approach. Furthermore, this could provide a new immunotherapeutic treatment option for patients with malignancies who currently show little response to immune checkpoint blockade, but which have a significant myeloid component in the TME, such as advanced prostate cancer (52–55).

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Disclosure of Potential Conflicts of Interest

W.C. Dougall reports receiving commercial research grants from Bristol-Myers Squibb. M.W.L. Teng reports receiving speakers bureau honoraria from Merck Sharp & Dohme. M.J. Smyth reports receiving commercial research grants from Aduro Biotech, Bristol-Myers Squibb, and Corvus Pharmaceuticals, and is a consultant/advisory board member for Arcus Biosciences, F-star, and Kymab. No potential conflicts of interest were disclosed by the other authors.

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