Supplementary Information

Supplementary Materials and Methods

Therapeutic mAbs
The rat α-OX40 mAb (OX86) was produced as described (2). The rat IgG2a isotype control 2A3 was from BioXCell.

Therapy of transplanted 4T1.2 tumors
Mice were anesthetized with ketamine (100mg/kg) and xylazine (10mg/kg) or isofluorane and injected with 4T1.2 (5x10^4/50μl PBS) subcutaneously on the right hind leg. Tumor size was measured every 2-3 days using electronic calipers and represented as tumor area (length x width). Radio-immunotherapy commenced when tumors reached 20-25mm^2 and mice were irradiated as described for AT-3 tumor-bearing mice.

Immunotherapy consisting of α-CD40 (25μg), α-CD137 (12.5μg), α-OX40 (100μg) alone or in combination or MAC4 (25-100μg) or 2A3 (100μg) mAbs as indicated was administered intraperitoneally on days 0, 4, and 8 relative to RT. Antibodies depleting CD4^+ (100μg) or CD8β^+ (50μg) T-cells were administered i.p. on days -1, 0, 4 and 8, α-asialoGM1 (100μg) was administered i.p. on days -1, 0, 6 and 12 relative to radio-immunotherapy. The efficacy of depletions was confirmed in each experiment.

Mice were sacrificed when tumors reached 100-200mm^2 or in some instances earlier due to outgrowth of metastatic lesions within the thoracic cavity.
Supplementary Results

Antibody-based targeting of CD40 and/or CD137 enhances the anti-tumor response to RT in 4T1.2 tumors.

Given the ability of α-CD40 and α-CD137 mAbs to enhance the anti-tumor effects of TRAIL-receptor agonists, HDACi and chemotherapy (3-4), we examined whether these immune-stimulatory agents could be effectively combined with RT to control and eradicate subcutaneous 4T1.2 tumors. Predetermined doses of α-CD40 and/or α-CD137 mAbs were used alone (Suppl. Fig.2A, top panels) or in combination with RT (12Gy; Suppl. Fig.2A, lower panels). While both RT and α-CD137 mAbs possessed single-agent activity, RT together with α-CD137 mAbs alone or in combination with α-CD40 therapy, was significantly more effective than RT in controlling 4T1.2 tumor growth (Suppl. Fig. 2A,B); achieving tumor rejection rates of greater than 40% and 85%, respectively. α-CD40 treatment demonstrated minimal single agent activity and failed to significantly augment the anti-tumor effects of α-CD137 therapy (Suppl. Fig.2A,B). Importantly, the majority of tumor-free mice treated with RT/α-CD40/α-CD137 rejected a secondary challenge of 4T1.2 tumor cells >80 days after the primary tumor had been eradicated (Suppl. Fig.2C), indicating that curative radio-immunotherapy induced immunological memory. Thus despite low levels of RT-induced apoptosis, radio-immunotherapy could successfully eradicate established 4T1.2 tumors and induce immunological memory capable of controlling the outgrowth of a secondary tumor challenge.

Rejection of 4T1.2 tumors by RT/α-CD40/α-CD137 therapy is critically dependent on CD8+ T-cells.
To examine the cellular mechanisms contributing to radio-immunotherapy (RT/α-CD40/α-CD137)-mediated 4T1.2 tumor rejection, tumor-bearing mice were depleted of CD8β+ and CD4+ T or natural killer (NK)-cells prior to therapy. The anti-tumor effects of radio-immunotherapy was critically dependent on CD8β+ T-cells, as depletion of these cells resulted in all RT/α-CD40/α-CD137-treated tumors growing out similarly to control-treated tumors (Suppl. Fig.3A). CD4+ T-cells and NK-cells also contributed to the therapeutic effect of radio-immunotherapy, as depletion of these subsets resulted in a 4-fold drop in cure-rate when compared to fully immunocompetent mice (Suppl. Fig. 3A). Notably, CD137 was expressed on tumor-associated CD8+ and CD4+ T and NK-cells but only CD137 expression on NK-cells was modulated by RT (Suppl. Fig. 3B). Importantly, RT did not deplete CD137+ immune-cells resident in 4T1.2 tumors (Suppl. Fig. 3B), further highlighting CD137 as a potentially important immunotherapeutic target in breast cancer.

**Antibody-based targeting of the checkpoint inhibitor PD-1 enhances the anti-tumor effects RT in 4T1.2 tumors.**

Given the high rejection rate achieved with RT/α-CD137/α-PD-1 in AT-3 tumors we next examined whether a similar level of therapeutic efficacy could be achieved with this therapeutic combination in the 4T1.2 tumor model. Notably 4T1.2 tumors, which supported a highly necrotic core (Suppl. Fig.4), were found to express comparable levels of PD-L1 to that seen on explanted AT-3 tumors (Suppl. Fig.5A). Further to this, 4T1.2 tumor associated CD45.2+, CD8+ T, CD4+ T and NK-cells expression PD-1 (Suppl Fig.5B). In contrast to what was observed in the AT-3 tumors PD-1 expression on these lymphocyte populations was largely homogeneous, which may relate the highly necrotic nature of the 4T1.2 tumor microenvironment. RT had
minimal to no effect on the expression levels of PD-1 on CD8+ T and NK cells but did induce a statistically significant increase in PD-1 on CD4+ T cells (Suppl Fig.5B). Having confirmed the PD-L1 and PD-1/CD137 (Suppl Fig.3B) status of the 4T1.2 tumors and infiltrating lymphocytes, respectively, this provided rational to examined whether α-PD-1 therapy, alone or in combination with α-CD137 could enhance the therapeutic efficacy of RT in this tumor model. For this experiment, mice bearing established subcutaneous 4T1.2 tumors were treated with α-PD-1 and/or α-CD137 mAbs alone (Suppl. Fig.4C, top panels) or in combination with RT (12Gy; Suppl. Fig.4C, lower panels). As previously observed, α-CD137 mAbs possessed single-agent activity and RT together with α-CD137 mAbs was more effective than RT alone in controlling 4T1.2 tumor growth (Suppl. Fig.5C,D). Interestingly in this model, while α-PD-1 treatment demonstrated minimal single agent activity, blockade of this inhibitory pathway did significantly enhance the anti-tumor effects of RT, achieving a rejection rate of 80% (Suppl. Fig.5C,D). Intriguingly, however, the anti-tumor effects of RT/α-CD137/α-PD-1 were less profound than that observed in RT/α-CD137 and RT/α-PD-1 treated mice. The reasons for this are unclear, however given the highly necrotic nature of the 4T1.2 tumour microenvironment in which immune cells are likely being chronically exposed to high antigen loads, the combined treatment of α-CD137 and α-PD-1 mAbs may have impacted upon the viability and/or anti-tumor activity of critical tumor-reactive cells. Antagonism between α-CD137 and α-PD-1 has been previously reported in mice chronically exposed to lymphocytic choriomeningitis virus (LCMV) (5). In this study, low-dose α-CD137 treatment in combination with high-dose α-PD-1 enhanced viral control as compared to α-PD-1 treatment alone, whereas the administration of high-dose α-CD137 and α-PD-1 mAbs reduced the anti-viral effects achieved with α-PD-1
treatment alone. Therefore, the effects of varying doses of α-PD-1 and α-CD137 mAbs need to be carefully evaluated in 4T1.2 tumor-bearing mice in order to arrive at doses that can optimally promote tumor control. More importantly, however, the striking efficacy of anti-PD-1 therapy alone in irradiated 4T1.2 further strengthens the rational to explore the importance of PD-1 as a viable immunotherapeutic target in breast cancers.
Supplementary Figure Legends

Supplementary Figure 1. Dose-finding experiments for RT treatment of 4T1.2 and AT-3 tumors. Established (20-25 mm²) s.c. (A) 4T1.2 and (B) AT-3 tumors were treated with 0 (Control), 8, 10, 12 or 14 Gy RT. For each treatment group individual tumor growth curves (grey lines) and mean tumor growth (black line) are shown. Numbers in parentheses indicate the fraction of tumor-free mice 30-40 days post tumor inoculation.

Supplementary Figure 2. Response of 4T1.2 tumors to RT and α-CD137/α-CD40 mAbs. (A) Mice bearing s.c. 4T1.2 tumors were mock-irradiated (upper panels) or treated with 12Gy RT (bottom panels) in combination with α-CD40 and/or α-CD137 mAbs as indicated. For each group, individual tumor-growth curves (grey lines) and mean tumor-growth (black line) are shown. Results are representative of 2 independent experiments. (B) Mean tumor sizes in each group from Fig. 3A at day 27 post tumor inoculation, where differences are indicated for *P<0.05 and **P<0.01. (C) Tumor-free mice were rechallenged with 5x10⁴ 4T1.2 cells (grey lines) >80 days post primary 4T1.2 tumor clearance. Growth of the secondary tumor inocula was assessed against primary 4T1.2 tumor-growth in naive mice (black lines). (A,C) Numbers in parentheses indicate the fraction of tumor-free mice 40 days post tumor inoculation.

Supplementary Figure 3. Contribution of tumor-associated CD137-expressing immune cells to the therapeutic efficacy of radio-immunotherapy in 4T1.2 tumors. (A) Mice bearing s.c. 4T1.2 tumors were treated with MAC4 (Ctr), or
depleting antibodies to CD4, CD8β or asialoGM1, prior to receiving mock-irradiation and MAC4 (Ctr) or radio-immunotherapy (RT/α-CD40/α-CD137, (IR+IT)). Results are presented as mean tumor-growth ±SEM. Significant differences in mean tumor size between radio-immunotherapy-treated groups of control-depleted animals versus mice depleted of CD4, CD8 or NK-cells are *P<0.05 and **P<0.01. #P<0.05 is indicated for differences in mean tumor size between control-treated groups (Ctr) of control-depleted mice versus mice depleted of CD4 T-cells. Numbers in parentheses indicate the fraction of tumor-free mice 40 days post tumor inoculation. (B) Mice bearing 4T1.2 tumors were mock-irradiated or treated with 12Gy RT. Four days (84h) post RT, 4T1.2 tumors were harvested and the indicated subsets of CD45.2+ tumor-infiltrating lymphocytes analyzed for CD137 expression. Left panels: Histograms represent a concatenated analysis of CD137 expression on each subset for all mice within each group (n=6-7). Isotype control: solid histograms, CD137 expression: lined histograms. Numbers within histograms represent the mean fluorescence intensity (MFI) of CD137 expression above background staining (Isotype). Right panels: MFI of CD137 expression, above background staining for each mouse analyzed in the mock and irradiated groups. Expression of CD137 on the indicated immune-cell subsets was compared against CD137 expression on splenocytes (Sp) isolated from the same tumor-bearing mice; **P<0.01. Results are representative of 2 independent experiments.

**Supplementary Figure 4. Established 4T1.2 but not AT-3 tumors support a persistent necrotic tumor microenvironment.** Established (20-25 mm²) s.c. 4T1.2 (left panel) and AT-3 (right panel) tumors were harvested and processed for
histological analysis. Formalin-fixed tissue sections were H&E stained and microscopically analyzed. Scale bar: 100 μM. Necrotic zones, N.

Supplementary Figure 5. Response of 4T1.2 tumors to RT and α-CD137/α-PD-1 mAbs. (A) Left panels: Surface expression of PD-L1 on live, explanted CD45.2+ 4T1.2 (left panel) and AT-3 (right panel) tumor cells. Isotype control: Solid histogram, PD-1: bold line. Right panels: MFI of PD-L1 expression, above background staining for each AT-3 and 4T1.2 tumor-bearing mouse (n=4-8). (B) Mice bearing 4T1.2 tumors were mock-irradiated or treated with 12Gy RT. Four days (84h) post RT, 4T1.2 tumors were harvested and the indicated subsets of CD45.2+ tumor-infiltrating lymphocytes analyzed for PD-1 expression. Left panels: Histograms represent a concatenated analysis of PD-1 expression on each subset for all mice within each group (n=4). Isotype control: solid histograms, PD-1 expression: lined histograms. Numbers within histograms represent the fold-change of mean fluorescence intensity (MFI) of PD-1 expression above background staining (Isotype). Right panels: Fold change of MFI of PD-1 expression, over background staining for each mouse analyzed in the mock and irradiated groups; *P<0.05. Fold-change was used rather than MFI itself, since the MFI of both background and PD-1 staining was ~10-fold lower in NK cells compared to CD8+ and CD4+ T cells. Results are representative of at least 2 independent experiments. (C) 4T1.2 tumor-bearing mice were treated with 12Gy RT (bottom panels) or mock-irradiated (upper panels) in combination with 2A3 (Ctr, 100μg), α-CD137 (12.5μg) and/or α-PD-1 (100μg) mAbs as indicated. For each group, individual tumor-growth curves (grey lines) and mean tumor-growth (black line) are shown. Numbers in parentheses indicate the fraction of
tumor-free mice 25 days post tumor inoculation. (D) Mean tumor sizes in each group in Suppl. Fig. 4C at day 25 post tumor inoculation; *P<0.05, **P<0.01.

Supplementary Figure 6. Combined effects of RT and α-OX40 therapy against established subcutaneous 4T1.2 and AT-3 tumors. Established (20-25 mm²) s.c. 4T1.2 (A) and AT-3 (B) tumors were treated with 12 Gy RT (bottom panels) or mock irradiated (upper panels) in combination with α-OX40 mAb (OX86, 100 μg) or MAC4 cIg (100 μg). For each treatment group, individual tumor growth curves (grey lines) and mean tumor growth (black line) are shown. Numbers in parentheses indicate the fraction of tumor free mice 40 days post tumor inoculation. Right panels: Graphical representation of the mean tumor sizes between each treatment group at day 28 (A) and 30 (B) post tumor inoculation; *P<0.05, **P<0.01 and results are representative of 2 independent experiments.
Supplementary References


