Radiotherapy and Cisplatin Increase Immunotherapy Efficacy by Enabling Local and Systemic Intratumoral T-cell Activity

Paula Kroon1, Elselien Frijlink1, Victoria Iglesias-Guimarais1, Andriy Volkov1, Marit M. van Buuren1, Ton N. Schumacher1,2, Marcel Verheij3, Jannie Borst1, and Inge Verbrugge1

Abstract

To increase cancer immunotherapy success, PD-1 blockade must be combined with rationally selected treatments. Here, we examined, in a poorly immunogenic mouse breast cancer model, the potential of antibody-based immunomodulation and conventional anticancer treatments to collaborate with anti–PD-1 treatment. One requirement to improve anti-PD-1–mediated tumor control was to promote tumor-specific cytotoxic T-cell (CTL) priming, which was achieved by stimulating the CD137 costimulatory receptor. A second requirement was to overrule PD-1–unrelated mechanisms of CTL suppression in the tumor microenvironment (TME). This was achieved by radiotherapy and cisplatin treatment. In the context of CD137/PD-1–targeting immunotherapy, radiotherapy allowed for tumor elimination by altering the TME, rather than intrinsic CTL functionality. Combining this radioimmunotherapy regimen with low-dose cisplatin improved CTL-dependent regression of a contralateral tumor outside the radiation field. Thus, systemic tumor control may be achieved by combining immunotherapy protocols that promote T-cell priming with (chemo)radiation protocols that permit CTL activity in both the irradiated tumor and (occult) metastases.

Introduction

Cancer immunotherapies include adoptive T-cell therapy, therapeutic vaccination, and/or antibody-based immunomodulation. From a technical perspective, antibody-based immunomodulation is relatively straightforward, because immunomodulatory antibodies can essentially be delivered in the same way as conventional anticancer drugs. Immunomodulatory antibodies approved for cancer immunotherapy are designed to target the T-cell coinhibitory receptors PD-1 or CTLA-4, and single or combined treatment induces durable responses in about one third of patients with solid tumors (1). Still, the majority of patients do not benefit from this treatment approach (2). Compared with targeting CTLA-4, targeting PD-1 is generally more successful and associated with fewer autoimmune symptoms (3). Therefore, targeting PD-1 currently serves as the backbone for developing new combination therapies. To choose combinations rationally, insight into their combined mechanism of action is required.

CD8⁺ cytotoxic T lymphocytes (CTL) can recognize (tumor-derived) intracellular peptides presented on the cell surface by MHC class I molecules. As MHC class I molecules are expressed on virtually all body cells, CTLs can in principle target any cancer type. CD4⁺ T cells also promote antitumor immunity, either by direct cytotoxic activity or by promoting the activity of CTLs and other immune cells (4). Several groups postulated that successful immunotherapy relies on a tumor-specific T-cell response that is self-sustained by continuous generation of new effector T cells (T-cell priming) and support of their activity (5, 6). To enable this cycle, the tumor must essentially act as its own “vaccine” by releasing both recognizable antigens and “danger” signals. Dendritic cells (DC) can then present these antigens to naïve T cells and provide appropriate costimulatory and cytokine signals needed to induce T-cell clonal expansion and effector differentiation. However, in immunogenic tumors that have given rise to a T-cell response throughout their development, negative feedback mechanisms reduce effector T-cell functions. These mechanisms include the activity of regulatory T cells (Tregs) and suppressive activity of myeloid cells, stromal cells, and even the tumor cells themselves (7). For example, PD-1 can be expressed on tumor cells and/or other (immune) cell types present in the tumor, and can inhibit T-cell function via PD-1 (8). Successful immunotherapy requires the elimination of such suppressive mechanisms.

Blocking CTLA-4 enables CD28 costimulation (9), which may promote new T-cell priming and effector T-cell activity. Blocking CTLA-4 and PD-1 promotes T-cell activity inside tumors in a...
complementary fashion (10), and blocking CTLA-4 promotes T-cell priming in patients with cancer (11). Concomitant targeting of CTLA-4 and PD-1 is associated with increased autoimmune destruction (12) and this combination should likely be avoided when developing new immunotherapy strategies. A potential alternative, targeting CD137 (also known as 4-1BB or TNFRSF9) using agonistic antibodies is currently in phase III clinical trials (13) and is being tested in combination with PD-1 blockade in phase Ib clinical trials (14). CD137 is a costimulatory receptor that belongs to the TNF receptor family, and its signaling promotes the priming and maintenance of CTL responses by delivering prosurvival and other signals to CD8+ T cells and DCs (15).

Both radiotherapy and chemotherapy induce tumor cell destruction, which leads to release of antigens and ‘danger’ signals (16). In principle, these events may lead to new T-cell priming. However, the likelihood that priming will occur without immunotherapy-based assistance is low, because radiotherapy almost never gives rise to an ‘abscopal’ effect, that is, regression of a tumor outside the radiation field (17). Extrapolating from mouse models (18), conventional chemotherapeutic drugs may have immunomodulatory actions in human, but thus far, this question has not been systemically addressed in the clinic.

Here, we examined the potential of using radiotherapy and a routinely coapplied conventional chemotherapy (cisplatin) to assist immunotherapy in evoking a systemic, tumor-eradicating T-cell response. We provide evidence that radiotherapy and chemotherapy make tumors permissive to CTL activity. These data argue that conventional anticancer regimens can be combined rationally with immunotherapy to improve systemic tumor control and increase tumor clearance rates and patient outcome.

Materials and Methods

Cells
AT-3 cells are derived from the MMTV-Polyoma virus middle-T (PyMT) transgenic mouse, back-crossed to C57BL/6 (19), and were received from the Peter MacCallum Cancer Centre (Melbourne, Australia) in 2012. AT-3 cells were cultured in DMEM, supplemented with 10% FCS, 0.1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, 2 mmol/L l-glutamine, 10 mmol/L HEPES, and 30 μmol/L β-mercaptoethanol at 37°C, 10% CO2. AT-3 cells were tested negative for Mycoplasma by PCR, and cells thawed from this "master stock" were routinely used within 6 passages (approximately 3 weeks) for in vitro and in vivo experiments. PyMT protein expression in AT-3 cells was validated by Western blot analysis, but the cells were not further authenticated in the past year.

Mice
Six- to 8-week-old female C57BL/6J (B6) mice were obtained from Janvier Laboratories (Le Genest Saint Isle, France) or from in-house breeding within the Netherlands Cancer Institute (NKI, Amsterdam, the Netherlands) and maintained in individually ventilated cages (Innovive) under specific pathogen-free conditions. All mouse experiments were performed in accordance with institutional and national guidelines and were approved by the Committee for Animal Experimentation at the NKI.

Therapeutic antibodies and reagents
Agonistic rat mAb to mouse CD137 (clone 3H3, IgG2a; ref. 20) was purified from hybridoma supernatant by affinity chromatography on protein-G. Rat anti-mouse PD-1 (clone RMP1-14, IgG2a) and isotype control (2A3) were purchased from BioXCell. FTY720 was purchased from Cayman Chemical and cisplatin from Pharmachemie BV (RVG 101430).

Tumor transplantation and therapy
AT-3 cell transplantation and therapy were performed essentially as described previously (21, 22), with minor modifications. Briefly, mice were anesthetized with isoflurane and injected with 1 × 106 AT-3 cells into the fourth mammary fat pad. In some experiments, mice were injected with 0.5 × 106 AT-3 cells into this fat pad on one side and with 2.5 × 106 AT-3 cells on the contralateral flank. The latter tumor was irradiated, and the other tumor served as the nonirradiated “abscopal” tumor. Tumor size was measured using a caliper, and treatment was initiated when the tumors reached 20–25 mm3. Therapy was done with n = 5–10 mice per group. Radiotherapy was applied using an XRAD225-Cx system (Precision X-Ray), as described previously (22, 23). In brief, the mice were anesthetized with isoflurane and a cone-beam CT scan of the mice was performed. The tumor(s) were localized on the CT scan and targeted with radiotherapy at 0.1-mm precision using round collimators 1.0 or 1.5 cm in diameter. A single fraction of 10–12 Gy (225 peak kilovoltage [kVp]), filtered with 0.3 mm of copper (3 Gy/minute) was delivered. Control mice were anesthetized and underwent a cone-beam CT scan, but were not exposed to radiotherapy. Immunomodulatory mAbs to PD-1 and CD137 or an isotype control mAb were diluted in PBS. The antibodies were administered twice weekly for 2 weeks either intraperitoneally (PD-1 mAb, 100 μg per injection), or intratumorally (CD137 mAb, 25 μg in 10 μL per injection), with the first dose delivered immediately after radiotherapy treatment. For some experiments, cisplatin was administered intravenously at 4 mg/kg on day 0 (i.e., immediately after RIT) and on day 14. Tumor transplantation and therapy for RNAseq experiments was performed identically, with the exception that CD137 mAb was delivered i.p. (100 μg). The sphinogosine-1-phosphate receptor-1 agonist FTY720 was diluted in saline (vehicle) and administered at 2 mg/kg by oral gavage. FTY720 treatment started one day prior to radiotherapy and was repeated three times per week throughout the duration of the experiment. All mice were sacrificed when the tumor(s) reached 100–200 mm3. A tumor size of 100 mm3 was set as a designated endpoint.

DNA vaccination
The DNA vaccination vector “SIG-HELP-SNPTYSM.VDEL” was generated by ligating annealed codon-optimized oligos (forward: 5'TCAGAGACCAACCCACCCTACAGCTGAAGAGGACGACGCTTAATAT3; reverse: 5'TCAGATATTACAGCTCGTCTTGCTC5' encoding SNPTYSM. KDEL and Xhol and Xbal restriction sites in the Xhol/Xbal linearized pVax-HELP vector designed by Oosterhuis and colleagues (24), and described in detail by Ahrends and colleagues (25). For DNA vaccination, the hair on a hind leg was removed using depilating cream (Veet; Reckitt Benckiser) on day −1. On days 0, 3, and 6, the mice were anesthetized with isoflurane, and 15 μL of a solution containing 2 mg/mL plasmid DNA in 10 mmol/L Tris and 1 mmol/L EDTA, pH 8.0, was applied to the hairless skin with a Permanent Makeup Tattoo machine (MT Derm GmbH), using a sterile disposable 9-needle bar with a needle depth of 1 mm and an oscillating frequency of 100 Hz for 45 seconds.
Flow cytometry

At the indicated time points, tumor-bearing mice were sacrificed, and tumor and lymphoid tissue were harvested. The tumors were mechanically chopped using a McIlwain tissue chopper (Mickle Laboratory Engineering), and a single-cell suspension was prepared by digesting the tissue in collagenase type A (Roche) and 25 µg/mL DNase (Sigma) in serum-free DMEM medium for 45 minutes at 37°C. Enzyme activity was neutralized by addition of DMEM containing 8% FCS, and the tissue was dispersed by passing through a 70-µm cell strainer. A single-cell suspension of lymphoid tissue was prepared by passing the tissue through a 70-µm cell strainer. Single cells were first stained with PE- or APC-conjugated H-2Kb/Db, and 25% viable cells were stained with Flodered780 (1:1,000, eBioscience), Zombie Red Fixed Viability Kit (1:5,000, BioLegend) or DAPI (Invitrogen) was added to exclude dead cells. All experiments were analyzed using a BD LSRII, BD Fortessa, or FlowJo software. RNA preparation and sequencing

Using flow cytometry, CD43+ CD8+ T cells (CTLs), CD45+ hematopoietic cells, and CD45+ (tumor/stromal) cells were isolated from both the irradiated and nonirradiated tumors of 9 mice per experimental group, and material from 3 mice was pooled per sample to retrieve sufficient RNA. Cells were collected in RLT lysis buffer (Qiagen) and total RNA was extracted using the RNeasy Mini Kit (Qiagen) in accordance with the manufacturer’s instructions. The integrity of the total RNA was assessed using a 2100 Bioanalyzer System (Agilent). Only RNA samples with an RNA Integrity Number (RIN) > 8 were used to create the library. Poly-A-selected RNA libraries were prepared using the TruSeq RNA library protocol (Illumina) and the resulting libraries were sequenced using an Illumina HiSeq2500 with V4 chemistry, with 50-bp single-end reads per lane.

Transcriptomics analysis of Illumina sequencing data

Sequencing reads in FASTQ files were mapped to the mouse genome (build GRCm38.77) using TopHat v2.1.8 (28), and the read summation program HTseq-count (29) was used to count uniquely mapped reads against annotated genes. Differential expression analysis was performed using the DESeq2 package in R (30). P values were corrected for multiple comparisons, based on the False Discovery Rate (FDR), with significance considered at a q-value < 0.01. Volcano plots were generated using ggplot2 (https://www.springer.com/gp/book/9780387981413).

Normalized read counts were used as input for Gene Ontology (GO) Gene Set Enrichment Analysis (GSEA) version 3.0 (31, 32) to identify groups of biological processes that were differentially expressed between cell populations obtained from the irradiated site and cell populations obtained from the nonirradiated site. We used the MSigDB C5 collection to identify enriched GO biological processes (BP). GSEA was performed with default parameters and gene set permutations were used. To gain a better overview of the linked biological processes, we generated enrichment maps using the Enrichment Map app v3.1.0, using cut-off values set at Q = 0.1 and Jaccard Overlap Combined = 0.375. We illustrated the largest gene set clusters and manually assigned the more general processes that these clusters represent.

The RNA-seq data reported in this article have been deposited in the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-6914.

IHC analysis

Harvested tumors were fixed for 24 hours in ethanol (50%), acetic acid (5%), and formalin (3.7%), embedded in paraffin, and then sectioned randomly at 5 µm. The sections were then stained

Kroon et al.
as described previously (23). In brief, fixed sections were rehydrated and then incubated with primary antibodies to CD8 (eBioscience; clone 4S115) and Foxp3 (eBioscience; clone FJK-16s). Endogenous peroxidases were blocked with 3% H2O2, and the sections were then incubated with biotin-conjugated secondary antibodies, followed by incubation with HRP-conjugated streptavidin–biotin (DAKO). The substrate was developed using diamobenzidine (DAB; DAKO). We included negative controls to determine background staining, which was negligible. The stained sections were digitally processed using an Aperio ScanScope (Aperio) equipped with a 20× objective. ImageJ software was used to quantify the number of positive cells in 3–5 random fields of view (FOV) per slide.

Statistical analysis
All summary data were analyzed using GraphPad Prism version 6 (GraphPad Software). Differences between various treatment groups were analyzed using the Mann–Whitney U test. Differences in survival curves were analyzed using the log-rank (Mantel–Cox) test. Differences with P values <0.05 were considered statistically significant.

Results
Immunotherapy with CD137 agonism and PD-1 blockade promotes T-cell priming
As a model system, we used mice with syngeneic AT-3 breast cancer cells implanted orthotopically into the fat pad and treated with immunotherapy and/or radiotherapy after the tumor reached >20 mm2. Standard immunotherapy consisted of a blocking antibody to PD-1 and an agonistic antibody to CD137 (Fig. 1A), targets which are expressed on DCs and on T cells in lymphoid organs and tumor tissue (Supplementary Fig. S1). In this setting, immunotherapy and radiotherapy as individual treatments merely delayed tumor outgrowth, whereas combined treatment (i.e., RIT) resulted in tumor clearance in the majority of the mice (Fig. 1B). We have previously shown that combined PD-1 blockade and CD137 agonism is more effective at enhancing radiotherapy-induced tumor control than single PD-1 blockade or CD137 agonism and that tumor control in this setting relies on CD8+ T cells (21, 22). Among single modality treatments, CD137 agonism, but not radiotherapy or PD-1 blockade induced a T-cell response (Fig. 1C), as determined by the appearance of CD45+ and CD8+ T cells with a CD43+ effector phenotype in blood posttreatment (33). PD-1 blockade further increased CD45+ and CD8+ T-cell responses when combined with CD137 agonism (Fig. 1C and D). Finally, when immunotherapy with both antibodies was combined with radiotherapy, CD45+ and CD8+ T-cell responses were also induced, as measured by a significant increase in effector phenotype T cells in the blood and a similar increase in the (inguinal) tumor-draining lymph node (dLN; Fig. 1D and E). These data suggest that immunotherapy with CD137 agonist antibody promotes T-cell priming, which is increased by PD-1 blockade and not impeded by concurrent radiotherapy.

Control of the irradiated tumor by RIT requires T-cell priming
To examine whether newly primed T cells contributed to tumor control after RIT, we treated mice with the drug FTY720 that induces the internalization of the sphingosine 1 phosphate receptor 1 (S1PR1). T cells use the S1PR1 to egress from secondary lymphoid organs and the drug prevents them from doing so (34). RIT was applied while the mice were treated with FTY720 or vehicle (Fig. 2A; Supplementary Fig. S2A). To assess T-cell priming and resulting effector T-cell generation, we measured the percentage of CD4+ and CD8+ T cells in the dLN that could produce effector cytokines TNFα and/or IFNγ. RIT increased the percentage of CD8+ effector T cells, and these cells significantly accumulated in the dLN upon FTY720 treatment (Fig. 2B, left). In contrast, TNFα-producing CD4+ effector T cells were not increased by RIT, nor did these cells accumulate in the dLN upon FTY720 treatment (Fig. 2B, right). These data indicate that RIT induced new priming of CD8+ T cells and that FTY720 treatment effectively “trapped” these newly primed T cells in the dLN.

Whereas 100% of the mice cleared their tumor and survived long term upon RIT, concurrent FTY720 treatment significantly increased tumor outgrowth (Fig. 2C) and reduced overall survival (Fig. 2D). FTY720 treatment did not reduce the therapeutic effect of radiotherapy or immunotherapy alone (Supplementary Fig. S2B). Thus, RIT leads to T-cell priming and these newly primed T cells make a critical contribution to regression of the irradiated tumor.

RIT does not induce regression of an abscopal tumor, despite infiltration with newly primed CTLs
Given that RIT induced T-cell priming, we hypothesized that the resulting systemic T-cell response could also act against a nonirradiated tumor in the same host. We tested this by implanting two tumors into the same mouse; one in the left fat pad and the other in the contralateral flank. Only the latter tumor was irradiated (Fig. 3A). The T-cell response and tumor regression were examined for both tumors. We found that after RIT the percentage of CD8+ T cells among total CD45+ (hematopoietic) cells increased significantly in both irradiated and nonirradiated tumors (Fig. 3B, left). The RIT-induced increase of CD8+ T cells in the nonirradiated tumor was largely prevented by FTY720 treatment (Fig. 3B, left), indicating that this increase was largely due to new T-cell priming. The CD4+ T-cell response following RIT was much less pronounced (Fig. 3B, right). Histologic analysis confirmed that CD8+ T cells accumulated to a similar extent following RIT in both irradiated and nonirradiated tumors (Fig. 3C). Infiltration by CTLs, capable of producing IFNγ and TNFα and the cytotoxic effector molecule granzyme B, was of similar magnitude in irradiated and nonirradiated tumors (Fig. 3D). In contrast, accumulation of CD4+ T cells that could produce TNFα or granzyme B was not evident (Fig. 3E). As compared with immunotherapy alone, RIT delayed outgrowth of the irradiated, but not of the nonirradiated tumor (Fig. 3F; Supplementary Fig. S3A). As overall survival was defined by the time for any of the two tumors to reach 100 mm2, RIT did not improve overall survival of mice as compared with immunotherapy alone (Fig. 3G). (Hypo)fractionated radiotherapy is more effective than single-dose radiotherapy in enhancing abscopal tumor control by immunotherapy in certain mouse models (35, 36). However, 3 × 8 Gy (hypo)fractionation also did not enhance immunotherapy-induced control of nonirradiated AT-3 tumors (Supplementary Fig. S3B and S3C). Thus, CTLs that are raised by RIT are present in equal measure in the irradiated and nonirradiated tumor, yet these CTLs can only eliminate the irradiated tumor.
The abscopal effect of RIT is not limited by T-cell priming nor intratumoral neutrophils/macrophages

We next addressed a number of potential factors that might prevent RIT-induced CTLs from eliminating the nonirradiated tumor. We first assessed whether the size of the tumor-specific CTL pool was a limiting factor. For this purpose, we identified the peptide SNPTYSVM from MMTV-Polyoma virus middle-T (PyMT) as an MHC class I–restricted antigen that could raise T-cell immunity to AT-3 tumor cells (Supplementary Fig. S4A–S4F). This enabled us to purposely generate tumor-specific CTL memory in vivo by vaccinating mice with plasmid (p)DNA encoding this epitope (Fig. 4A), designed according to ref. 24. Vaccinated mice were challenged with two AT-3 tumors and treated with RIT (Fig. 4B). Also in this setting, RIT did not enhance control of the nonirradiated tumor (Fig. 4C) or improve survival of mice (Fig. 4D), as compared with immunotherapy alone. These data suggest that the magnitude of the tumor-specific CTL response was not the limiting factor for systemic tumor control following RIT.
We next examined which mechanisms of T-cell suppression other than PD-1/PD-L1 interaction may operate in the nonirradiated AT-3 tumors. Treg frequency was low in the irradiated and nonirradiated tumors and did not change significantly following RIT (Fig. 4E), suggesting that it did not correlate with CTL-mediated tumor control.

Tumor-resident neutrophils and macrophages can also locally impair CTL function (37). Following RIT, a decrease in the frequency of F4/80+ macrophages was observed in irradiated, but not in nonirradiated tumors (Fig. 4F, left). The frequency of Ly6G+Ly6Clow neutrophils did not change after RIT (Fig. 4F, right). In addition, antibody-mediated depletion of neutrophils or TAMs (Supplementary Fig. S4G) did not improve control of nonirradiated tumors (Supplementary Fig. S4H), nor did it increase overall survival following RIT (Fig. 4G).

Following RIT, the frequency of NK and NK-T cells was decreased in irradiated and nonirradiated tumors to a similar extent. There was also no difference in the frequency of Ly6C+Ly6G− inflammatory monocytes, CD103+ DCs, and CD11b+ DCs in irradiated as compared with nonirradiated tumors (Fig. 4H). Thus, the presence of these cell types did not correlate with CTL-mediated tumor control.

Although radiotherapy can upregulate cell surface expression of MHC class I (38), it did not increase MHC class I expression on nonhematopoietic cells in the AT-3 tumor in vivo, as determined on day 3 and 8 postradiotherapy (Supplementary Fig. S4I). Taken together, these data suggest that the magnitude of the tumor-specific CTL response, PD-1 signaling, Tregs, neutrophils, TAMs, NK(T) cells, inflammatory monocytes, DCs, or MHC class I expression were not key factors that limited CTL activity in the nonirradiated tumor after RIT.

RIT induces a TME characterized by reduced cell proliferation and increased tissue repair.

RIT led to the same degree of CTL infiltration in the irradiated and nonirradiated tumors, whereas only the irradiated tumor regressed, suggesting that CTLs can exert their activity on tumor cells only after the tumor has been altered by irradiation. To understand the immunomodulatory effect of irradiation in the context of immunotherapy, we performed mRNA sequencing (RNA-seq). Eight days after RIT (allowing sufficient time for T cells to infiltrate both tumors: see Fig. 3), we sorted the effector (CD43+CD8+ T cells (i.e. CTLs), CD45+ hematopoietic cells (excluding CD43+CD8+ T cells) and CD45+ tumor/stromal cells (Fig. 5A). Statistical analysis of normalized read counts revealed the differential expression of 805 genes in CTLs (Fig. 5B), 1,107 genes in the hematopoietic cells (Fig. 5C), and 3,045 genes in the tumor/stromal cells (Fig. 5D). These genes encode a wide diversity of proteins (Supplementary Table S1) that perform a multitude of cellular functions.

We identified groups of biological processes that were differentially modulated between the cell populations at the irradiated and nonirradiated tumor sites. In all three cell populations, gene sets associated with cell division, DNA replication and repair, and...
chromatin remodeling were significantly downregulated in the irradiated tumor (Fig. 5E–G), congruent with the cells receiving a DNA-damaging input in the form of irradiation.

In the CTLs, we additionally identified small gene sets associated with negative regulation of cytokine expression (Fig. 5E), which included both Foxp3 and Il10 (Supplementary Table S1), which may report effects of irradiation. We did not identify gene sets associated with increased CTL-intrinsic effector function that could explain the increased CTL efficacy in the irradiated tumor. This finding is consistent with our functional

Figure 3.
RIT leads to comparable infiltration of irradiated and nonirradiated tumors with T cells, but the nonirradiated tumor does not regress. A, Experimental set-up. B, Percentage of CD4+ and CD8+ T cells within the CD45+ cell population in the irradiated (Irr) and nonirradiated (non-Irr) tumors before (D0) or 8 days after the start of RIT, in the presence or absence of FTY720. *P < 0.05. C, AT-3 tumor sections (n = 3 mice/group) were stained for CD8 before treatment (D0) or 4, 8, or 14 days after the start of RIT. Left:, representative images; right, summary data. Quantification in the right represents the average (±SD) of 5 fields of view (FOVs) for 3 irradiated (filled circles) and 3 nonirradiated tumors (open circles). Images represent 1/6th of a FOV; scale bar, 100 μm. D and E, Percentage of TNFα+IFNγ+ or Granzyme B (GzB)+CD8+ T cells (D) and CD4+ T cells (E) within the CD45+ cell population isolated from irradiated and nonirradiated tumors before (D0) and 8 days after starting RIT. Each symbol represents a single tumor, and the mean is indicated. *P < 0.05. F, Mean tumor growth in mice (n = 5–6/group) that received no therapy (Ctr), radiotherapy (10 Gy, R), alone or in combination with immunotherapy (IT). G, Survival curve for mice treated as indicated. *P < 0.05, between the Ctr and IT groups; **, P < 0.01 between the Ctr and RIT groups and between the radiotherapy (RT) and RIT groups.
data regarding CD8\(^+\) T cells, showing that both irradiated and nonirradiated tumors are infiltrated with effector-phenotype \(\text{CD}^+\) T cells. In the hematopoietic and tumor/stromal cells, we identified several biological processes that were significantly different between the irradiated and nonirradiated tumor sites (Fig. 5F and G). These included overlapping processes and genes in the hematopoietic and tumor/stromal cells, such as increased cell migration (e.g., \(\text{Cxcl}17, \text{Cxcl}14\)), vasculogenesis (e.g., \(\text{Vegfc}, \text{Egfr}\)), and cell adhesion/extracellular matrix (ECM; e.g., \(\text{Selp}, \text{Mmp3}\), see also Supplementary Table S1). In addition, and unique to the tumor/stromal cell population, we identified increased expression of gene sets associated with RNA/ribosome processes (e.g., \(\text{Rps19}, \text{Rps12}\)) and wound healing (e.g., \(\text{Pdgfb}, \text{Cxc12}\)) in the irradiated tumor as compared with the nonirradiated tumor (Fig. 5F and G). Increased expression of proapoptotic \(\text{Bax}\) was observed specifically in the tumor/stromal cells of the irradiated tumor (Supplementary Table S1).

**Figure 4.** Optimizing tumor-specific T-cell priming, depletion of neutrophils or TAMs is insufficient to control nonirradiated AT-3 tumors following RIT. A, The vaccine encodes the H-2K\(^b\)-binding PyMT epitope SNPTYSVM and MHC II "helper" epitopes. ***,** Double stop codon. B, Experimental set-up. Mean tumor size (C) and survival curves D of 5–7 mice/group that received the indicated treatments. ***, P < 0.005; **, P < 0.05. E, FoxP3\(^+\) regulatory T cells in irradiated (Irr) and nonirradiated (non-Irr) tumors before treatment (D0) and on the indicated days after the start of RIT (n = 3/group). Images, representative FoxP3 staining of 10% of a FOV, scale bar, 100 μm. Quantification (right): mean (±SD) of 5 FOVs for 3 irradiated (filled circles) and 3 nonirradiated tumors (open circles). F and H, TAMs, neutrophils (F), NK cells, NKT cells, inflammatory monocytes, CD103\(^+\) DCs, CD11b\(^+\) DCs (H) in untreated (D0), irradiated (Irr), and nonirradiated (non-Irr) tumors on day 8 (D8) after RIT. Each symbol represents an individual tumor, and the mean (±SD) is shown (**, P < 0.01, *** P < 0.001). G, Survival curve of tumor-bearing mice that receive RIT in the presence or absence of antibodies targeting Ly6G or CSF1R (n = 5/group).
Figure 5.
RIT induces a CTL-permissive TME that is characterized by gene signatures associated with reduced cell proliferation and increased response to tissue injury. 

A, Experimental set-up. B–D, Volcano plots of the indicated cell populations showing significant (adjusted $P < 0.01$) transcriptomic changes in the irradiated compared with the nonirradiated tumor. Purple, orange, and gray dots represent downregulated, upregulated, or unchanged genes, respectively. E–G, Enrichment Maps showing significantly enriched gene sets (biological processes) in blue (enriched in the irradiated tumor) and red (enriched in the nonirradiated tumor). Gene sets that share a high number of genes are clustered together, and the thickness of the green lines represents the number of shared genes. Clusters of similar biological processes are labeled.
Taken together, these RNA-seq data revealed that the TME of the irradiated tumor was different from that of the nonirradiated tumor. Radiotherapy inflicted a DNA damage response in all cell populations in the tumor and led to tissue repair, as suggested by increased protein translation, angiogenesis, and cell migration. This gene expression profile was associated with increased CTL activity against the tumor cells, most likely through CTL-extrinsic effects.

Cisplatin functionally mimics the radiotherapy-induced, T-cell-permissive TME and increases RIT efficacy

Next, we aimed to create a "CTL-permissive" TME in the nonirradiated tumor to allow for systemic CTL-based tumor eradication following RIT. We tested low-dose cisplatin chemotherapy to achieve this effect (Fig. 6A) for the following reasons: (i) cisplatin has partially the same mode of action as radiotherapy by inducing DNA damage, (ii) cisplatin combined with radiotherapy is standard-of-care in the treatment of different types of cancer and (iii) (low-dose) cisplatin has been shown to support T-cell function in (pre-)clinical vaccination studies (18).

We found that low-dose cisplatin delayed tumor outgrowth, and that adding cisplatin treatment to RIT further improved control of nonirradiated tumors (Fig. 6B and C) and increased overall survival (Fig. 6D). This enhanced therapeutic effect was CD8⁺ T-cell-dependent (Fig. 6D), even though cisplatin modestly reduced the magnitude of the T-cell response following RIT (Supplementary Fig. S5A and S5B). In the absence of radiotherapy, cisplatin treatment also enhanced the antitumor effect of this immunotherapy approach (Supplementary Fig. S5C and S5D). Thus, systemic cisplatin treatment functionally mimicked the localized effects of radiotherapy, allowing CTL-mediated growth delay of the nonirradiated tumor and prolonging overall survival following RIT.

Figure 6.
Cisplatin increases the therapeutic efficacy of RIT. A, Experimental set-up. B, Mean tumor growth (±SEM) of irradiated and nonirradiated tumors. Mean tumor size (±SEM) of nonirradiated tumors on day 20 (C) and survival curves of the indicated groups of mice; where indicated a CD8-depleting antibody (α-CD8) was administered one day before the start of treatment (D). Data shown are pooled data from 3 independent experiments of 4–7 mice/group in each experiment. **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; n.s., not significant.
Discussion

There is an unmet clinical need to improve responses to PD-1 blockade, which currently forms the backbone for immunotherapy combinations (2). The PD-1 coinhibitory receptor is associated with tyrosine phosphatase activity that inhibits CD3/CD28 signaling (8). In this way, the PD-1 "checkpoint" can impede both T-cell priming and effector function. In patients with cancer, PD-1 blockade thus far seems to primarily relieve effector T cells from PD-L1/2–based suppression in the TME (39). Therefore, this approach is likely to be most effective as stand-alone treatment for immunogenic cancers in which T cells have already infiltrated the tumor (40). Immunotherapy of poorly immunogenic cancers that have not raised a T-cell response will by definition require interventions that induce tumor-specific T-cell priming. Even in immunogenic cancers that respond to PD-1 blockade alone, new T-cell priming is expected to strengthen and broaden the antitumor immune response, thereby increasing efficacy and combating resistance (6). In addition, immune suppression within the TME will preexist in immunogenic tumors and may develop in poorly immunogenic tumors once a T-cell response is raised, resulting from negative feedback control. PD-1/PD-L1 interactions are only a small part of this feedback control, which is exerted by diverse immune- and nonimmune cells in the TME. Effective antitumor immunity requires both priming of tumor-specific T cells and a CTL-permissive TME. Here, we show that radiotherapy and conventional chemotherapy can promote intratumoral CTL activity by modulating the TME and by synergizing with an immunotherapy that enables T-cell priming.

We here identified that the murine AT-3 breast cancer cell line carries a foreign MHC class I–restricted antigen "SNPTSYSM." Few T cells were present within AT-3 tumors at steady-state. PD-1 blockade alone had no therapeutic effect, but CD137 agonism induced CTL priming and antitumor immunity. CD137 triggering on activated CD8+ T cells stimulates proliferation, survival, and possibly effector differentiation (15), supporting CTL priming. Furthermore, CD137 triggering on DCs and other myeloid cell types can lead to the upregulation of costimulatory ligands CD80/CD86 (e.g., ref 41), which may help to overcome peripheral tolerance and induce T-cell responses to tumor antigens. In the TME, CD137 agonism may support CTL function by similar mechanisms. CD137 mAb can also stimulate hypoxic, CD137-expressing endothelial cells to recruit T cells into the tumor (42). We found that PD-1 blockade aided CD137-stimulated CTL priming, supporting evidence that the PD-1 checkpoint can also limit T-cell priming, as observed previously (e.g., ref 43).

We predict that combining PD-1 blockade with any form of immunomodulation that induces CTL priming will be generally useful clinically. CTLA-4 blockade (e.g., ref 36) and CD27 agonism (25) can exert similar effects in distinct tumor models. In our current study and previous ones (21–23), agonistic antibody to CD137 administered either intratumorally or intraperitoneally, did not lead to weight loss or other overt pathology of the mice in the context of RIT. In humans, in which CD137 agonist antibody is applied systemically, combination with PD-1 blockade has comparable side-effects as PD-1 blockade alone, suggesting the approach is feasible (14).

In the immunotherapy setting with combined PD-1 blockade and CD137 agonism, AT-3 tumors were not eliminated, despite a robust CTL response. In adoptive tumor-specific T-cell therapy, a robust CTL response is often also not sufficient for tumor control (44), highlighting that CTL suppression in the TME can pose an additional bottleneck for systemic antitumor immunity. Our study demonstrates that radiotherapy can alter the state of the TME to permit effective CTL activity, under conditions where PD-1 blockade cannot. Newly primed CTLs raised by our RIT protocol contributed to control of the irradiated tumor. Having a second, nonirradiated tumor in the same mouse allowed us to pinpoint the immune modulating effects of radiotherapy. The nonirradiated tumor was similarly infiltrated by newly primed CTLs as the irradiated tumor, but did not regress, indicating that impediments beyond PD-1 signaling hampered abscopal tumor control. In a CT26 transplantable tumor model, control of the irradiated tumor by combined radiotherapy and PD-1 blockade was also found to be partly dependent on newly primed T cells. In that model, control of a simultaneously implanted nonirradiated tumor was also improved by PD-1 blockade (45). In that case, PD-1 signaling was the key impediment for CTL activity in the TME, whereas in our AT-3 model, additional impediments were in place. In PyMT-induced tumors, stimulation of TAMs with TLR7/9 agonists (imiquimod, Cpg) allowed them to reinvade tumor-resident T cells (46). However, in the AT-3 model, the CTL-enabling effect of radiotherapy could not be reproduced by depletion of neutrophils or TAMs. TAMs can also phagocytose dead tumor cells and enable antigen cross-presentation by DCs. Altering the functional state of TAMs may be preferred over their depletion to enhance intratumoral CTL activity.

Comparative transcriptome analysis of cell populations from the irradiated and the nonirradiated tumors in the same mice revealed that a "CTL-permissive" TME was associated mostly with changes in CTL-extrinsic, rather than CTL-intrinsic gene signatures. We did not identify gene sets within the CTLs that could explain enhanced efficacy in the irradiated tumor. This indicates that the intrinsic quality of the CTLs that infiltrated the irradiated and nonirradiated tumors after RIT was similar and of good quality, which we also validated by ex vivo flow cytometry. Differentially expressed genes identified in the CTLs were associated with negative regulation of cytokine production and included Foxp3 and Il10. We speculate that this is an immune regulatory signature that arose in CTLs that experienced and survived radiotherapy. It is unlikely that this population contributed to enhanced tumor control. Instead, our data suggest that CTL-extrinsic parameters (an altered TME) were decisive for CTL efficacy in the irradiated tumor after RIT. The differentially expressed genes only allow speculation regarding the mechanisms involved. Increased vasculogenesis identified in the irradiated tumor did not alter CTL infiltration into the irradiated tumor as compared with the nonirradiated tumor, as measured 8 days after RIT. Reduced proliferation of the tumor cells might have improved CTL-mediated tumor cell death by allowing T cells more time to complete killing. Potential sensitization of tumor cells to apoptosis by upregulation of Fas may have contributed to increased CTL-mediated tumor control in the irradiated tumor. RNA- and ribosome-associated processes were upregulated in irradiated tumor/stromal cells, suggestive of increased protein synthesis. Radiotherapy enhances protein synthesis in an mTOR-dependent manner and increases peptide presentation by MHC and tumor cell immunogenicity (38). We accordingly found that mTOR inhibition reduced the therapeutic efficacy of our RIT
regimen (22). Finally, processes that were altered nontranscriptionally may have allowed increased CTL efficacy in the irradiated tumor in our experimental setting.

In the rapidly developing RT field, tumor cell destruction by radiotherapy is seen as a mode of vaccination, due to the release of antigens and 'danger' signals. Thus, the field emphasizes the potential of radiotherapy to contribute to CTL priming, which may result in systemic antitumor immunity and "abscopal effects" on nonirradiated tumor masses, when adequately supported by additional interventions (35, 36, 47). Radiotherapy may help to release danger-associated molecular patterns such as calreticulin or HMGB1 and/or cytosolic double-stranded DNA that can activate type I IFN signaling (36). Such signals activate DCs from a "tolerogenic" into an "immunogenic" state (48). In tumors that fail to deliver sufficient tumor antigens to DCs de novo, radiotherapy-induced debulking of the tumor could help to reach the "antigen threshold" required for inducing a CTL response. Our study emphasizes that radiotherapy also modulates the TME to overcome T-cell suppression. Combination of immunotherapy and radiotherapy may lead to regression of tumor masses outside of the field of radiation (e.g., refs. 49, 50). However, to qualify systemic tumor regression as "abscopal" effect of radiotherapy, it is required that radiotherapy also contributes to the systemic treatment effect, that is, is synergistic with immunotherapy. Most likely, this can only be achieved when T cells are newly primed as a result of the combined treatment and exert their cytotoxic activity within the nonirradiated tumor.

We show that low-dose cisplatin can facilitate CTL activity in nonirradiated AT-3 tumors in mice treated with PD-1/CD137-targeting therapy, thereby functionally mimicking the immunomodulatory effects of radiotherapy. On the basis of our findings, "repurposing" cisplatin at low-dose as an immunomodulatory drug may help to convert a CTL-suppressive TME into a CTL-permissive one. It will be of interest to test whether the immunomodulating effects of radiotherapy and cisplatin that are revealed here and their effective combination with CD137/PD-1 targeting therapy can also lead to increased and systemic antitumor effects in other mouse tumor models, such as the poorly immunogenic MMTV-PyMT model (19). In general, our findings indicate that systemic tumor control may be achieved by combining immunotherapy protocols that promote T-cell priming with chemoradiation protocols that permit CTL activity in both the irradiated tumor and (occult) metastases.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: P. Kroon, M. Verheij, J. Borst, I. Verbrugge
Development of methodology: P. Kroon, T.N. Schumacher
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P. Kroon, E. Frijlink, V. Iglesias-Guiramais, I. Verbrugge
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): P. Kroon, E. Frijlink, V. Iglesias-Guiramais, A. Volkov, M.M. Van Buuren, J. Borst, I. Verbrugge
Writing, review, and/or revision of the manuscript: P. Kroon, E. Frijlink, A. Volkov, M.M. Van Buuren, M. Verheij, J. Borst, I. Verbrugge
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P. Kroon, E. Frijlink
Study supervision: J. Borst, I. Verbrugge

Acknowledgments
We thank animal facility personnel for mouse husbandry, J.-J. Sonke and A. Khmelinski for help in the small-animal radiotherapy facility, the Intervention Unit for help with the mouse experiments, R. Kerkhoven, A. Velds, M. Nieuwenland, and I. de Rink of the genomics core facility for assistance and bioinformatics support with RNA sequencing, H. Yagiya (Juntendo School of Medicine, Tokyo, Japan) for anti-CD137 (3H3), M. Toebes for assistance in generating tetramers, H. Hillman and D. El Attnoiou at the Peptide Production Facility for generating the peptides, J. Walker and N. Groene for technical assistance, K. de Visser and the Borst laboratory for helpful discussions, M. van den Broek for critical reading of the manuscript. Supported by the Dutch Cancer Society (grants NKI 2013-5951, 10764, to I. Verbrugge; 10894, to I. Verbrugge and J. Borst) and a Health Holland public-private partnership grant in collaboration with Elekta (grant L5HM15036; to J.-J. Sonke).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 18, 2018; revised December 18, 2018; accepted February 11, 2019, published first February 19, 2019.

References
Radiotherapy and Cisplatin Increase Immunotherapy Efficacy by Enabling Local and Systemic Intratumoral T-cell Activity


Updated version: Access the most recent version of this article at: doi:10.1158/2326-6066.CIR-18-0654

Supplementary Material: Access the most recent supplemental material at: http://cancerimmunolres.aacrjournals.org/content/suppl/2019/02/19/2326-6066.CIR-18-0654.DC1

Cited articles: This article cites 50 articles, 18 of which you can access for free at: http://cancerimmunolres.aacrjournals.org/content/7/4/670.full#ref-list-1

Citing articles: This article has been cited by 4 HighWire-hosted articles. Access the articles at: http://cancerimmunolres.aacrjournals.org/content/7/4/670.full#related-urls

E-mail alerts: Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions: To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions: To request permission to re-use all or part of this article, use this link http://cancerimmunolres.aacrjournals.org/content/7/4/670. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.