Low Dose Radiotherapy Reverses Tumor Immune Desertification
and Resistance to Immunotherapy

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Abstract

Developing strategies to inflame tumors is critical for increasing response to immunotherapy. Here we report that low-dose radiotherapy (LDRT) of murine tumors promotes T-cell infiltration and enables responsiveness to combinatorial immunotherapy in an interferon-dependent manner. Treatment efficacy relied upon mobilizing both adaptive and innate immunity and depended on both cytotoxic CD4$^+$ and CD8$^+$ T cells. LDRT elicited predominantly CD4$^+$ cells with features of exhausted effector cytotoxic cells, with a subset expressing NKG2D and exhibiting proliferative capacity, as well as a unique subset of activated dendritic cells expressing the NKG2D ligand Rae1. We translated these findings to a phase I clinical trial administering LDRT, low-dose cyclophosphamide and immune checkpoint blockade to patients with immune desert tumors. In responsive patients, the combinatorial treatment triggered T-cell infiltration, predominantly of CD4$^+$ cells with Th1 signatures. Our data support the rational combination of LDRT with immunotherapy for effectively treating low-T cell infiltrated tumors.

Statement of significance: Low dose radiation reprogrammed the tumor microenvironment of tumors with scarce immune infiltration and together with immunotherapy induced simultaneous mobilization of innate and adaptive immunity, predominantly CD4$^+$ effector T cells, to achieve tumor control, dependent on NKG2D. The combination induced important responses in patients with metastatic immune-cold tumors.
Introduction

Response to immune checkpoint blockade (ICB) is robust and durable in a proportion of patients. Patients with so-called cold or immune desert tumors are however less likely to respond to ICB (1,2). Important efforts are thus underway to identify effective and feasible approaches for inflaming these tumors (3). Moreover, the inherent plasticity of tumors and their microenvironment enables dynamic upregulation of a diverse range of inhibitory mechanisms, hence necessitating combinatorial treatment approaches to sustain tumor control by T cells.

Along with its direct tumoricidal effects, hypofractionated (high-dose) radiation therapy (RT) can mediate important immunomodulatory effects including, (i), in situ vaccination through release of tumor-associated antigens (4); (ii) the activation of dendritic cells (DCs) (5); (iii) the release of danger signals and the upregulation of cytokines and chemokines (6); and (iv) normalization of the tumor vasculature (7). In addition, local RT can activate deoxyribonucleic acid (DNA) sensing pathways in host (5) and tumor cells (8), triggering production of type I interferon (IFN) (9) and mobilizing innate and adaptive immunity. Numerous studies have convincingly shown that RT can promote T-cell generation, migration into the tumor bed, tumor-cell recognition, and effector function (3). Moreover, hypofractionated RT may trigger systemic antitumor immunity enabling control of distal metastases, the so-called abscopal effect, (3) and synergizes with ICB in preclinical studies (5,10-13) and in patients (4,14).

The effects of LDRT (i.e., up to 2 Gy per fraction) remain largely unexplored in the context of cancer immunotherapy. Early evidence in a mouse model of localized neuroendocrine pancreatic tumors suggested that low-dose irradiation (LDRT, i.e., 0.5-2 Gy) can reprogram the tumor microenvironment, inducing macrophage M1 polarization. In turn, iNOS-positive M1 macrophages produce relevant chemokines to recruit effector T cells, while they induce tumor vasculature normalization and inflammation, allowing T-cell infiltration (15). More recently, Barsoumian et al (16) have shown that high-dose irradiation to primary murine lung tumors combined with low-dose irradiation to secondary metastases and ICB were effective in controlling metastatic tumors through the engagement of innate and adaptive immunity, and downregulation of immunosuppressive TGFβ (16).
We sought to investigate treatment options for ovarian cancer, a disease which has so far eluded ICB combinations. While high-dose RT has been previously used together with ICB against other tumor types, the diffuse spread of ovarian cancer throughout the peritoneal cavity puts the abdominal viscera at risk from conventional RT administered to large abdominal volumes (17,18), which has thus been abandoned due to toxicity. Inspired by previous clinical evidence that weekly low-dose RT can be however administered safely to the entire abdominal cavity (19,20), we tested LDRT for its ability to safely reprogram the TME (21) and facilitate response to immunotherapy in advanced ovarian cancer. Here we present novel evidence that LDRT transiently inflames tumors, rendering them vulnerable to immunotherapy. Since LDRT elicited upregulation of adaptive immune resistance mechanisms in newly inflamed tumors, a rational orthogonal combinatorial immunotherapy approach was pursued to address simultaneously immune checkpoints on effector T cells, T regulatory (Treg) cells and antigen-presenting cells (APCs) to control tumors. The effect of the combination was predicated on simultaneous mobilization of both innate and adaptive immunity. Importantly, high-dimensional interrogation of tumors with single-cell RNA sequencing (scRNAseq) revealed that effective LDRT profoundly reprogrammed the TME, newly enlisting DCs and activated effector CD4\(^+\) and CD8\(^+\) T cells executing a cytolytic transcriptional program that was in part hinged on NKG2D expression. These results were translated in a phase I clinical trial, where LDRT resulted in de novo inflammation, and regression of metastatic solid tumors when combined with orthogonal immunotherapy.
Results

Low-dose whole abdominal radiotherapy induces immune-cell infiltration in advanced orthotopic ovarian cancer

To evaluate the impact of low-dose whole abdominal radiotherapy (LD-WART) in ovarian cancer, we chose the orthotopic intraperitoneal (i.p.) murine ID8 model (22), characterized as being propense to losing intraepithelial T-cells with progression in vivo (23). In order to gauge the schedule of LDRT delivery, we performed experiments looking at the time-course of cell response (Fig. 1A). We ascertained that LDRT induced acute stress in ID8 cells (evidenced by calreticulin exposure), without significant effect on survival in vitro (Supplementary Fig. S1A, B). In vivo it induced tumor-cell DNA damage (revealed by γH2AX foci) without impacting tumor growth (Supplementary Fig. S1C, D). However, 1 Gy irradiation of tumors was sufficient to induce important transcriptional changes in vivo, notably a significant upregulation of inflammation, including IFNα and IFNγ responses; complement activation; IL6/JAK/STAT3 signaling (Fig. 1B); expression of key chemokines known to attract T and NK cells, as well as cross-presenting DCs (23-25) (Fig. 1C, D); and other inflammatory markers (Supplementary Fig. S1E-I).

Advanced ID8 tumors exhibited minimal inflammation at the steady state, but we detected an important influx of lymphocytes, NK cells, macrophages and DCs on day 5 post-RT by gene signatures, validated by immune staining on day 7 following 1 Gy-RT (Fig. 1E, F). At the steady state, there were very few intraepithelial CD8+, CD4+ or CD11b+ cells, with most immune cells largely confined to the peritumoral stroma, while post-RT CD8+, CD4+ and CD11b+ cells localized in the intraepithelial tumor compartment (Supplementary Fig. S1J). Comparing 0.5, 1 or 2 Gy-RT by immunohistochemistry, we observed the highest infiltration of CD8+, CD4+, and CD11b+ cells and the highest CD8+:Foxp3+ cell ratio following 1 Gy (Fig. 1G & Supplementary Fig. S1J); this dose was thus chosen for all subsequent experiments. T-cell inflammation tended to subside within a week, but repeat administration of 1 Gy in weekly intervals resulted in sustained recruitment of immune cells into ID8 tumors (Supplementary Fig. S1K) and was used in all subsequent experiments. T-cell influx was specific to tumor deposits, as we did not observe any changes in T-cell content in retroperitoneal lymph nodes, nor in the spleen, which also received 1 Gy-RT (Supplementary Fig. S1L). Demonstrating its dependence on IFN signaling, infiltration of
CD8$^+$ cells was abrogated in vivo by IFN$\alpha$ receptor blockade or IFN$\gamma$ depletion (Fig. 1H). Thus, cyclical LD-WART reprograms the TME to inflame advanced ID8 tumors in a mechanism involving IFN activation.

**Metronomic radiotherapy confers tumor responsiveness to combinatorial immunotherapy**

We sought to develop a combinatorial treatment strategy addressing immune targets upregulated by LDRT. Thus, we administered ICB with $\alpha$PD1 and $\alpha$CTLA4 blocking Ab, to activate T cells given the detection of increased $Pd1$ and $Ctla4$; agonistic $\alpha$CD40 Ab, to activate APCs (26) given the increase in $Cd40$ (Supplementary Fig. S2A); and low-dose cyclophosphamide (CP), which attenuated Treg cells (27) (Supplementary Fig. S2B). The combinatorial treatment comprising CP on day 0, and LD-WART, ICB and $\alpha$CD40 antibody on day 1 (henceforth dubbed radio-combinatorial immunotherapy, RACIM), was administered weekly, thrice (Fig. 2A), starting at a time when mice had obvious i.p. ID8 tumors by imaging (luminescence). Strikingly, 83.5% of mice receiving RACIM exhibited tumor response by imaging while on therapy (14% complete response and 11% deep partial response with 97-98% reduction) by day 20. Most mice with partial response however progressed after discontinuation of therapy (Fig. 2B & Supplementary Fig. 2C), with median overall survival of 69 days (Fig. 2C). On day 90, all surviving mice were disease-free by imaging and by pathologic examination, yielding an overall cure rate of 15%. Strikingly, the combination immunotherapy lacking LDRT (i.e. CP+ICB+$\alpha$CD40, dubbed CIM) exhibited no therapeutic effect (0% response or tumor cure; median survival 50 days) (Fig. 2B, C). RACIM therapy was associated with no obvious toxicity; mice experienced no weight loss nor systemic inflammation (Supplementary Fig. S2D, E). Importantly, foregoing LDRT at the second and/or third cycle reduced the therapeutic efficacy of the combination (Supplementary Fig. S2F).

We validated the RACIM treatment in the subcutaneous (s.c.) Lewis Lung Carcinoma (LLC) model, which is reportedly non-responsive to ICB (28) and exhibits scarce T-cell infiltration (29). We found that unlike early LLC tumors (∼10 days, ∼100 mm$^3$), advanced tumors (∼15-20 days, 200-400 mm$^3$) are depleted of T cells (Supplementary Fig S2G). We treated s.c. LLC tumors of 300-350 mm$^3$ with RACIM and observed significant control and survival
benefit, similarly to the ID8 model (Supplementary Fig. S2H). Thus, orthogonal combinatorial immunotherapy leveraged the immunomodulatory effect of LDRT and led to marked therapeutic response in advanced low T-cell infiltrated tumors.

We used gene expression analysis (NanoString) to analyze the effects of RACIM 48 hrs. after cycle 2 (i.e., the peak of therapeutic response) in ID8 tumors. We observed significant upregulation of genes associated with T cells, monocytes, and DCs following RACIM relative to CIM or to untreated control tumors (Fig. 2D). By immune staining, we confirmed a marked influx of CD11b⁺ myeloid, CD8⁺ and CD4⁺ T cells following RACIM or LDRT relative to CIM or control tumors (Fig. 2E), with most pronounced infiltration of CD11b⁺, CD8⁺ and CD4⁺ T cells seen after RACIM.

To unveil the individual contributions of the components of RACIM, we administered take-one-out combinations, i.e. RACIM without one component. Importantly, the frequency of CD4⁺ and CD8⁺ T cells decreased significantly when ICB was omitted (Fig. 2F). Underscoring the key role of effector T cells, no animal cure was observed when ICB was omitted from RACIM (Fig. 2F). Furthermore, highlighting the importance of attenuating Treg with RACIM, we observed a significant increase in Tregs and a decrease in the CD8/Foxp3 ratio, and no mouse cure when CP was omitted (Fig. 2G). Notably, in the absence of agonistic αCD40 we found a decrease in the frequency of MHC-II expressing CD11b⁺CD11c⁺ cells and an increase in M2 macrophages (F4/80⁺CD206⁺, Fig. 2H), confirmed by a significant decrease in Nos2 expression in CD11b⁺ cells (Supplementary Fig. 2I). Each perturbation of RACIM converged to a common effect, i.e. collapse of effector T cells, revealed by a significant decrease in Tnfa and Ifng gene expression in ID8 tumors (Fig. 2I). Hence, leveraging the proinflammatory effect of LDRT, RACIM exhibited a dramatic therapeutic effect on originally low-T cell inflamed tumors, and all components of the combinatorial treatment contributed to mobilizing an effective antitumor immune response.

**RACIM expands tumor-rejecting CD4⁺ and CD8⁺ TILs with activation and exhaustion features**
We sought to understand whether in addition to important quantitative differences in T-cell infiltration, the curative effects of RACIM were further associated with qualitative differences in the TILs. We analyzed TILs five days after the second LDRT cycle by scRNAseq and used ProjectTIL (30) to assign T cells to previously described T-cell states. We identified nine distinct T-cell states (Fig. 3A). TILs from tumors treated with RACIM were highly enriched in activated effector T-cell populations, which were assigned largely to exhausted (Tex), progenitor-exhausted (Tpex) and effector-memory (T_{EM}) states (Fig. 3A) (31). The Th1/Treg as well as the Tex/Treg cell ratio were significantly increased by RACIM over CIM or control (Supplementary Fig. S3A). Relative to CIM-treated tumors, CD4⁺ Tex cells were the most differentially expanded following RACIM (Fig. 3B). Importantly, by concomitant T-cell receptor sequencing (TCRseq) we determined that CD4⁺ Tex and Tpex were the most clonally expanded cells, suggesting tumor specificity (Supplementary Fig. S3B) (31-34). Within the top expanded CD4⁺ T cell clonotypes, we found the same clonotype largely in a Tex or Tpex state, but less frequent cells from the same clonotypes were also T_{EM}, early activated, T helper 1 (Th1) or follicular helper (Tfh) cells (Supplementary Fig. S3B), suggesting that CD4⁺ Tex cells may evolve from all these precursor states. Indeed, among all expanded clonotypes, CD4⁺ Tex and Tpex compartments shared numerous T cell clones, and both Tex and Tpex shared clones with the T_{EM}, Th1 or Tfh compartments (Fig. 3C). To the best of our knowledge, precursor states for exhausted CD4⁺ TILs have not been described to date.

CD4⁺ Tex TILs following RACIM were characterized by significantly higher expression relative to CIM of Ifng, Prf1, and Gzmb, associated with effector function and cytolytic capacity; Pdcd1, Lag3, Haver2 and Tox, associated with exhaustion; and costimulatory receptors Cd28, Cd27, Icos and Tnfrsf4; while lacking expression of Tcf7 and Tbet transcription factors, indicating a terminal Tex state (Fig. 3D, E). Pseudotime analysis supported a state evolution model in which pre-exhausted Th1-like cells differentiate into intratumoral CD4⁺ Tex cells through an intermediate CD4⁺ Tpex state (Fig. 3F). Along this trajectory, there was gradual upregulation of exhaustion associated genes (Tox, Nr4a2, Pdcd1, Haver2, Lag3, Tigit), chemokines/chemokine receptors (Ccl2, Ccl3, Ccl5, Cxcr6), and cytotoxicity related genes (Prf1, Klrd1, Nkg7), along with downregulation of progenitor-associated genes (Tcf7 and Il7r). Notably, in the intervening Tpex state we observed peak gene expression of the chemokine Xcl1, as well as of various activation markers (Tnfsfr9/CD137, Cd81, Cd200 and Crtam) (Supplementary Fig. S3C).
We confirmed by flow cytometry a significant enrichment in CD4+PD1+TCF1− cells expressing markers of exhaustion and costimulatory receptors in RACIM-treated tumors (Supplementary Fig. S3D-S3F). We also identified a higher frequency of functional CD4+PD1+TCF1− TILs expressing IFNγ, with a small fraction of them also expressing IL-2, TNFα or GzmB (Fig. 3G). Finally, we confirmed polyfunctional cytokine production ex vivo upon stimulation in a proportion of CD4+ Tex cells (Fig. 3G).

These findings suggest that RACIM recruits a large amount of oligoclonal CD4+ cells to tumors, which acquire exhaustion but also exhibit important effector functions. We thus asked whether these cells partake in the therapeutic effect of RACIM. Indeed, CD4+ depletion abrogated the therapeutic effect of RACIM, with no cures seen (Fig. 3H).

We also identified CD8+ TIL populations exhibiting Tpex and Tex states with higher exhaustion, costimulation and effector function following RACIM relative to CIM (Fig. 3I, J). Similar to their CD4+ counterparts, RACIM-treated tumors harbored the highest proportion of expanded CD8+ T-cell clonotypes, with the largest clonal expansion observed among CD8+ Tex cells (Fig. 3K), which shared numerous TCR clones mostly with the CD8+ TEM compartment (Fig. 3L). Expansion of PD1+TCF1− CD8+ TILs expressing coinhibitory receptors but also CD28, CD27 and CD40L was confirmed by flow cytometry analysis (Supplementary Fig. S3G-S3I). Relative to CIM, CD8+PD1+TCF1− TILs from RACIM-treated tumors comprised higher frequencies of functional cells secreting Gzm, IFNγ and/or TNFα (Fig. 3M), and TOX+ cells were still functional upon ex vivo restimulation (Fig. 3M). We confirmed the critical contribution of CD8+ T cells to the therapeutic efficacy of RACIM, as animals previously depleted of CD8+ T cells with anti-CD8 Ab lost significant survival benefit (Fig. 3N). Thus, LDRT synergizes with combinatorial immunotherapy by enlisting T cells, and specifically by expanding a population of activated, functional effector CD4+ and CD8+ T cells with tumor-rejecting capacity which acquire features of progenitor exhausted and exhausted cells in the TME, consistent with effective antigen engagement.

RACIM reprograms tumor antigen-presenting cells and enlists NKG2D as a key costimulatory signal
We next examined the effects of therapy on the myeloid compartment. Single-cell RNAseq analysis of CD11b+ cells from untreated, CIM- or RACIM-treated tumors revealed 29 myeloid cell transcriptomic states, which we could assign to three main populations: macrophages, DCs and monocytes (Supplementary Fig. S4A) (35). Therapy drove important reprogramming of the tumor myeloid compartment (Fig. 4A), with RACIM inducing profound changes in all three myeloid populations relative to CIM alone (Fig. 4B).

Canonical macrophages have been classified as M1 and M2, with purported pro-inflammatory and anti-inflammatory/immunosuppressive roles, respectively (36). We identified 22 monocytes/macrophages clusters according to Immgen signatures (37) (Fig. 4A and Supplementary Fig. S4B). Macrophages at baseline could be assigned collectively to M2 as they expressed Mrc1 (CD206) (Supplementary Fig. S4C). Conversely, CIM or RACIM induced a clear shift in macrophage states, among which we could identify M1 macrophages expressing Nos2 (iNOS, Supplementary Fig. S4D). Differential gene expression analysis showed a further shift in macrophage transcriptional programs by RACIM relative to CIM, with upregulation of genes linked to the inflammasome pathway (Malat1, Tnfrsf1a/TNFα, Cd14, Lrp1, Dusp1, C3ar1, Calr, Itgb2, Nr4a, Il1b, Nfkia); glycolysis (mt-Nd2); antigen presentation (H2k1, H2d1); type-I IFN (Irf8, Ifrd1); IFNγ sensitivity (ifngr1); chemotaxis and leukocyte migration (Icam1, Cxcl10, Cxcl2, Ccr2); and wound repair (Socs3, Klf6, Gna12), in addition to downregulation of M2 genes (Cd5l), fatty acid metabolism and prostaglandin synthesis (Fabp5, Prdx1, Tmsb4x), and iron-induced oxidative stress (Ftl1, Fth1, Ftl1-ps1, Prdx1; Supplementary Fig. S4E). Moreover, RACIM-associated macrophages displayed higher Cd40 and Cd86 (Supplementary Fig. S4F). The above changes were corroborated by flow cytometry, with a substantial reduction of Ly6G- CD11b+F480+CD206+ M2 macrophages observed following RACIM (Supplementary Fig. S4G).

We also found different intratumoral DC states, which could be further annotated as being either conventional (c)DC1 (Xcr1hi; cluster 25), cDC2 (Ilr2hi; cluster 21), cDC2/mono-like (Mo)DC (cDC2/MoDC; Clec10ahi; clusters 9 and 19), cDC3 (Ccr7hi; cluster 23), or plasmacytoid (p)DC (Cox6a2hi; cluster 28) (Fig. 4C & D). These resembled DC states previously reported in other mouse tumor models, indicating that similar to their human counterparts (35,38,39), mouse DC states are largely conserved across tumor
types (Supplementary Fig. S4H). However, the cDC2/MoDC state (clusters 9 and 19) was specifically associated with RACIM treatment in the ID8 tumor model (Fig. 4C & D).

Although typically found at low frequency in tumors, Batf3-expressing cDC1 cross-present efficiently tumor antigens to CD8+ T cells (40) and are critical mediators of antitumor immunity and response to ICB (41). We found cDC1 (cluster 25) in RACIM-treated tumors, while they were almost absent in CIM (Fig. 4C & E). Notably, while cDC1 were also present in control tumors, (Fig. 4C & E) upon RACIM-treatment they exhibited higher expression of MHC class I (H2K1 and H2D1), suggesting an improved ability to cross-present antigens (Fig. 4F). Batf3 is a key transcription factor driving the development of cDC1 (40,42,43). To assess the contribution of Batf3 to tumor control, we administered RACIM to Batf3−/− mice. Loss of Batf3 abrogated the therapeutic benefit of RACIM (Fig. 4G). Since tumor control associated with Batf3-expressing DCs depends on T-cell migration from draining lymph nodes to tumors (44), we administered fingolimod (FTY720), an inhibitor of lymphocyte egress from lymph nodes. This also abrogated the effect of RACIM (Fig. 4H). TCF1−PD1+CD8+ cells were decreased in the tumors of Batf3−/− mice, indicating that cross-presenting DCs serve to maintain the pool of terminally differentiated effector TCF1− cells (Supplementary Fig. S4I).

Given the dramatic expansion of CD4+ tumor-rejecting TILs upon RACIM, we next interrogated cDC2 (cluster 21) and cDC2/MoDC (clusters 9 & 19), which may interact with CD4+ T cells and support their antitumor activity (45). We found that RACIM induced profound reprogramming of the DC compartment which explains its impact on adaptive immunity seen above (Fig 4C). For example, RACIM suppressed DC cluster 21, enriched in anti-inflammatory genes such as Mt, Tgfb1, and Nr4a2, while it expanded clusters 9 and 19, enriched in genes involved in MHC class-I presentation such as Psmb8/10, Psma1/4/5/7 and Tap1/2; Cd40 and Cxcl9/Cxcl10; and genes related to type-I IFN (Gbp2, Isg15, Ift205, Irf7, Irf8) associated with antitumor immunity (46) (cluster 9); and class-II presentation such as H2Ab1, H2Aa, H2DMA, and Cd74 (cluster 19) (Fig 4I).

We confirmed the important shifts in DCs induced by RACIM via flow cytometry, as we found a significant increase in activated CD11b+CD11c+MHC-II+CCR2+ DCs (Supplementary Fig. S4G) with increased co-expression of costimulatory ligands CD40, CD70, CD80 and CD86 relative to CIM (Supplementary Fig. S4J). Importantly, we noted
that RACIM expanded cDC1 and cDC2 expressing the stress response marker Rae1, a ligand to the natural killer group 2D (NKG2D) costimulatory receptor (Fig. 4J). Rae1 upregulation was quite specific, since we did not detect transcripts for other NKG2D ligands in the DCs. This was corroborated by flow cytometry and tissue immune staining, where we identified higher levels of RAE1 on CD11b+CD11c+MHC-II+ DCs (Fig. 4K, L) in RACIM-treated tumors.

We thus asked whether the NKG2D receptor was upregulated in tumor-rejecting lymphocytes mobilized by RACIM. We found by scRNAseq that both CD4+ and CD8+ Tex cells significantly upregulated Klrk1 (NKG2D), in RACIM-treated tumors (Fig. 4M & N). By flow cytometry we confirmed that CD4+TCF1+PD1+ T cells, and to a lesser extent CD8+TCF1+PD1+ TILs from RACIM expressed significantly higher levels of NKG2D relative to CIM-treated tumors (Fig. 4O & P).

NKG2D serves as an important costimulatory receptor for effector T cells in peripheral tissues (47,48). We observed significantly increased Ki67 expression in NKG2D+ TCF1− PD1+ CD4+ and CD8+ T cells as compared to their NKG2D− counterparts (Fig. 4Q & R), indicating that NKG2D expression defines a subset of canonical CD4+ or CD8+ exhausted TILs that retain proliferative capacity. Remarkably, the increase in NKG2D expression in CD4+PD1+ T cells, and to a lesser extent CD8+PD1+ T cells, was abrogated when anti-CTLA4 or anti-PD1 blockade were omitted from the treatment cocktail (Fig. 4S & T), revealing the important interdependencies that underpinned the synergies in RACIM. We thus asked whether NKG2D supports the function of tumor-rejecting T cells upon RACIM. Indeed, NKG2D blockade by antibody attenuated NKG2D+TCF1+PD1+ CD4+ as well as CD8+ Tex cells (Fig. 4O & P) and abrogated the therapeutic efficacy of RACIM (Fig. 4U), without however affecting RAE1-expressing DCs (Fig. 4K).

**Low-dose radiotherapy combined with immune checkpoint blockade induces responses in advanced immune desert human tumors**

We sought to translate the above advances to the clinic. We thus conducted a phase I clinical study (RACIN, NCT03728179), details available in (Supplementary Notes), in which patients with solid tumors exhibiting <5 intraepithelial CD8+ cells per high power field
(HPF), otherwise interpreted as “immune desert” tumors, were treated with LDRT (at 0.5 or 1 Gy per fraction, every two weeks, total dose 6 Gy or 13 Gy, respectively) delivered to all (target and non-target) metastatic deposits in combination with ICB, which similar to the mouse comprised low-dose CP (200 mg/m² every two weeks) to attenuate Treg cells (27), combined with anti-PD1 (nivolumab), 240 mg every two weeks (q2wk), and anti-CTLA4 (ipilimumab), 1 mg/kg every 6 weeks (q6wk), for up to 24 weeks. Doses of ipilimumab and nivolumab were inspired by the previous Checkmate 227 study (49), which demonstrated clinical activity of ipilimumab 1mg/Kg q6wk and nivolumab 3mg/Kg q2wk in patients with advanced non-small-cell lung cancer (NSCLC). The 240 mg flat dose q2wk of nivolumab was subsequently approved by the Food and Drug Administration as equivalent to the 3mg/kg q2wk dose (50).

In the absence of available drugs to activate myeloid cells, we elected to administer at least aspirin (300 mg orally, daily) to suppress prostaglandin E₂ (PGE₂), a major immunosuppressive factor elaborated by tumor myeloid cells (51). Following completion of the 4 cycles with ipilimumab/nivolumab, all eligible patients could receive nivolumab (240 mg q2wk) with daily aspirin till progression or toxicity. LDRT was applied to all metastatic deposits identified by radiologists as pathologic, sparing the bone marrow as much as possible (52). We treated all patients with the same dose of CIM but varied the dose of RT: The first three patients received 0.5 Gy and 5 additional patients received 1 Gy at each lesion (Fig. 5A). The primary endpoint was dose-limiting toxicity (DLT), defined as grade 4 or worse by Common Terminology Criteria of Adverse Events (version 4.03), during the period from cycle-0/day-1 (C0D1) to C2D1, eight weeks later (Supplementary Table S1).

Eight immunotherapy-naïve patients with advanced metastatic prostate (n=4), high-grade serous ovarian (n=2) or gastrointestinal tract tumors (n=2), with no other therapeutic options after a median of three prior lines of chemotherapy, were recruited between March and August 2019. The median number of intraepithelial CD8⁺ cells at baseline was 2.4 cells (range 2-4) per HPF, and 7 patients (87.5%) had <1% PDL1⁺ tumor cells at baseline. The median number of non-synonymous somatic mutations per Mb for the tumors was 2.41 Mb (range: 0-6.9). None of the tumors exhibited mismatch repair deficiency or BRCA mutation. At data cut-off 17th August 2020, the median follow-up was 11.9 months (range 4.4 – 17.2 months). Patient and tumor characteristics are summarized in (Supplementary Table S2).
Adverse events (AEs) of any grade occurred in all 8 patients, the most frequent being related to clinical investigations or gastrointestinal events (Supplementary Table S3). None of the adverse events were attributed to LDRT. Immune-related serious adverse events (SAE) of grade 3 or more occurred in two patients (25%). A grade 3 colitis occurred after two cycles of combination treatment in a prostate cancer patient. The patient was taken off study and was treated with methylprednisolone (1 mg/kg i.v. bolus once), followed by oral prednisone 25 mg/day for 2 months. The patient progressed based on bone scan one month after treatment discontinuation. Two months after the initial episode, the patient developed off-study a second episode of grade 3 colitis, managed with infliximab (5mg/kg i.v. once), oral steroids and vedolizumab (300 mg i.v. thrice), with complete resolution. A grade 4 myocarditis occurred during the first cycle of treatment in another prostate cancer patient (the only DLT). The patient (1EEY) was taken off study and was treated with intravenous methylprednisolone (1 gr/day for 5 days) followed by oral prednisone 1 mg/kg, in association with mycophenolate mofetil (1 gr for 5 days orally, followed by 500 mg for 30 days). A pacemaker was implanted. As troponin levels remained elevated after 20 days of immunosuppressive treatment, 3 doses of tocilizumab (8 mg/kg) were administered with resolution of the event. The patient died 3 months after treatment discontinuation. One more patient with gallbladder cancer died during the study, both deaths attributed to disease progression. Two other deaths also occurred due to other reasons.

Ipilimumab alone was discontinued in two prostate cancer patients (25%) for G2 colitis and G3 hepatitis, respectively, both with slow improvement on oral steroids. In both cases, toxicity occurred after two cycles of treatment and did not recur with the continuation of radiation, CP and nivolumab. Only one patient was eligible for maintenance with nivolumab and aspirin, which was discontinued after one cycle due to disease progression.

Tumor responses were evaluated by Immune Response Evaluation Criteria in Solid Tumours (iRECIST) (53), and when available, relevant serum tumor markers. We observed tumor size reduction in targeted irradiated lesions in three patients overall (37.5%) (Fig. 5B). Four other patients experienced SD, with overall disease control rate (PR+SD) of 87.5%, while one patient (12.5%) had confirmed disease progression (Fig. 5C). The overall response rate by iRECIST was 12.5%, with 1 out of eight patients achieving PR. Notably, a patient with prostate cancer and another with ovarian cancer were regarded as SD and PD, respectively, by iRECIST, but all irradiated lesions demonstrated dramatic metabolic response in fields
that received LDRT, based on molecular imaging with $^{68}$GaPSMA-PET and $^{18}$FDG-PET/CT, respectively. In both cases disease progression was related to new lesions emerging uniquely outside of the irradiated areas (Fig. 5D and E). Another gallbladder cancer patient had PR according to iRECIST; this patient subsequently progressed, also outside the irradiated volume (Fig. 5F). Emphasizing the importance of irradiating all lesions, we observed tumor progressions only outside the irradiated volumes in all three patients where responses were documented by imaging (Fig. 5G). Changes in radiographic appearance of tumors overtime, biochemical responses, radiation dosimetry and the location of new metastases are illustrated in (Fig. 5D-G).

**Immune desert tumors are reprogrammed following low-dose radiotherapy**

We sought to assess whether LDRT induced similar biological effects in human tumors as in the mouse tumor model. We analyzed biopsies obtained from the same metastatic deposits at baseline and 7-10 days following the first administration of LDRT, prior to initiating ICB. We considered as responding tumor lesions those that exhibited subsequent reduction in size following combined LDRT-immunotherapy and compared responding tumor lesions from three patients who experienced PR or SD (patient 19F7 with cholangiocarcinoma, 1EEY with prostate cancer and 02F5 with ovarian cancer in Fig. 5B) to non-responding lesions from four other patients. Similar to the mouse model, we observed a marked influx of T cells, which was mainly composed of CD4$^+$ cells in responding tumors (representative images in Fig. 6A). Differential gene expression analysis of matched pre- and post-irradiation biopsies revealed different patterns of response to LDRT in responding vs. non-responding tumors. For example, in responding tumors, LDRT triggered activation of DNA damage response ($POLB$, $NEIL1$, $MLH1$), type-I IFN response ($TBK1$, $IFIH1$, $EIF2AK$), immune-cell activation ($NFKB$, $NTF3$, $ATF1$), antigen presentation and innate immune activation ($CD83$, $CIQBP$, $ZIC2$) as well as TCR activation and effector-memory ($CD44$, $SOS1$, $RICTM$), while we also detected downregulation of genes related to epithelial-mesenchymal transition ($COL1A2$, $COL1A1$, $PLAT$, $COL6A3$, $COL5A1$, $FN1$). Conversely, non-responding tumors upregulated genes associated with immune suppression ($HAVCR2$, $IL10$, $LILRB4$ and $LAIR1$) and downregulated genes associated with DNA repair ($PRKDC$, $RAD21$, $RAD50$, and $DDIT4$), and inflammation ($IL6$, $ISG20$, $IFNB1$, $TNFA$, Fig 6B and Supplementary Fig. S5A). Furthermore, responding tumors exhibited a significant increase in Th1 signatures following LDRT, while non-responding tumors were characterized by increasing M2 macrophage and
tolerogenic DC signatures (Fig 6C). Thus, like in mice, LDRT successfully reset in responding tumors the immune TME, recruiting innate and adaptive immune cells. Indeed, we noted an important overlap in the gene signatures of responding mouse and human tumors, with Th1 signatures reaching statistical significance in both (Fig 6D).

We used spatial transcriptional profiling (GeoMx) to test whether immune activation in responding tumors involved the intraepithelial tumor compartment. We confirmed that spatial transcriptional profiling accurately captured immune cells by correlating gene expression data and cell counts of CD3$^+$ and CD8$^+$ cells by multispectral immunofluorescence (mIF) (Supplementary Fig. S5B). We acquired topologic transcriptional immune profiles of mIF-guided regions of interest (54) from responding and non-responding tumors, profiling tumor islets and stroma at baseline and following LDRT. Using single-sample gene-set enrichment (ssGSEA) analysis of such signatures (55), we observed distinct immune profiles associated with therapeutic outcome. Responding tumors exhibited an increase in Th1, CD8$^+$ and T$_{EM}$ signatures located mainly in tumor islets following LDRT (Fig. 6E). Conversely, non-responding tumors displayed an upregulation of M2 macrophage and neutrophil signatures following RT, which were detected mainly in tumor stroma (Fig. 6E).

Reinvigorated clonal T-cell responses have been reported in peripheral blood of patients undergoing successful ICB (56). Since in the mouse we observed that mobilization of antitumor T cells from lymph nodes was necessary for response to the combined treatment, we used deep sequencing to evaluate TCR repertoire changes in peripheral blood following LDRT. We observed increased TCR clonality, confirmed by a reduced Shanon entropy index, and a significant increase in inequality of clonal frequencies by Gini coefficient, all revealing the mobilization of expanded peripheral blood T-cell clones following LDRT, specifically in patients with responding tumors (Fig. 6F, and Supplementary Fig. S5C). Furthermore, we noted a significant increase in the frequency of the largest dominant TCRs clones between pre- and post-LDRT blood samples in patients with responding tumors. Thus, similar to mouse, RACIN mobilized systemic immunity successfully in patients who benefitted from the combination.

**Discussion**
T-cell exclusion from the TME represents a major mechanism of intrinsic resistance to ICB (1,2). Here we show that LDRT drives T-cell inflammation and creates immune vulnerabilities which can then be successfully exploited with rational combinatorial immunotherapy. While high-dose radiation (>5 Gy per fraction delivered to small tumors as stereotactic treatment) increases antigen release and presentation, and primes immune cells (3), we chose to deliver LDRT (i.e., doses below the threshold to directly kill cancer cells) to enable administration of large volumes in order to irradiate all metastatic deposits and promote immune-cell infiltration into them without causing toxicity.

Two prior preclinical studies have explored the use of LDRT to enhance immunotherapy, one in combination with adoptive T-cell transfer and one with high dose RT plus ICB (15,16). Here we demonstrate in both the mouse and humans that a dose of 0.5-1 Gy elicits dramatic reprogramming of the TME. In advanced murine ID8 ovarian tumors this included the triggering of DNA damage and IFN response, the upregulation of numerous cytokines and inflammatory chemokines, as well as of druggable targets including immune checkpoints and CD40, thus offering a window of opportunity to rationally intervene with immune modulation. Based on these TME changes upon 1Gy-irradiation, we devised a combinatorial treatment including αCD40 agonist antibody, ICB with PD1 and CTLA4 blockade, and depletion of Tregs via CP (57). We demonstrated that all components of the cocktail (referred to as RACIM) and repeat LDRT were required for therapeutic efficacy. Moreover, deconvolution experiments, along with comprehensive characterization of the immune TME revealed a dynamic interdependence of innate and adaptive immunity activation, a fundamental requisite for eradicating cancers (58).

Given the critical need for ICB in the RACIM cocktail for tumor control in conjunction with LDRT, it is not surprising that adaptive immune mechanisms were central in mediating tumor rejection in mice. Both CD4+ and CD8+ cells emerged as necessary, as the elimination of either compartment led to therapeutic collapse. Importantly, we identified CD4+ TILs with cytolytic features following RACIM in mice. Cytolytic CD4+ T cells recognize cognate peptides in the context of class-II MHC, normally presented by APCs, and have been implicated in antiviral immunity (59,60), autoimmune pathology (61), and recently in antitumor responses in the mouse (45,62,63) and in patients (64,65). Transcription factors such as T-bet and Eomes (66,67), as well as Runx3, ThPOK (68), Hobit (69) and Blimp-1
(70) are involved in the cytolytic differentiation of CD4+ cells. However, cytolytic CD4+ cells with exhausted features have not been described to date. Here we report cytolytic CD4+ cells with polyfunctional effector properties exhibiting features of canonical exhausted cells, including downregulation of Tcf7 and upregulation of Tox and Pdcd1. Importantly, we also detected precursor exhausted CD4+ cells coexpressing Tcf7 and Tox in these tumors. Interestingly, clonotype analysis revealed that the Tpex (Tcf7+Tox+) and canonical Tex (Tcf7- Tox+) CD4+ compartments shared numerous expanded TCR clones, presently interpreted as tumor-specific (71,72), indicating that exhausted cytolytic CD4+ cells derive from such precursors, similar to what has been reported previously for CD8+ T cells (31,32,73). Some of these same clones were also distributed within the TEM, Th1 or Tfh compartments, suggesting that these may also serve as precursors for CD4+ Tex cells. In the CD8+ T-cell compartment, TCF1+PD1+ Tpex cells retain high proliferative potential and undergo long-term self-renewal, while also replenishing the dominant population of TCF1- exhausted effector T cells (34,74). Response to ICB has been in fact associated with the detection of Tpex CD8+ T cells that can proliferate and give rise to polyfunctional TCF1+PD1+CD8+ effector cells (31,75). In this context, Tox ensures stable commitment to the exhausted state (33,76,77). Our data suggest a similar evolution of CD4+ precursor-exhausted cells, and a role in the efficacy of the LDRT/immunotherapy combination.

In mouse and human T cells, NKG2D serves as an important costimulatory receptor (47,48), which enhances CD8+ T-cell cytolytic function (78) and prevents Fas-mediated apoptosis (79). Since its ligands are primarily upregulated in sites of peripheral tissue damage or inflammation, the NKG2D pathway likely plays a key role in regulating effector T-cell responses in the periphery. Interestingly, NKG2D is not expressed by CD4+ T cells at the steady state, but important frequencies of tissue-destructive NKG2D+ CD4+ T cells have been detected in patients with destructive autoimmune disease (80), as well in virally-induced cancers (81). The NKG2D pathway likely plays an important role in tumor immune elimination, since tumors develop numerous mechanisms to evade NKG2D (82-85), and neutralization of soluble NKG2D ligands enhances response to ICB (86). Importantly, we identified a subset of CD8+ and especially CD4+ Tex cells expressing NKG2D in tumors treated with RACIM. These Tex cells exhibited higher proliferative capacity relative to Tex cells that did not express NKG2D, suggesting that polyfunctional features are the result partly of in situ NKG2D costimulation. Induction of NKG2D in CD4+ and to a lesser extent CD8+ Tex cells was dependent on activation by CTLA4 or PD1 blockade, explaining in part how
the interdependency of RT and ICB interventions drove therapeutic synergy in RACIM. Interestingly, NKG2D has been implicated in the acquisition by TIL of the ability to engage tumor target and be retained to radiated tumors upon CTLA4 blockade (87).

The combined treatment also produced profound reprogramming of the myeloid compartment, which explains the effective mobilization of adaptive immunity. DCs underwent important reprogramming upon RACIM, with activation and acquisition of molecular states consistent with competent APCs capable of eliciting T-cell immunity. Importantly, a high frequency of RACIM-induced tumor DCs were found to overexpress the NKG2D ligand RAE1, matching the higher frequency of NKG2D-expressing CD8+ and CD4+ TILs in the same tumors. DCs expressing NKG2D ligands have been described in autoimmune diseases and infection (88), but not in tumors. Our findings support an important functional crosstalk between myeloid cells expressing NKG2D ligands and NKG2D+ T cells driving tumor rejection in the mouse. Indeed, supporting a key role of NKG2D in sustaining immune-rejecting T cells, administration of NKG2D antibody abrogated the efficacy of RACIM.

BATF3-expressing cDC1 have been identified as key APCs for antigen cross-presentation to CD8+ T cells (40) and effective response to ICB (41), while cDC2 are purportedly required to drive CD4-mediated antitumor responses (45). We observed profound reprogramming of both compartments. Consistent with the key role of CD8+ T-cells in our model, the combination of LDRT and immunotherapy lost its therapeutic efficacy in a Batf3−/− genetic background lacking cDC1, where we also observed an attenuated mobilization of CD8+PD1+TCF1− cells in tumors. Importantly, both DC1 and DC2 upregulated NKG2D following RACIM, and indeed a new state of DC2 (cDC2/MoDC), endowed with features of competent APCs and expressing RAE1, emerged in RACIM treated tumors. The reprogramming of the DC compartment was accompanied also by marked repolarization of macrophages, with significant activation and acquisition of states that could be collectively assigned to M1, with important implications for T-cell homing (15). CD40L likely contributed to these changes in an important fashion, as the absence of αCD40 agonist antibody in the cocktail was associated with significantly fewer CD11b+CD11c+MHC-II+ DC2s and more F4/80+CD206+ M2 macrophages, resulting in the collapse of T-cell attack (89-91).
We translated the preclinical findings to the clinic with a pilot study in 8 patients yielding a response rate of 12.5\% by iRECIST, while two additional patients achieved a dramatic response by $^{68}$GaPSMA-PET and $^{8}$FDG-PET/CT, respectively. This is quite remarkable in this patient population (92-94), especially considering that we treated only patients with immune desert tumors. Our pilot study was not designed to directly compare the two RT doses tested. However, even with this limitation, paired biopsies confirmed the proinflammatory effect of LDRT at both doses, compatible with simultaneous activation of innate and adaptive immunity, which was associated with tumor response. While not all immune reprogramming observed in our preclinical study was documented in patient tumors, interestingly, similar to the mouse, the predominant T-cell population infiltrating tumors post-RT were largely CD4$^+$ cells. Although in the mouse we documented the critical role of the NKG2D pathway, unfortunately tumor biopsies in patients were performed as originally planned 7-10 days post RT, to capture immune infiltration changes, but this fell outside of the short window of upregulation of NKG2D ligands observed in the mouse.

Geospatial resolution localized these responses within epithelial tumor deposits, as required for effective tumor control. Consistent with effective mobilization of immunity and migration of T cells from draining lymph nodes seen in the mouse, responder patients exhibited important mobilization of oligoclonal T-cell response in blood immediately after LDRT.

Importantly, higher than expected persistence of side effects was observed with RACIN. Immune-related SAEs in our trial (25\%) were similar to CheckMate 227 (24.5\%) (49). However, in CheckMate 227 only 18\% of the patients discontinued treatment (49), while in our trial all patients with toxicity discontinued treatment after an average of 2 cycles, and immune toxicity was rather refractory to immune suppression. Low-dose CP combined with anti-PD1 has been reported as well tolerated (95), suggesting that the addition of ipilimumab to the combination contributed importantly to immune toxicity. Low-dose CP attenuates human Tregs (57,96,97), while ipilimumab activates systemic effector T cells at the expense of Tregs (98,99). Therefore, this combination may expose important autoimmune vulnerabilities that remain otherwise compensated in patients. Pavlick et al also found that combining low-dose CP (300 mg/m$^2$) with ipilimumab 10 mg/kg q3wk resulted in severe toxicity in 10 melanoma patients, with 40\% grade 4 AEs, including steroid-refractory colitis in three patients requiring anti-TNF\(\alpha\) therapy, and myasthenia gravis in one patient (100).
In summary, we have demonstrated a novel and important synergy between LDRT and rationally developed combinatorial immunotherapy for the treatment of tumors with poor immune infiltration, hinged on simultaneous activation of multiple innate and adaptive immune pathways revealing interdependencies between LDRT and immune modulation. These led to powerful mobilization of antitumor immunity, with both effector CD4+ and CD8+ T cells implicated, which in the mice led to tumor eradication and in patients to regression of lesions that had been included in the radiation plan. Importantly, our preliminary clinical experience suggests the importance of irradiating all metastatic deposits, since in patients who experienced an objective response, we observed durable responses only in irradiated lesions, while lesions that were initially considered non-pathologic (and therefore not radiated) eventually were proven to be metastatic deposits which progressed. Future research should focus on improving the combination strategies to further enhance such synergies and generate important protective memory. For example, in our clinical study we were unable to use a CD40 agonist. To attenuate macrophage suppression, we used aspirin, which prevents prostaglandin E2 (PGE2)-mediated inhibition of DCs, attenuates Tregs and MDSCs (101,102), and reduces endothelial FasL mediated killing of homing effector T cells (51). Although delivering systemic CD40 ligands and anti-CTLA4 antibodies may prove intolerable in combinations in humans, strategies focusing on targeted delivery of these agents in the TME may offer improved approaches to achieve effective and safe immune modulation in combination with enabling LDRT.
Materials and Methods

1. Preclinical Study Experiments

a. Mouse strains and cell lines

Female C57BL/6 (MGI Cat# 5658455, RRID:MGI:5658455) mice aged 6-8 weeks were purchased from Harlan (Envigo, Netherlands). Female Batf3\(^{-/-}\) (IMSR Cat# JAX:013755, RRID:IMSR_JAX:013755) mice backcrossed onto a C57BL/6 background, and Foxp3-eGFP mice (IMSR Cat# EM:01945, RRID:IMSR_EM:01945), kindly provided by Prof. Pedro Romero (University of Lausanne; UNIL), were bred and housed in pathogen free conditions in the UNIL animal facility in Epalinges.

All in vivo animal experiments were performed in accordance with relevant guidelines and regulations of the University of Lausanne Ethic Committee for the human care of laboratory animals and were approved by the Service de la Consommation et des Affaires Vétérinaires of the Canton of Vaud (SCAV).

The mouse ovarian epithelial papillary serous adenocarcinoma cell line ID8 was a gift from Dr. K.F. Roby (University of Kansas Medical Center, USA) (22). The Lewis cell carcinoma cell line was purchased from American Type Culture Collection (ATCC Cat# CRL-1642, RRID:CVCL_4358). Both cell lines tested negative for mycoplasma contamination (last mycoplasma test April 2021). Tumor cell lines were authenticated by high polymorphic short tandem repeat loci (Microsynth report # 01230_007070). The median number of passages between thawing and collection was 10 (range 2-16).

Tumor cell lines were cultured in Dulbecco’s Modification of Eagle’s Medium w/L-glutamine (DMEM; Gibco-Thermo Fisher), 4% fetal bovine serum (FBS), 0.09 mg/ml penicillin-streptomycin. ID8 cells were gene-engineered to express luciferase by transduction with retrovirus prepared with the MSCV-Luciferase-PGK-hygro retroviral plasmid, a gift from Scott Lowe, (RRID:Addgene_18782). Retrovirus was prepared and stored as described in (103). Transduction was performed with viral supernatant and Protamine sulfate (Sigma-Aldrich) when the cells reached a confluence of 30-40%. Medium was replaced after 24 hours and at 48 hours transduction hygromycin (400 \(\mu\)g/ml final concentration) was added to select for transduced cells. After two weeks luciferase expression was assessed by
Bioluminescent imaging (BLI) performed using Xenogen IVIS® Lumina II imaging system and the photons emitted by the luciferase-expressing cells were quantified using Living Image software v.3.2 (Living Image software, RRID:SCR_014247) following manufacturer's instructions.

**b. Tumor engraftment**

For the ovarian cancer model, $5 \times 10^6$ ID8 tumor cells (luciferase+) were injected intraperitoneally (i.p.) in female C57BL/6 mice, typically aged 6-8 weeks on day -21. Successful engraftment of the i.p. tumors was defined as $\geq 7 \times 10^7$ photons/sec BLI emission. On day -2, mice were blindly randomized into treatment groups based on BLI emission (average of $7 \times 10^7$ photons/sec in controls and treated groups). Attrition: mice exhibiting BLI emission of $<5 \times 10^7$ or higher than $1 \times 10^9$ were not included in the experiments. The reason to exclude them was either lack of tumor when the bioluminescence was low or presence of ascites when the bioluminescence is higher than $1 \times 10^9$. This strategy was followed for all in vivo work. ID8 tumor bearing animals were weighed twice a week and euthanized if they exhibited clinical signs of disease or distress (cachexia, anorexia, respiratory problems, ascites, etc.).

For the Lewis lung carcinoma model, $1 \times 10^6$ LL/2 tumor cells were injected s.c in C57BL/6 mice. Once tumors reached an average volume of 350 mm$^3$, mice were randomized and treatments initiated. Attrition: mice harboring tumors smaller than 300 mm$^3$ were not included due to the presence of T cells in the tumor microenvironment. Tumors were measured with caliper at randomization and five times a week thereafter. Euthanasia was performed when tumors reached 1000 mm$^3$. Weight was monitored twice a week as per standard practice.

**c. Tumor imaging by bioluminescence**

BLI images were taken with Xenogen IVIS using D-luciferin, (In Vivo Imaging Solutions) as described by the manufacturer. Images were normalized using Living Image software (PerkinElmer). Maximum luminescent intensity and total flux in photons per second were calculated and reported for the abdominal region of each mouse in photons/sec. Significance
was determined using one-way ANOVA for BLI. All time points were compared to the earliest time point of day -2.

d. Clonogenic survival assay
Cells were seeded in triplicate into six-well plates at 100-400 cells/well in 4mL DMEM medium. Once cells were attached to the wells a single dose of irradiation (0, 0.5, 1, 2, 3, 4, 6, or 8 Gy) was applied. Cells were then incubated at 37°C in 5% CO2 for 24 hours. Colonies were fixed and stained with crystal violet. All colonies of 50 cells or more were then counted. The survival fraction (SF) was estimated according to the formula: $SF = \frac{\text{number of colonies formed}}{\text{number of cells seeded}} \times \text{plating efficiency of the control group}$.

e. Immunogenic cell stress assay
Five hundred thousand cells were seeded in a T25 cm$^2$ flask with 5 mL DMEM medium. To determine the kinetics of calreticulin upregulation, ID8 cells were treated with 1 Gy RT or Doxorubicin (positive control, 25 μM; Sigma) and harvested 4, 6, 12 and 24 hours after exposure as previously described, washed twice with cold PBS, followed by staining with a calreticulin-specific antibody (Abcam Cat# ab83220, RRID:AB_1859755), Annexin-V (Thermo Fisher Scientific Cat# 88-8007-72, RRID:AB_2575165) which recognizes phosphatidylserine on the surface of apoptotic cells, plus vital dye 4,6-diamidino-2-phenylindole (DAPI) which stains dead cells. Isotype-matched IgG antibody was used as a negative control (Abcam, Cat# ab91357, RRID:AB_2888649) and the analysis was limited to living (DAPI negative) tumor cells (104).

f. Mouse monoclonal antibody and chemotherapy treatment
After successful tumor implantation, C57BL/6 mice were treated with various combinations of 100 μg of agonistic CD40 mAb (Bio X Cell Cat# BE0016-2, RRID:AB_1107647), 100 μg of α-mouse PD1 (Bio X Cell Cat# BE0146, RRID:AB_10949053), 100 μg of α-CTLA4 (Bio X Cell Cat# BE0164, RRID:AB_10949609). Antibodies were administered i.p. in weekly cycles thrice, with and without 1 Gy low dose irradiation delivered to the whole mouse abdominal cavity (low-dose whole abdominal irradiation; LD-WART). Metronomic cyclophosphamide was used at 100 mg/Kg per mouse (27) and was administered once a week for 3 cycles the day preceding immunotherapy treatment.
For the *in vivo* depletion or blocking experiments, specific antibodies as well as isotype controls were i.p. injected twice a week starting at day -2. Depletions were confirmed by flow cytometric analysis of peripheral blood. Antibodies and drugs used for in vivo studies are listed in Supplementary Table S4.

**g. Low-dose RT**

RT was delivered to the whole peritoneal cavity (ID8) or subcutaneous tumors (LLC). Briefly, mice were anesthetized with isofluorane and the abdomen or the skin was irradiated with 0.5, 1, and 2 Gy depending on the experiment using the Small Animal Radiation Research Platform (x-Rad-iR-225, North Branford, Connecticut) using 12 Gy/min at 225 KV, 13.0 mA, 30 cm SSD. Radiation was focalized using a collimator of 4x4 cm or 2x2 cm depending on the location. For radiotherapy combinatorial immunotherapy (RACIM), LD-WART was provided with metronomic cyclophosphamide as well as anti-PD1 and anti-CTLA4 immune checkpoint blockade antibodies and anti-CD40 agonist antibody once per week, thrice.

**h. Flow cytometry analysis**

Analysis of the tumor microenvironment throughout all results and Figures was performed exclusively on tumor deposits that were collected, mixed and analyzed as one sample per mouse, and we did not analyze ascites fluid. Tumor samples were dissociated in DMEM medium supplemented with 200 μg/ml of liberase TL (Roche) and 1 mg/ml of DNase-I (Sigma-Aldrich) at 37°C for 1 hour. The same protocol was used for spleen and mesenteric lymph node but with an incubation of 15 minutes. Uniform single-cell suspensions were obtained after smashing digested tissues with a syringe plunger on a 100 μm filter. Subsequently, dead cells were stained with LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit according to the manufacturer’s protocol, followed by blocking of Fc receptors during an incubation of 15 min at 4°C with purified anti-CD16/CD32 mAb (BD Biosciences Cat# 553141, RRID:AB_394656). Cells were then stained for 30 min at 4°C with the fluorochrome-conjugated monoclonal antibodies of interest in 50 μl PBS supplemented with 2% fetal bovine serum, 2mM EDTA. The cells were then washed twice and fixed in 1%-paraformaldehyde (Sigma, St. Louis, MO) in PBS or in Fix/Perm buffer.
(eBioscience) for intracellular staining. Cells were prepared for intracellular staining using a permeabilization buffer set (eBioscience) before adding antibodies against intracellular markers according to the manufacturer’s instructions (eBioscience). Fluorescence minus one (FMO) control were stained in parallel using the panel of antibodies with sequential omission of one antibody. For intracellular cytokine secretion detection, cell suspensions were restimulated in vitro in the presence of 500 ng of Ionomycin/ 50 ng of PMA (Sigma-Aldrich) or after TCR engagement with 10µg/ml, immobilized anti-CD3 (BioLegend Cat# 100340, RRID:AB_11149115) in association with 2µg/ml soluble anti-CD28 (BioLegend Cat# 102116, RRID:AB_11147170) and GolgiPlug Brefeldin A solution (BD Biosciences Cat# 555029, RRID:AB_2869014) for 4 hours. Analysis of stained cells was performed using a LSR-II cytometer (BD) supplied with BD Diva interface and FlowJo software (FlowJo, RRID:SCR_008520). Antibodies used for flow cytometric analysis are listed in Supplementary Table S5.

i. **Cell sorting and single cell RNA sequencing**

Single cell suspensions for scRNAseq were prepared and stained as for flow cytometric analysis (described above). For the control, cells were pooled from 4 tumors (due to low levels of immune infiltrate) while for the treatment groups (CIM and RACIM), 3 independent single cell suspensions were evaluated for each (i.e., N=3 tumors/treatment). DAPI was added at a final concentration of 0.5 µg/ml immediately prior to running samples on the Aria II or Aria III sorter (BD). Sorted cells were collected in cold DMEM, 10% FBS. After the sort, cells were assessed for viability by trypan blue staining and their concentration adjusted to 1000 live cells /µl to process to the single cell encapsulation using a Chromium Single Cell Instrument and reagents (10x Genomics, Pleasanton, CA). A Chromium Next GEM Chip G was loaded with the appropriate number of cells and the sequencing libraries prepared with the Chromium Next GEM Single Cell V(D)J Reagent Kits v1.1 following the manufacturer’s recommendations. Briefly, an emulsion encapsulating single cells, reverse transcription reagents and cell barcoding oligonucleotides was generated. After the actual reverse transcription step, the emulsion was broken, and double stranded cDNA generated and amplified in a bulk reaction. For 5’ gene expression library this cDNA was fragmented, ligated to a sequencing adaptor, and PCR amplified. For V(D)J library preparation, a similar approach was followed except that 2 steps of PCR based V(D)J target enrichment were performed prior to fragmentation.
Libraries were quantified by a fluorimetric method and their quality assessed on a Fragment Analyzer (Agilent Technologies). Cluster generation was performed with 140 to 165 pM of an equimolar pool from the resulting libraries using the Illumina HiSeq 3000/4000 PE Cluster Kit reagents. Sequencing was performed on the Illumina HiSeq 4000 using HiSeq 3000/4000 SBS Kit reagents according to 10X Genomics recommendations (26 cycles read1, 8 cycles i7 index read, and 91 cycles read2). Sequencing data were demultiplexed using the bcl2fastq2 Conversion Software (bcl2fastq, RRID:SCR_015058) and primary data analysis performed with Cell Ranger (Cell Ranger, RRID:SCR_017344).

j. NanoString analysis
Gene expression profiling was performed using a custom 770 gene NanoString Cancer Immunology panel comprising immune-related genes, and genes pertaining to common cancer signaling pathways. Briefly, for each NanoString assay, 1 μg of RNA was isolated from 30 mg of frozen tissue using RNeasy Mini Kit (QIAGEN) as described by the manufacturer. Samples were first lysed in buffer RLT and disrupted/homogenized using the TissueLyser II system from Qiagen. RNA was quantified using the NanoDrop ND1000 Spectrophotometer (Thermo Scientific); average RNA was 734.38 ng/mL (range 298-1003 ng/mL), and RNA quality was checked with the Fragment Analyzer (Advanced Analytical technologies Inc., Ames, IA). Samples were run by the Centre for Integrative Genomics (CIG) at the University of Lausanne. Class comparison was performed using nSolver 2.6, PanCancer Analysis module, normalization from selected set of housekeeping genes using GeNorm algorithm (geNORM, RRID:SCR_006763) (the number of housekeeping genes to use is automatically chosen), then normalized by geometric mean of the selected genes.

k. Gene expression analyses
The R software (version 3.6.2) was used for all bioinformatics analyses. Similar methods were used for human and mouse gene and pathway analyses. A database of gene orthologues was built using the R biomaRt package (biomaRt, RRID:SCR_019214) and was used when interrogating human-derived gene signatures on mouse data.

The signatures used in this study include immune gene signatures from Bindea et al. (55), cell types signatures from the mouse single-cell atlas Tabula Muris (105), biological “Hallmarks” signatures from MSigDB (https://www.gsea-msigdb.org/gsea/msigdb/index.jsp)
and some manually curated pathway (i.e., RAE/NKG2D, cytokine, costimulation and immune checkpoint pathways). Gene signature scores were computed using the single-sample gene-set enrichment analysis (ssGSEA) as implemented in the GSVA R package (with default parameters except mx.diff = FALSE). The absolute composition of the immune component was also assessed using the CIBERSORTx (106) on-line tool using the LM22 immune signature collection. Differential analyses for gene expression and pathway scores were performed using the lmFit function of the limma R package (LIMMA, RRID:SCR_010943). Differential analyses performed in human patients were done by using the patient origin as a covariate (paired analyses). Gene expression, signature/pathway scores and P-values of statistical testing were represented under the form of heatmaps using the pheatmap R package (pheatmap, RRID:SCR_016418). In the P-value heatmap scenario, the P-values were –log10-transformed before plotting and directionality of the comparison was kept and color-coded. Pie charts were plotted using the ggplot2 R package (ggplot2, RRID:SCR_014601).

1. **Multispectral immunofluorescent imaging**

All tumor samples were fixed in 10% neutral buffered formalin (NBF) for a minimum of 16 hours before being dehydrated and formalin fixed paraffin embedded (FFPE). The following primary antibodies were used: mouse anti-NK1.1 (Thermo Fisher Scientific Cat# MA1-70100, RRID:AB_2296673), rat anti-FoxP3 (Thermo Fisher Scientific Cat# 14-5773-82, RRID:AB_467576); rabbit anti-CD4 (Abcam Cat# ab183685, RRID:AB_2686917), rabbit anti-CD8 (Bioss Cat# bs-0648R, RRID:AB_10857537), rabbit anti-CD11b (Abcam Cat# ab133357, RRID:AB_2650514), rabbit polyclonal against FoxP3 (Abcam Cat# ab54501, RRID:AB_880110), rabbit polyclonal against human CD8 (Bioss Cat# bs-0648R, RRID:AB_10857537), rabbit polyclonal against CD11b (Abcam Cat# ab133357, RRID:AB_2650514), rabbit anti mouse CD4 (Abcam Cat# ab183685, RRID:AB_2686917), rabbit polyclonal to yH2AX (Novus Cat# NB 100-384, RRID:AB_350295), goat polyclonal to Foxp3 (GeneTex Cat# GTX89752, RRID:AB_10725476), rabbit polyclonal to Rae-1 (LifeSpan Cat# LS-B3539-50, RRID:AB_10608707).

Multiplex IF IHC was performed on 5-µm paraffin sections on Ventana Discovery Ultra staining module (Ventana, Roche). The following panels were performed: CD4-Opal 690, CD8-Opal 520, CD11b-Opal 480, DAPI; yH2Ax-Opal 620, DAPI; RAE-Opal 620, DAPI;
RAE-Opal 620, CD11b-Opal480, DAPI. Slides were placed on the staining module for deparaffinization, epitope retrieval and endogenous peroxidase quenching. The multiplex staining method/procedure consists of several/multiple rounds of staining, each round includes non-specific sites blocking (Ventana, Discovery Inhibitor and Discovery Goat Ig Block), incubation with unlabeled primary antibody, with HRP-conjugated secondary antibodies (Discovery OmniMap anti-Rabbit, anti-Goat and anti-Mouse, Ventana), with Opal™ (Akoya) reactive fluorophore (Opal 480, 520, 620, 690) detection that covalently label the primary epitope, followed by an antibody (both primary and secondary) heat denaturation step prior to the next round of antibody staining. Finally, nuclear staining was performed with spectral DAPI (Akoya). Multispectral immunofluorescent (mIF) images from the stained slides were acquired at 20X and 40X magnification using Akoya Vectra® POLARIS multispectral microscope.

m. RNA extraction, cDNA preparation, and real-time q-PCR

Tissue samples from tumors and purified cells were kept frozen (−80°C) until mRNA extraction. As needed, samples were disrupted with a TissueLyser and homogenized in RLT buffer (Qiagen). RNA extraction was performed using micro or mini RNase kit (Qiagen) using the DNAse treatment step (Qiagen), and cDNA preparation were conducted following standard procedures using the PrimeScript™ 1st strand cDNA Synthesis Kit (Takara). Quantitative PCR was performed using TaqMan™ Fast Universal PCR Master Mix (2X), no AmpErase™ UNG (Life technologies) on the 7500 real-type system or QuantStudio™ 6 (Applied Biosystems) as indicated by the supplier. Primers and probes for the quantitative qPCR were analyzed with the following assay: GAPDH (Mm9999915_g1), IFNa4 (Mm00833969_s1_m1), IFNb (Mm00439552_s1), XCL1 (Mm00434772_m1), CXCL1(Mm04207460_m1), CXCL9 (Mm00434946_m1), CCL5 (Mm01302427_m1), TNFa (Mm00443258_m1), IFNg (Mm01168134_m1), Nos2 (Mm01309897_m1), Perforin 1 (Mm00812512_m1), klrk1 (Mm01183328_m1), Rae (Mm00558293_g1), IL-18 (Mm00434226_m1), H60a (Mm01311160_m1 ), IL12a (Mm00434169_m1 ), H2K1 (Mm01612247_mH). All primers obtained from Life technologies.

n. Cytokine and chemokine beads assays.

Mice bearing ID8 tumors treated or not with RACIM were bled at cycle 2 day 5. Sera diluted 5 times in PBS. Cytokines and chemokine were quantified according to the
manufacturer protocol: IFNγ (BD Biosciences Cat# 558296, RRID:AB_2869141), TNFα (BD Biosciences Cat# 558299, RRID:AB_2869144), IL-1α (BD Biosciences Cat# 560157, RRID:AB_2869318), IL-1β (BD Biosciences Cat# 562278, RRID:AB_2869415), IL-2 (BD Biosciences Cat# 558297, RRID:AB_2869403),IL-6 (BD Biosciences Cat# 562236, RRID:AB_2869412), IL-10 (BD Biosciences Cat# 562263, RRID:AB_2869410), CXCL9 (BD Biosciences Cat# 558341, RRID:AB_2869166).

o. Statistical analyses

All statistical analyses were performed using GraphPad Prism program (GraphPad  (RRID:SCR_000306). When indicated we performed statistical analyses using SPICE software (SPICE, RRID:SCR_016603). We used $\chi^2$ permutation test for pie chart comparison according to Roederer et al (107). When more than two groups were compared, one-way analysis of variance (ANOVA pairwise comparisons) with Tukey-Kramer post hoc test to compare among all pairs of means was used. Unpaired t tests with Welch correction were performed only in experiments having two groups of animals from the same experiment. Survival curves were estimated by using the Kaplan Meier method, log-rank test. Time to death was measured from the day tumor cells were injected. Confidence intervals (CI) were calculated from standard errors.

The number of animals per experiment (sample size) was calculated using the standardized effect size (SES). SES is the magnitude of the difference between the means of two groups in units of standard deviations. This is the effect of the size / pooled SD. SD of 2.0 were used with an 80%-90% power, a 5% significance level and a one-sided or two-sided t-test. Based on this, our experiments had between 5 and 10 animals per group (108).

p. Single cell RNA sequencing data analysis

**Data processing of scRNA-seq libraries:** The scRNA-seq reads were aligned to the GRCh38 reference genome and quantified using Cell Ranger count (10x Genomics, version 3.0.2). TCR reads were aligned to the GRCh38 reference genome and consensus TCR annotation was performed using Cell Ranger vdj (10x Genomics, version 2.1.0, (Cell Ranger, RRID:SCR_017344)). All additional analyses were performed using R 4.0.2 and Seurat v3.0 (109) (SEURAT, RRID:SCR_007322), unless it is indicated otherwise. For analyzing
shared clonotypes, The Jaccard index was calculated using the R package scRepertoire, (110) (R package version 1.0.0).

**Single cell data filtering and normalization:** In order to retain high quality transcriptomes, total ribosomal and mitochondrial count filters were applied. For the T cells, the maximum percentage counts coming from ribosomal and mitochondrial genes were 60% and 10% respectively. In addition, the acceptable number of detected genes ranges between 250 and 600. For the myeloid compartment, cells were prefiltered in a different (more permissive) way with a lower limit of 200 detected genes in the attempt retain the neutrophils which naturally have less mRNA. After this initial filtering, data was normalized by total counts using the ‘LogNormalize function’ from the ‘Seurat v3.0’ package, which normalizes the feature expression measurements for each cell by the total expression, multiplies this by a scale factor of 10,000 and log-transforms the result.

**Unsupervised clustering:** Scaled z-scores for each gene were calculated using the ScaleData function and regressed against the number of UMIs per cell. T cells and myeloid cells were analyzed using different parameters. Concerning T cells, scaled data was used as the input of a principal component analysis (PCA) on the top 1000 most variable genes. The first 30 principal components were used to generate UMAP projections, with a minimum distance of 30 neighbors. Within the UMAP space, neighbours were found using the FindNeighbors function from the ‘Seurat v3.0’ package using 2 dimensions. Then, Shared Nearest Neighbor (SNN) clustering method (111) implemented in Seurat 3 as the FindClusters function was applied, with parameters resolution=0.05, reduction="umap”, k.param=10. Concerning the myeloid cells, the expected vast heterogeneity of this population made us to perform a whole genome PCA and the UMAP analysis was based on the first 100 principal components. Within the UMAP space, neighbors were found using the FindNeighbors function using 20 dimensions The SNN clustering method was applied, with parameters resolution=2.5, reduction = ”umap”, k.param =10.

**Cell annotation:** In order to classify T cells, each cell from the dataset was projected onto a reference T cell atlas following the methodology proposed by Andreatta M. et al. (30). For the classification of the myeloid cells into the main subpopulations, namely, macrophages, monocytes, neutrophils and dendritic cells, we used the R package ‘SingleR’ (112) and the immune cells signatures from the Immgen database (37). In order to further classify dendritic
cells into distinct subpopulations, namely, DC1, DC2, DC3, pDC and MDDC, we used the signatures proposed by Zilionis et al (35). To compare the resulting clusters with DC states across mouse tumor models, a reciprocal similarity score between each tumor-infiltrating DC state comparison pair was calculated using a linear support vector classifier fitted to log2 transformed data from each scRNA-seq study.

2. Clinical Study and Evaluation of Patient Samples

a. RACIN clinical trial
RACIN (protocol identification NCT03728179) is approved by the Ethical Committee of Canton Vaud and it is conducted in accordance with the Declaration of Helsinki. All patients signed a written informed consent. RACIN is a single-arm, phase I trial of dose-escalation and safety evaluation, testing the combination of nivolumab plus ipilimumab, aspirin and low-dose cyclophosphamide, along with escalating doses of low-dose ionizing radiation (0.5-1 Gy), followed by nivolumab maintenance. Inclusion criteria: patients with advanced, TIL-negative solid tumors. Experimental subjects were not randomized into groups because this was deemed irrelevant to this study.

The current translational paper presents the experience of the 3 patients that comprised the first cohort, and the first 5 patients comprising the second cohort.

The interventions used in the study were:

- Low dose ionizing radiation: one fraction of 0.5, 1, 2, or 3 Gy q2wk on day 2 of each cycle (from Cycle 0 to Cycle 4).
- Immune checkpoint blockade antibodies; nivolumab 240 mg flat dose q2wk and ipilimumab 1 mg/kg q6wk, both administered i.v. and sequentially (when administered concurrently), starting on day 2 of each cycle, for four cycles (Cycle 1 to Cycle 4).
- Cyclophosphamide 200 mg/m² q2wk administrated i.v. one day prior to each combination of radiation and immunotherapy treatment (day 1 of each cycle C0 to C4).
- Aspirin (300 mg) administrated p.o. once daily for 4 cycles, from C1D1. Aspirin is continued during nivolumab maintenance, according to tolerance. An H2 antagonist is used in combination with aspirin to prevent gastric damage.
At the end of the fourth cycle, patients who did not progress by RECIST receive nivolumab maintenance (240 mg flat dose q2wk), until progression or unacceptable toxicity. Aspirin is maintained according to tolerability.

According to protocol version 4, in the Phase Ia (dose escalation) part of the study, patients received low-dose irradiation at escalating doses, according to rules of the classical Phase I escalation design avoiding a dose limiting toxicity (DLT) in more than 17% (1/6) of subjects (algorithm adapted to multidrug combination). The first cohort of 3 (or 9) patients received nivolumab, ipilimumab, cyclophosphamide, and aspirin at predefined doses with 0.5 Gy. Two subsequent cohorts of six patients have been planned, with radiation administered at escalating doses (2, 3 Gy), together with nivolumab, ipilimumab, aspirin and low-dose cyclophosphamide. The expected maximum number of patients to be treated at the Phase Ia was 21, and up to 27 patients.

After choosing the recommended phase Ib dose of radiation, an additional group of 19 patients would enter an expansion cohort in the Phase Ib part of the study, until a maximum of 40 or 46 patients have been treated.

The primary outcomes of the study, include the following:

- For phase Ia:
  1. Treatment safety and toxicity measured during the DLT period, the backbone limiting toxicity (BLT) period and all along the trial period using National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (CTCAE v.4.03, refer to Appendix 4: Adverse Event Grading Scale).
  2. Maximum tolerated dose (MTD) or recommended phase Ib dose (RP1bD) of low-dose irradiation for radio-immunotherapy combination.

- For Phase Ib:
  Safety and tolerability of low-dose radiation at the RP1bD, in combination with the backbone treatment (measured during the full trial follow-up).

Statistical analyses of the RACIN clinical data were carried out in SAS version 9.4.

b. Low dose ionizing irradiation
One fraction of 0.5 or 1 Gy q2wk was administered to all metastatic deposits (total radiation dose 6 Gy or 13 Gy, respectively). Before starting radiation treatment, patients underwent a computed tomography (CT) scan (planning scan) without intravenous contrast medium. They were scanned in a supine position with a head and knee support. For the CT-planning scan, standard acquisition parameters were used (tension, 120 kV; tube rotation time 1 s; tube current, 160 mA; helical acquisition with pitch of 0.938; reconstructed image thickness, 2 mm). CT planning scan was automatically fused with the volumetric information of either $[^{18}\text{F}]$FDG-PET/CT or $^{68}$GaPSMA-PET/CT and the largest combined tumor volume identified by each imaging method served as the gross tumor volume (GTV). Velocity Advanced Image Software; Velocity Medical Solutions, Atlanta, GA from Varian Medical Systems, Inc. or Raystation® 9.2 planning system software© from Raysearch Laboratories AB, Sweden was used for fusion and contouring. Gross tumor volume with 5 mm expansion constituted the clinical target volume. The planning target volume (PTV) was automatically derived from the CTV with a 5mm expansion. All the organs at risk were drawn (active bone marrow, bowel, sigmoid, rectum, bladder, femoral heads, heart, lungs, spleen, liver, duodenum) and protected from the low dose irradiation. Treatment plans were performed using the Tomotherapy treatment planning system (Accuray Inc, Sunnyvale, CA) with a field width of 5 cm and a pitch of 0.287. Image guidance before each fraction was implemented using the MV fan-beam CT of Tomotherapy. The prescribed total dose to the PTV was 6.5 Gy for the cohort 1 (0.5 Gy per fraction) and 13 Gy for the cohort 2 (1 Gy per fraction) administered every 2 weeks for 26 weeks.

c. Antibodies and chemotherapy

Immune checkpoint blockade antibodies, namely nivolumab (anti-PD1) and ipilimumab (anti-CTLA4) antibodies, both products of Bristol-Myers Squibb (BMS); nivolumab 240 mg flat dose q2wk and ipilimumab 1 mg/kg q6wk, were both administered i.v. and sequentially (when administered concurrently), starting on day 2 of each cycle, for four cycles (C1 to C4). Cyclophosphamide 200 mg/m$^2$ q2wk administrated i.v. one day prior to each combination of radiation and immunotherapy treatment (D1 of each cycle C0 to C4). Aspirin (300 mg) administrated p.o. once daily for 4 cycles, from C1D1. Continued during nivolumab maintenance, according to tolerance. An H2 antagonist used in combination with aspirin to prevent gastric damage. At the end of the fourth cycle, patients started on nivolumab maintenance at 240 mg flat dose q4wk, until progression or excessive toxicity.
**d. RNA extraction and library preparation**

RNA from snap frozen tissue biopsies was extracted using the RNA easy kit (Qiagen, Hilden, Germany). RNA quality was assessed using the Fragment Analyzer (Advances Analytical Technologies Inc., Ankeny, USA). RNA-seq libraries were prepared from 250 ng of total RNA. The RNA samples were depleted for ribosomal RNAs with the Illumina TruSeq Stranded Total RNA Gold kit. Ribosomal-RNA depleted RNA was then converted in cDNA and amplified with the kit Nugen Ovation® RNA-Seq System V2. It was used to generate sequencing libraries with the Illumina TruSeq DNA Nano kit. Libraries were quantified by a fluorimetric method and their quality assessed on a Fragment Analyzer (Agilent Technologies). Cluster generation was performed with 2 nM of an equimolar pool from the resulting libraries using the Illumina HiSeq 3000/4000 SR Cluster Kit reagents and sequenced on the Illumina HiSeq 4000 using HiSeq 3000/4000 SBS Kit reagents for 150 cycles. Sequencing data were demultiplexed using the bcl2fastq2 Conversion Software (bcl2fastq, RRID:SCR_015058).

**e. Tumor RNA sequencing processing**

Illumina single-end sequencing reads were aligned to the human reference GRCh37.75 genome using STAR aligner (113) and the 2-pass method as briefly followed: the reads were aligned in a first round using the --runMode alignReads parameter, then a sample-specific splice-junction index was created using the --runMode genomeGenerate parameter. Finally, the reads were aligned using this newly created index as a reference. The number of counts was summarized at the gene level using htseq-count (114). The number of uniquely mapped, non-mitochondrial and non-ribosomal reads averaged 22'532'108 ± 3’732’619 (s.d.). Read counts were normalized into reads per kilobase per million (RPKM) and log2 transformed after addition of a pseudo count value of 1.

**f. NanoString NanoGeoMX processing**

The NanoString® GeoMx® platform was used for spatial profiling of pre- and post-treatment tumor biopsies from 5 patients. Briefly, slide mounted 5 μm FFPE tissue sections were subjected to antigen retrieval with Tris-EDTA pH 9 under heat and pressure, then incubated with a cocktail of RNA-binding probes targeting approximately 1400 unique genes with 5 probes/gene and containing UV photocleavable unique molecular identifiers (UMIs). In
parallel, the same tissue section was coincubated with fluorescent antibodies for pan-cytokeratin, CD3e, and CD68, as well as the DNA dye Syto13 for visualization. Geometric areas of interest (AOI) were selected for spatial molecular profiling, and each of the regions was sequentially illuminated with UV light to release the UMI-containing oligos, which were captured in corresponding microtiter plates. A next generation sequencing library was then prepared, during which AOI-specific unique dual indices (UDIs) were added to each molecule. Libraries were sequenced using an Illumina NextSeq, which performed on-instrument demultiplexing. Sequencing reads were then mapped back by to the corresponding genes based on the UMIs by an internal data processing pipeline. The AOI were initially annotated as being either tumor, immune (T cell and macrophage) or mixed (tumor and immune) AOIs. The number of genes represented in the NanoGeoMX data equals 1’404.

g. RNA expression analyses

NanoGeoMX and RNA-sequencing data were merged together in order to gain in statistical power and in the number of post-irradiation versus pre-irradiation comparisons. To do so, NanoGeoMX data were first rebulked using the following methodology: The gene expression of ROIs from a same patient, treatment and type (Immune, Tumor or Mixed) were first averaged. Then we further averaged Tumor, Mixed and Immune averaged profiles together per patient and treatment, which resulted in one expression profile per patient and per treatment (as for bulk RNA-sequencing). RNA-sequencing data were narrowed down to the genes represented in the NanoGeoMX platform (1’404 genes). We then merged RNA-sequencing and NanoGeoMX data in a single expression matrix and applied a batch correction algorithm using the ComBat function of the sva R package. Finally, we performed Pearson correlation tests between samples and found that they cluster primarily by patient origin, which indicated that our merging method was appropriate.

The gene expression analysis and pathway score analyses were either performed on the rebulked data (merge of RNA-sequencing and NanoGeoMX) or on the NanoGeoMX data alone as indicated in the figures. Differential expression analyses and pathway/signature scoring were performed as described in the methodology for the mouse data. For the differential gene expression and pathway analysis, a significant result was defined as an unadjusted \( p \)-value lower than 0.05. Gene Ontology (GO) term enrichment and Reactome pathway enrichment analyses of the differentially expressed genes was achieved using the
on-line tool (http://geneontology.org/), only the five best pathways are shown in the supplementary figures.

For the Bindea et al (55) immune signature analyses, M1 and M2 macrophage signatures were manually added in order to deconvolute the Bindea macrophage signature. The M1 macrophage signature was defined as LCN2, SAA3, IL1B, CCL5, IRG1, FPR2, CFB, IL1A, CD38, CD274, STAT1 and the M2 macrophage signature as CCND1, MRC1, CKB, GSN, CD300LD, TREM2, CADM1, IGF1, CD36, GM23766, CLEC7A, FLT1, BCAR3, EGR2, STAT6.

h. Multispectral immunofluorescent imaging

All patient tumor samples were fixed in 4% neutral buffered formalin (NBF) for a minimum of 6 hours before being processed in VIP5Jr (Vacuum Infiltration Processor Tissue-Tek, SAKURA), for dehydration and paraffinization of the tissue. This process consists of 1 hour immersion in 4% formalin, then tissues were dehydrated in 2 baths of 1 hour in alcohol 96°, followed by 3 baths of 1 hour in alcohol 100°, then 3 times 1 hour bath in Xylene. Finally, the tissue was placed in paraffin for 3 hours before being embedded (FFPE). Multiplex IF IHC was performed on 3.5- µm paraffin sections on Ventana Discovery Ultra staining module (Ventana, Roche).

The following primary antibodies were used: rabbit anti-CD4 (Cell Marque Cat# 104R-14, RRID:AB_1516770), rabbit anti- FoxP3 (Abcam Cat# ab99963, RRID:AB_10675258), rabbit anti-CD56 (Cell Marque Cat# 156S, RRID:AB_1516783), rabbit anti-CD3 (Agilent Cat# A0452, RRID:AB_2335677), mouse anti-cytokeratin (LifeSpan Cat# LS-C95422-1, RRID:AB_10565578), rabbit anti-CD8 (Abcam Cat# 4207-1, RRID:AB_764503). The following panel was performed on patient tumors: CD4-Opal 570, FoxP3-Opal 480, CD3-Opal 520, cytokeratin-Opal 690, CD8-Opal 780, DAPI.

Slides were placed on the staining module for deparaffinization, epitope retrieval and endogenous peroxidase quenching. The multiplex staining method/procedure consists of multiple rounds of staining, each round including non-specific site blocking (Ventana, Discovery Inhibitor and Discovery Goat Ig Block), incubation with unlabeled primary antibody, incubation with HRP-conjugated secondary antibodies (Discovery OmniMap anti-
Rabbit, anti-Goat and anti-Mouse, Ventana), with OpalTM (Akoya) reactive fluorophore (Opal 480, 520, 620, 690, 780, 570) detection that covalently label the primary epitope, followed by an antibody (both primary and secondary) heat denaturation step prior to the next round of antibody staining. Finally, nuclear staining is performed with Spectral DAPI (Akoya). Multiplex IF images from the stained slides were acquired at 20X and 40X magnification using Akoya Vectra® POLARIS multispectral microscope.

i. TCRα and TCRβ Sequencing

mRNA was isolated using the Dynabeads mRNA DIRECT purification kit (Lifetechnologies) and was then amplified using the MessageAmp II aRNA Amplification Kit (Ambion) with the following modifications: \textit{in vitro} transcription was performed at 37°C for 16 hours. First strand cDNA was synthesized using the Superscript III (Thermofisher) and a collection of TRAV/TRBV specific primers. TCRs were then amplified by PCR (20 cycles with the Phusion from NEB) with a single primer pair binding to the constant region and the adapter linked to the TRAV/TRBV primers added during the reverse transcription. A second round of PCR (25 cycles with the Phusion from NEB) was performed to add the Illumina adapters containing the different indexes. The TCR products were purified with AMPure XP beads (Beckman Coulter), quantified and loaded on the MiniSeq instrument (Illumina) for deep sequencing of the TCRα/TCRβ chain. The TCR sequences were further processed using \textit{ad hoc} Perl scripts to: (i) pool all TCR sequences coding for the same protein sequence; (ii) filter out all out-frame sequences; (iii) determine the abundance of each distinct TCR sequence. TCR with a single read were not considered for the analysis.

To calculate the TCR metrics of a given repertory we took the frequencies of such repertory, and applied different formulas. For a set of TCR sequences we have a set of frequencies \( f_1 \) to \( f_n \) (where \( n \) correspond to the maximum number of unique TCR sequences in that set). In the formula, we refer to each of them as \( f_i \). The metrics we have calculated are respectively: Shannon entropy (eq. 1), Gini index (eq. 2), clonality (eq.3), richness, and shared frequency.

1) \( E = -\sum_{i=1}^{n} f_i \times \log_2(f_i) \)

2) \( G = \sum_{i=1}^{n} \sum_{j=1}^{n} \frac{|x_i-x_j|^2}{2n^2} \)

3) \( C = 1 + \frac{\sum_{i=1}^{n} f_i \times \ln(f_i)}{\ln(n)} \)
The “richness” estimates the number of unique words of a given repertory, i.e. the parameter “n”. Among the various metrics the “shared frequency” is the only one that need two (or more) sets. This estimator needs, in fact, to obtain a list of sequences that are present on two (or more) sets. With this list of shared sequences, it is possible to evaluate their size in terms of sum of frequencies, in each set.

**Data availability:** All data are available from the authors upon reasonable request. For genomic analysis data can be consulted in GSE169742:

Authors’ contributions
FH designed and performed pre-clinical experiments, analyzed and interpreted data, wrote the manuscript, cowrote the clinical study with GC and interpreted findings with GC. GC and MI conceived and supervised the study, analyzed and interpreted data, wrote and edited the manuscript. CR designed and performed pre-clinical in vitro and in vivo experiments, analyzed and interpreted data, and participated to manuscript writing. MO: performed patient immunohistological evaluation and analyses, contributed to patient RNA and TCR sequencing analysis and interpretation, contributed to interpretation of patients’ translational data in the clinical context. DB, IC, SC, MA: performed bioinformatics and statistical analyses. FB: performed TCR sequencing analysis and interpretation. RG: performed TCR sequencing. JCO: designed flow cytometry staining and analyses. AS: performed experiments. AO, MI, EG, BNR, DB, AS, KZ, RD, CD, JP, NS, JB, CS: were part of the RACIN clinical study and contributed substantially to the design, conception, conduct, acquisition, and analysis of the human data. GD, ZT, and UD: performed statistical design and analysis of the RACIN clinical trial. TS, and SW: performed NanoString GeoMx experiments and analysis. PF and SR: performed immunohistological evaluation and analyses. MJP and MM: analysed and interpreted scRNAseq of the myeloid compartment. AH and DD: performed experiments and human data analysis. LK: supported the RACIN study. All authors provided substantial revisions, edited, and approved the final manuscript.

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References


Requires BATF3


Figure 1: Low-dose radiotherapy induces immune-cell infiltration in orthotopic ID8 tumors. (A) Treatment schema of mice engrafted with intraperitoneal ID8 ovarian tumors. Arrow represents administration of low-dose whole abdominal radiation (LD-WART, 1 Gy). (B & C) NanoString analysis of LD-WART treated vs. control tumors. MSigDB pathways (B) and intratumoral levels of cytokines and chemokines (C) are displayed as heatmaps. Red: upregulated, blue: downregulated. (D) mRNA levels of intratumoral cytokines and chemokines. (E) Heatmap of cell density changes in tumors based on NanoString analysis. The heatmap legend applies to B, C and E. (F) Flow cytometry quantification of tumor infiltrating leukocytes. (G) CD8\(^+\):Foxp3\(^+\) cell ratio by multispectral immunofluorescence imaging 5 days post-LD-WART. (H) Flow cytometry quantification of CD8\(^+\) TILs in control mice and mice subjected to IFN\(\alpha\) receptor blockade or IFN\(\gamma\) depletion. (Symbols represent individual tumors and bars the mean. Data are representative of 3 independent experiments and are presented as mean +/- SEM. *P ≤ 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; Student’s unpaired t-test).
Figure 2: Metronomic radiotherapy enables combinatorial immunotherapy. (A) Schema of *in vivo* study evaluating treatment by LD-WART (1Gy) vs. CIM vs. RACIM. (B) Left: waterfall plot representing the percentage change in tumor BLI levels at day 20 for mice treated in the different groups (RACIM, n=36 mice; control, n=41; CIM, n=26; LD-WART, n=24). Complete response (CR), partial response (PR; at least 30% decrease in BLI from baseline), stable disease (SD), progressive disease (PD; at least 20% increase in BLI from baseline). Right: tumor growth curves evaluated by bioluminescence (BLI). (C) Kaplan-Meier analysis in representative mice treated in 6 different experiments (RACIM, n=80 mice; control, n=92; CIM, n=30; LD-WART, n=24). *P* values were determined by a one-sided log-rank Mantel–Cox test. (D) Heatmap of cell density changes in tumors based on Nanostring analysis. (E) Multispectral immunofluorescence imaging reveals immune cell infiltration in tumors at cycle 2, day 5 (20X magnification, DAPI nuclear counterstaining, images are representative of n=5 mice/group). Number of cells per high-power field (HPF) plotted as mean ± SEM; *P* was calculated using unpaired two-tailed Student’s *t*-tests. (F-H) Immune cell phenotypes evaluated on single cell suspensions of control, RACIM or RACIM one component, ID8 tumors (n=5-7 mice per group). Kaplan-Meier analyses of overall survival following RACIM in the absence of (F) anti-PD1 or anti-CTLA-4 antibody, (G) cyclophosphamide (CP), or, (H) anti-CD40 agonist antibody for n=10 mice per group. *P* values were determined by a one-sided log-rank Mantel–Cox test. (I) mRNA levels of *Tnfa* and *Ifng* in differently treated ID8 tumors. *In vivo* data are representative of 3 independent experiments. (*P* ≤ 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.)

Figure 3: Low-dose irradiation and combinatorial immunotherapy expands tumor-rejecting CD4+ and CD8+ TILs exhibiting states of activation and exhaustion. (A) UMAP plots of tumor lymphocyte scRNAseq data (n=3 tumors/treatment, n=4 tumors pooled for control, all collected on day 5 of cycle 2). Left; reference map for all groups. Right; contour plots reveal cell density/group. Supervised T-cell state classification by TILPRED identifies functional T-cell subsets; precursor exhausted (Tpex), terminal exhausted (Tex), effector memory (EM), early activated (EA), T helper 1 (Th1), T follicular helper (Tfh), regulatory T cell (Treg), and naïve like T cells. (B) Fold-change in T-cell subsets following RACIM vs.
CIM. (C) Cord diagram of the Jaccard similarity coefficient shows the relative number of common TCRs shared between CD4+ T-cell subsets following RACIM. (D) Violin plots representing the expression of various activation and cytotoxicity markers in CD4+ T-cell subsets. (E) CD4+ T cells expressing indicated genes across subsets and their corresponding average expression (size of dot indicates the percentage of cells in each subset; expression intensity is indicated by color). (F) Pseudotime trajectory analysis of CD4_Tpex, CD4_Tex and Th1 clusters identified by unsupervised single-cell analysis. (G) Left: SPICE graphic representing flow cytometric analysis of GzmB and cytokine production by CD4+ TCF1+ PD1+ TILs. Right: Bar plots representing cytokine production by CD4+ TCF1+ PD1+ TOX+ cells after phorbol myristate acetate (PMA)/ionomycin or anti-CD3/anti-CD28 TCR stimulation. (H) Kaplan-Meier analysis of overall survival of RACIM-treated mice, depleted or not of CD4+ T cells. P values determined by a one-sided log-rank Mantel–Cox test. (I) % CD8+ T cells expressing indicated genes across subsets and their corresponding average expression (as in E). (J) Violin plots showing expression of Ifng, Gzmb, Prf1 in CD8+ T cells following CIM vs. RACIM. (K) Bar plots representing the most clonally expanded CD8+ T-cell clonotypes (by TCRseq) following RACIM treatment (TCRs in all 3 tumors: #1; in individual tumors: #2–4). (L) Cord diagram of the Jaccard similarity coefficient shows the relative number of common TCRs between CD8+ T-cell subsets following RACIM. (M) Left: SPICE graphic representing flow cytometric analysis of GzmB and cytokine production by CD8+PD1+TCF1+ TILs. Right: bar plots representing cytokine production by CD8+ TCF1+ PD1+TOX+ cells after phorbol myristate acetate (PMA)/ionomycin or anti-CD3/anti-CD28 TCR stimulation. (N) Kaplan-Meier analysis of overall survival of RACIM-treated mice depleted or not of CD8+ T cells. P values were determined by a one-sided log-rank Mantel–Cox test. Data are representative of n=3 biologically independent experiments (n=5-10). (*P ≤ 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.)

Figure 4: RACIM reprograms tumor antigen-presenting cells and enlists NKG2D as a key signal. (A) UMAP plots of intratumoral myeloid cell scRNAseq data (n=3 tumors/treatment, n=4 tumors pooled for control, all collected on day 5 of cycle 2). Left: reference map for all groups. Red: DCs, blue: monocytes, green: macrophages. Right: 29 myeloid states amongst groups. (B) Fold-change in myeloid-cell subsets for RACIM vs. CIM. (C) Quantification of DC clusters amongst groups. (D) Rose plot of differentially expressed
genes corresponding to DC clusters amongst groups. (E & F) Violin plots showing expression of (E) Batf3 and (F) H2K1 and H2D1 (MHC class I) transcripts in cDC1 cells amongst groups. (G & H) Kaplan-Meier analysis of control vs. RACIM in (G) Batf3−/− mice, and (H) in wild-type mice in the presence of Fingolimod (FTY-720) treatment. P values were determined by a one-sided log-rank Mantel–Cox test. (I) Heatmap showing expression of the most representative genes for clusters 9, 19 and 21. Gene expression was normalized to median expression value per gene across all clusters shown in the heatmap. (J) Percentage of cells expressing Rae1, Ulbp1, H60b, and H60c, and average expression in the myeloid compartment by scRNAseq (size of dot indicates the percentage of cells in each subset; expression level is indicated by color). (K) RAE1 expression on intratumoral CD11b+CD11c+MHC-II+ cells determined by flow cytometric analysis on day 5 of cycle 2. (L) Left: Multispectral immunofluorescence imaging reveals RAE1 expression (red) by CD11b+ cells (yellow; 20X magnification, DAPI nuclear counterstaining, representative of n=5 mice/group). Right: number of CD11b+RAE+ cells per HPF plotted as mean ± SD; P was calculated using unpaired two-tailed Student’s t-tests. (M-N) % CD4+ and CD8+ exhausted T cells expressing NKG2D at the transcriptional (M & N, klrk1 gene by scRNAseq analysis) and protein levels (O & P, flow cytometry analysis) on day 5 of cycle 2. (Q & R) % intratumoral Ki67+proliferating CD4+TCF1+PD1+ (Q) and CD8+TCF1+PD1+ (R) cells upon RACIM on day 5 of cycle 2. (S-T) NKG2D expression on intratumoral CD4+TCF1+PD1+ (S) and CD8+TCF1+PD1+ (T) T cells determined by flow cytometry on day 5 of cycle 2 in control or RACIM or RACIM without ICB, treated tumors. (U) RACIM survival with NKG2D blockade. P values were determined by a one-sided log-rank Mantel–Cox test. Data are representative of 2 to 3 independent experiments (n=5-10 mice/group). Unless otherwise indicated, statistical analysis was performed using Student’s unpaired t-test, error bars represent mean ± standard deviation. (* P ≤ 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.)

Figure 5: Low-dose irradiation plus immune checkpoint blockade induce responses in advanced human immune desert tumors. (A) Therapeutic schema of the phase I RACIN study. (B) Spider plot depicts the percentage change in the sum of targeted irradiated metastases compared to baseline. (C) Swimmer plot depicts patients’ response to RACIN over time; each bar: one patient, light orange: time on combination treatment, green: time on maintenance treatment, cohort 1: 0.5 Gy, cohort 2: 1 Gy, orange triangles: completed
treatment, asterisk: treatment termination due to toxicity or progression, black circles: death. Immune Response Evaluation Criteria in Solid Tumors (iRECIST v1.1) was used to indicate partial response (iPR, green diamond), stable disease (iSD, light blue square), confirmed progressive disease (iCPD, maroon circle) or unconfirmed (iUPD, maroon empty circle). (D) $^{68}$Ga-PSMA PET/CT images of irradiated tumors (white arrows) before and after treatment from a patient with metastatic castration resistant prostate cancer having SD according to Prostate Cancer Clinical Trials Working Group 3 (PCWG3) but an important response on $^{68}$Ga-PSMA PET/CT images. Changes in PSA tumor marker. Progression observed outside the irradiated areas 24 weeks after treatment initiation. (E) $^{18}$FDG-PET/CT images of irradiated tumors (white arrows) before and after treatment from a patient with high grade serous ovarian carcinoma having by iRECIST iPd, but an important response on $^{18}$FDG-PET/CT imaging. Changes in CA125 tumor marker. Progression outside the irradiated areas 24 weeks after treatment initiation. (F) Computed tomography images of irradiated tumors (white arrows and circles) before and after treatment from a patient with gallbladder cancer having PR by iRECIST and 70% reduction from baseline in targeted irradiated lesions. Changes in CA 19-9 tumor marker. Progression outside the irradiated areas 17 weeks after treatment initiation. (G) Anatomical location of irradiated target and non-targeted lesions in responder patients and the anatomical location of tumor recurrence (D2: second dorsal vertebrae; D12: dorsal 12; R: right; L: left; LN: lymph node; liver segments identified with roman numbers: III, IV, V).

Figure 6: Effect of low-dose irradiation on tumor immune landscape. (A) TILs before and after LDRT revealed by multispectral immunofluorescent imaging in two representative responding tumors. Left, representative multispectral immunofluorescence images (20X magnification, CK: pancytokeratin); right, quantification of CD4$^+$ and CD8$^+$ cells. (B) Scatter plot showing differential gene expression between baseline and post-irradiation biopsy in responding (x-axis) vs. non-responding tumors (y-axis). The log2 of the fold-change in median gene expression (log2FC) is shown (positive values indicate upregulation post-LDRT). Genes displaying a significant change (unadjusted $P$-value < 0.05) are color-coded as shown in legend. (C) Line plots showing the progression of immune gene signature scores from baseline to post-LDRT biopsies in responding vs. non-responding tumors. (D) Scatter plot showing differential immune signature score analysis between baseline and post-
irradiation biopsy in responding human tumors (x-axis) vs. responding mouse (RACIM) ID8 tumors (y-axis) (top panel) and between non-responding human vs. non-responding (CIM) mouse tumors (bottom panel). (E) NanoString GeoMx analysis of intraepithelial tumor immune infiltrates vs. tumor stroma immune infiltrates in responding vs. non-responding tumors. The log2 of the fold-change in the median of the signature score (log2FC) is shown. Immune signature score displaying a significant change (unadjusted P-value < 0.05) are color-coded as depicted in the legend of panel B. (F) Comparison of T-cell receptor (TCR) CDR3 diversity by clonality, Shannon diversity entropy, Gini coefficient, richness and shared frequency in three patients with responding tumors.
Figure 1
Figure 2
Figure 4

A: UMAP plots showing different subtypes of immune cells across three conditions: Control, CIM, and RACIM.

B: Heatmap showing fold change in gene expression across different conditions.

C: DC clusters quantification (counts).

D: Heatmap showing expression of different immune cell markers.

E: Heatmap showing expression of different kinase markers.

F: Heatmap showing expression of different transcription factors.

G: Survival analysis showing differences in survival rates across different conditions.

H: Flow cytometry analysis showing differences in cell surface markers.

I: Heatmap showing expression of different cell surface markers.

J: Heatmap showing expression of different immune cell markers.

K: Heatmap showing expression of different transcription factors.

L: Heatmap showing expression of different cell surface markers.

M: Heatmap showing expression of different kinase markers.

N: Heatmap showing expression of different immune cell markers.

O: Heatmap showing expression of different transcription factors.

P: Heatmap showing expression of different cell surface markers.

Q: Heatmap showing expression of different immune cell markers.

R: Heatmap showing expression of different transcription factors.

S: Heatmap showing expression of different cell surface markers.

T: Heatmap showing expression of different kinase markers.

Figure 4