



# Antitumor efficacy of radiation plus immunotherapy depends upon dendritic cell activation of effector CD8+ T cells

DOI:

[10.1158/2326-6066.CIR-15-0253](https://doi.org/10.1158/2326-6066.CIR-15-0253)

## Document Version

Accepted author manuscript

[Link to publication record in Manchester Research Explorer](#)

## Citation for published version (APA):

Dovedi, S., Lipowska-Bhalla, G., Beers, S., Cheadle, E., Mu, L., Glennie, M. J., Illidge, T., & Honeychurch, J. (2016). Antitumor efficacy of radiation plus immunotherapy depends upon dendritic cell activation of effector CD8+ T cells. *Cancer Immunology Research*, 4(7). <https://doi.org/10.1158/2326-6066.CIR-15-0253>

## Published in:

Cancer Immunology Research

## Citing this paper

Please note that where the full-text provided on Manchester Research Explorer is the Author Accepted Manuscript or Proof version this may differ from the final Published version. If citing, it is advised that you check and use the publisher's definitive version.

## General rights

Copyright and moral rights for the publications made accessible in the Research Explorer are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

## Takedown policy

If you believe that this document breaches copyright please refer to the University of Manchester's Takedown Procedures [<http://man.ac.uk/04Y6Bo>] or contact [uml.scholarlycommunications@manchester.ac.uk](mailto:uml.scholarlycommunications@manchester.ac.uk) providing relevant details, so we can investigate your claim.



## **Antitumor efficacy of radiation plus immunotherapy depends upon dendritic cell activation of effector CD8<sup>+</sup> T cells**

Simon J. Dovedi<sup>1\*</sup>, Grazyna Lipowska-Bhalla<sup>1</sup>, Stephen A. Beers<sup>2</sup>, Eleanor J. Cheadle<sup>1</sup>, Lijun Mu<sup>3</sup>, Martin J. Glennie<sup>2</sup>, Timothy M. Illidge<sup>1,4†</sup>, Jamie Honeychurch<sup>1†</sup>.

<sup>1</sup> Targeted Therapy Group, Institute of Cancer Sciences, & <sup>4</sup>Christie NHS Trust, University of Manchester, Manchester Academic Health Sciences Centre, United Kingdom. <sup>2</sup>Antibody and Vaccine Group, Cancer Sciences Unit, University of Southampton Faculty of Medicine, Southampton General Hospital, Southampton, United Kingdom. <sup>3</sup>The Second Affiliated Hospital, Dalian Medical University, Dalian 116027, China.

\*Current address: Simon J. Dovedi, MedImmune Ltd, Granta Park, Cambridge, United Kingdom

†These authors contributed equally to this work.

**Running Title:** Efficacy of radiation plus immunotherapy is DC-dependent

**Key Words:** Lymphoma; CD40; TLR7; macrophage; radiation therapy; dendritic cell; immunotherapy

**Financial support:** S.J.D, T.M.I and J.H, Cancer Research UK (A17737), Leukaemia and Lymphoma Research (08075); E.J.C and L.Mu, Cancer Research UK (A17737); G.L.B Leukaemia and Lymphoma Research (08075); S.A.B and M.J.G Cancer Research UK (A12343).

**Corresponding authors:** Prof. Tim Illidge, [tmi@manchester.ac.uk](mailto:tmi@manchester.ac.uk) and Dr. Jamie Honeychurch, [Jamie.Honeychurch@ics.manchester.ac.uk](mailto:Jamie.Honeychurch@ics.manchester.ac.uk)

The authors declare no conflict of interest.

**Word count:** 4996; 5 figures

## **Abstract**

Tumor cells dying after cytotoxic therapy are a potential source of antigen for T-cell priming. Antigen-presenting cells (APCs) can cross-present MHC I–restricted peptides after the uptake of dying cells. Depending on the nature of the surrounding environmental signals, APCs then orchestrate a spectrum of responses ranging from immune activation to inhibition. Previously, we had demonstrated that combining radiation with either agonistic monoclonal antibody (mAb) to CD40 or a systemically administered TLR7 agonist could enhance CD8 T-cell–dependent protection against syngeneic murine lymphoma models. However, it remains unknown how individual APC populations impact on this antitumor immune response. Using APC depletion models, we now show that dendritic cells (DCs), but not macrophages or B cells, were responsible for the generation of long-term immunological protection following combination therapy with radiotherapy and either agonistic CD40 mAb or systemic TLR7 agonist therapy. Novel immunotherapeutic approaches that augment antigen uptake and presentation by DCs may further enhance the generation of therapeutic antitumor immune responses, leading to improved outcomes after radiotherapy.

## Introduction

The emergence of immunomodulatory agents that lead to durable antitumor immune responses has generated considerable enthusiasm that targeting key molecular regulators on T cells or APCs are important in controlling cancer (1-4). Preclinical studies evaluating the activation of CD40, a member of the TNF receptor super-family, with agonistic monoclonal antibody (mAb) and stimulating TLR7 with small molecule agonists have shown promising therapeutic activity against lymphoma (3, 4) and other cancer types (5, 6), through the generation of antitumor CD8<sup>+</sup> T-cell responses. Efficacy in these lymphoma models can be further enhanced by combination with radiotherapy (RT) or chemotherapy (3, 7-9). These cytotoxic treatments presumably act by debulking tumors and stimulating inflammation, thus creating a pool of dying tumor cells that serve as a source of antigen for cross-presentation of MHC I-restricted peptides; and enhancing immunogenicity (10-13).

The tumor cells dying from effective cytotoxic therapy are engulfed by APC that, dependent on local micro-environmental signals, could lead to suppression, tolerance, or immunity. Each of the three bone marrow-derived professional APC subsets, namely B-cells, macrophages (MØs) and DCs, are capable of cross-presenting exogenously acquired antigen *in vitro* (14). In the mouse, the most competent APC for presenting exogenous cellular antigen for T-cell priming *in vivo* appears to be a subpopulation of CD8<sup>+</sup>DEC<sup>-</sup>205<sup>+</sup> DCs (15), although MØs are also capable of priming naïve CD8<sup>+</sup> T cells after antigen capture (16). Conversely, tumor-associated DCs can function to impair CD8<sup>+</sup> T-cell responses through expression of inhibitory molecules and the induction of T-cell tolerance or anergy (17). Likewise, upon recognition of apoptotic cells, MØs produce a range of inhibitory molecules, including immunosuppressive cytokines such as IL10

and TGF $\beta$ , and are phenotypically polarized towards immune suppression within the tumor microenvironment (18). Malignant B cells can present antigen to both CD4<sup>+</sup> and CD8<sup>+</sup> T cells and after CD40 ligation upregulate adhesion and costimulatory molecules, resulting in enhanced T-cell activation (19). Thus, the decision to initiate immune activation rather than inhibition is regulated by APCs and is likely to vary according to the diversity of environmental signals perceived.

Previously we have shown that combining RT with either CD40 mAb or systemically administered TLR7 agonists can induce long-term CD8<sup>+</sup> T cell–dependent tumor protection (3, 7). However, it is currently unclear how different APC populations orchestrate priming of the immune response against tumors after combination therapy. In the present study we have investigated the importance of various APC populations to therapeutic outcomes, using depletion models to ablate either DCs, M $\phi$ s or B cells from the tumor environment at the time of treatment. Our results provide insights into the therapeutic opportunities that exist in combining RT with immunomodulatory agents and highlight the importance of the host immune system and DC populations to the generation of durable therapeutic antitumor CD8<sup>+</sup> T-cell responses that lead to long-term clearance of tumors.

## **Materials and methods**

**Animals and cell lines.** C57Bl/6 and BALB/c mice were obtained from Harlan, U.K. CD11c-diphtheria toxin receptor (DTR) and CD169-DTR mice (kindly provided by M. Tanaka, Riken Yokohama Institute, Japan) were maintained on BALB/c and/or C57BL/6 backgrounds. Animal experiments were approved by a local ethical committee and performed under a United Kingdom Home Office license. Further details on experimental animals, housing and sample size can be found in the Supplementary Methods. The syngeneic BCL<sub>1</sub> lymphoma (and  $\pi$ BCL<sub>1</sub> variant) were provided by M. Glennie, University of Southampton, and are maintained by routine *in vivo* passage (7); T-cell lymphoma line EL4 (and its ovalbumin expressing derivative EG7) were purchased from ATCC in 2011 (catalogue number TIB-39 and CRL-2113 respectively). On receipt, cells were expanded in culture to passage 3 and aliquots frozen in liquid nitrogen to create a batch of authenticated stock lines. Cell lines were screened for Mycoplasma contamination prior to freezing. Aliquots of stock cell lines were defrosted for use as required and cultured as previously described (3, 7). Defrosted cell lines were regularly re-screened for Mycoplasma contamination during culture.

**Tumor therapy.** Mice were inoculated with either  $3 \times 10^6$  EG7,  $1 \times 10^5$  EL4 (both s.c.) or  $1 \times 10^6$  BCL<sub>1</sub> cells (i.v.). For the s.c. models, local tumor irradiation was performed 7 days after inoculation (when tumors were approximately 100 mm<sup>3</sup>) as previously described (3). For the BCL<sub>1</sub> model, total body irradiation (TBI) was performed 15 days after inoculation at a dose rate of 1.15 Gy/min. TBI-treated mice were fed acidified water (pH 2.5; 1N HCl) supplemented with neomycin sulfate (2 g/L) (Sigma Aldrich, United Kingdom), starting 1 week prior to TBI and continuing for 4 weeks afterward. Mice were treated with CD40 mAb either i.v. (100  $\mu$ g, BCL<sub>1</sub> model) or s.c. (500  $\mu$ g, EL4 and EG7 models) 4 h after irradiation. R848 was administered i.v. at

a dose of 3 mg/kg in a dose volume of 50  $\mu$ L/10 g, in PBS, and repeated once per week for up to 5 weeks. For tumor rechallenge experiments, long-term surviving (LTS) mice were implanted contra-laterally with either EG7 or EL4 cells at least 60 days after previous tumor implantation. Additional control mice were implanted to confirm tumor growth. Experimental groups contained at least 5 mice/group and are representative at least 2 independent experiments.

**Immune cell depletion studies.** For B-cell depletion, mice received CD20 mAb (250  $\mu$ g, mouse IgG2a clone 18B12, a gift from Robert Dunn, Biogen-Idec, U.S.) 1 day prior to tumor cell inoculation. For M $\emptyset$  depletion, liposomes encapsulating dichloromethylene-bisphosphonate (Cl<sub>2</sub>MBP; clodronate) were prepared according to the method of Van Rooijen et al. (20). PBS-liposomes were used as control. M $\emptyset$  depletion was achieved using serial injections of clodronate-liposomes (400  $\mu$ L followed by up to 2 doses of 200  $\mu$ L either i.v. or i.p.) 48 h apart. Peritoneal M $\emptyset$  depletion was achieved by a single 250  $\mu$ L injection, i.p. For CD11c depletion, 8 week old BALB/c or C57Bl/6 mice were irradiated (10 Gy low-dose rate ionising radiation) and reconstituted with  $2 \times 10^6$  CD11c-DTR/GFP bone marrow cells i.v. After 6 weeks chimerised mice were randomised and used in therapeutic studies. DC or CD169<sup>+</sup> M $\emptyset$  depletion was achieved with i.p injection of 100 ng diphtheria toxin up to 3 times per week starting 1 day before tumor cell implantation (Sigma, UK). For CD8<sup>+</sup> or CD4<sup>+</sup> depletion, mice were treated with a depleting antibody, YTS169 or YTA1.3.2, respectively (Antibody and Vaccine Group, University of Southampton), as previously described (7). Peripheral blood was sampled during therapy and cellular depletion confirmed by flow cytometry. Experimental groups contained at least 5 mice/group and are representative at least 2 independent experiments.

**Cytokine produced by CD8<sup>+</sup> T cells from long-term surviving mice.** Splenocytes were isolated from either LTS or control mice and cocultured with irradiated tumor cells (25 Gy) as described previously (3). Experimental groups contained 3-5 mice and are representative of 2 independent experiments.

**Phagocytosis assays.** Mice were inoculated i.p with  $5 \times 10^6$  BCL<sub>1</sub> cells labeled with PKH-26 (Sigma Aldrich, Poole, UK) and treated with external-beam irradiation; 24 h later MØs were isolated from peritoneal lavage, sedimented on to glass coverslips, counter stained with Alexa-fluor phalloidin and phagocytosis visualized by fluorescence microscopy. Uptake was quantified by flow cytometry following counter-staining with F4/80 Ab (AbD Serotec, Oxford, UK). For DC uptake, CD11c<sup>+</sup> cells were isolated from the spleens of BALB/c mice using MACS as described (21). DCs ( $10^5$ ) were cocultured with PKH-26-labeled irradiated lymphoma cells (10 Gy; +72 h) at an E:T ratio of 20:1 and uptake assessed after 3 h by flow cytometry using a FACScan (BD Biosciences, CA, USA). Sodium azide was added to some wells to inhibit uptake and allow discrimination between tumor cells which have been truly internalized versus those which may be externally adhered to DC.

**Immunohistochemistry.** Tissues were harvested from mice immediately *post mortem* and frozen in isopentane cooled in liquid nitrogen. Staining of CD11c (clone N418, AbD Serotec, U.K.) used a goat anti-hamster biotin-conjugated F(ab)<sub>2</sub> (Jackson ImmunoResearch, U.K.) with amplification by the ABC method (Vector Lab, Peterborough, UK) and diaminobenzidine (DAB; Sigma, Poole, UK) visualization.



## Results

**RT plus CD40 mAb generates durable CD8<sup>+</sup> T-cell immunity.** Radiotherapy modulates the immunogenicity of tumor cells but is rarely able to initiate systemic antitumor responses. In models of T- and B-cell lymphoma, we quantitated the therapeutic efficacy of RT in combination with CD40 mAb. In mice bearing established EG7 tumors, the number of long-term survivors (LTS) after therapy increased from 20% treated with 10 Gy RT, and 40% treated with CD40 mAb, to 80% with the combination (Fig. 1A). Long term survivors were protected against contralateral rechallenge with EG7 cells by a tumor-specific memory immune response (Fig. 1B), which also significantly delayed tumor development when the mice were rechallenged with the parental EL4 tumor cells (Fig. 1C), demonstrating that immune responses were generated against multiple tumor antigens and not restricted to ovalbumin (expressed by EG7). A similar pattern of response was observed in mice bearing established EL4 tumors, with ~6-fold more tumor antigen-specific CD8<sup>+</sup> T-cells in LTS mice compared to tumor naïve control mice (Fig. 1D and E). Enhanced efficacy after combination treatment was CD8<sup>+</sup> T-cell–dependent, as observed in the BCL<sub>1</sub> model of established systemic lymphoma, with survival abrogated when CD8<sup>+</sup>, but not CD4<sup>+</sup> T cells were depleted (efficiency of depletion, >95%; Fig. 1F and G). In summary, these data demonstrate that RT in combination with CD40 mAb generated CD8<sup>+</sup> T-cell responses capable of both improving survival and preventing recurrence of T- and B-cell lymphoma.

**B cells do not contribute to antitumor CD8<sup>+</sup> T-cell responses.** Given the capacity of B cells to prime CD8<sup>+</sup> T-cell responses we sought to determine the impact of B-cell depletion on the

efficacy of concurrent RT plus CD40 mAb therapy. Using a depleting CD20 mAb, we observed no significant impact on therapeutic response, either in terms of overall survival or tumor volume, of combination therapy in mice bearing established T-cell lymphoma (EL4, Fig. 2A and EG7, Fig. 2B). Depletion of B cells was >95% as confirmed by flow cytometry (Fig. 2C). Given the expression of CD20 on the BCL<sub>1</sub> cells, we were unable to selectively deplete B cells in mice bearing this tumor model. These data demonstrated that B cells do not influence the generation of antitumor immune responses after RT plus CD40 mAb therapy.

**Macrophages do not impact the efficacy of combination RT plus CD40 mAb therapy.** To determine the level of uptake of irradiated tumor cells by MØs *in vivo*, PKH-26–labeled BCL<sub>1</sub> lymphoma cells were injected into the peritoneal cavity of BALB/c mice. Animals were then treated with 5 Gy RT. Peritoneal cells were harvested 24 h later by lavage and uptake by MØs assessed by microscopy (Fig. 3A). MØs (labeled green with Alexa-fluor phalloidin) could clearly be seen to phagocytose irradiated tumor cells (labeled red with PKH-26). The degree of uptake was quantified by flow cytometry and found to correlate with RT-dose, with approximately 60% of tumor cells engulfed by MØs after 5 Gy, compared with 40% after 2 Gy RT (Fig. 3B). A similar RT dose-dependent increase in uptake by peritoneal MØs was observed when tumor cells were irradiated *in vitro* and injected into mice (data not shown).

To assess the influence of MØs on therapeutic outcome following RT plus CD40 mAb therapy, clodronate-encapsulated liposomes (Clod-lip) were used to deplete MØs in mice bearing established B- and T-cell lymphomas. Initially we confirmed *in vitro* that treatment with Clod-lip was not directly impacting tumor cell viability (Supplementary Fig. S1A) and that treatment *in vivo* led to successful depletion of MØs (confirmed in parallel cohorts sacrificed after treatment)

(Supplementary Fig. S1B). Depletion of MØs had no effect on the combination RT plus CD40 mAb in either the BCL<sub>1</sub> (Fig. 3C; frequency of LTS: 43% vs. 57%; combined therapy + Clod-lip vs. combined therapy + PBS-lip;  $P = 0.31$ ) or EL4 models (Fig. 3D; frequency of LTS: 100% vs. 80%; combined therapy + Clod-lip vs. combined therapy + PBS-lip;  $P = 0.44$ ). In addition, MØ depletion had no significant effect on therapeutic outcome after treatment with RT alone (data not shown). These observations demonstrate that MØs were dispensable for T-cell priming in response to combination therapy with RT and CD40 mAb.

**Dendritic cell depletion abrogates the therapeutic effect of combination therapy.** Ligation of CD40 is one of the key signals involved in DC activation facilitating effective T-cell priming (6). Thus, DC are an attractive candidate for being the principle antigen cross-presenting cell involved in generating the CD8<sup>+</sup> T-cell response following combination therapy, particularly as both B cells and MØs appear dispensable (Figs. 2 and 3, respectively). Using a phagocytosis assay whereby CD11c<sup>+</sup> DCs isolated from the spleen were cocultured with PKH-26–labeled tumor cells, we demonstrated that DCs efficiently engulfed irradiated B-lymphoma cells (Fig. 4A). Uptake was significantly reduced in the presence of sodium azide, which inhibits DC phagocytosis (21). The effect on therapeutic outcome of depleting DCs was assessed using the well-established CD11c-DTR transgenic model, whereby administration of diphtheria toxin (DT) results in the temporal ablation of DCs (22). To circumvent problems of toxicity associated with expression of the receptor on nonhematopoietic cells (22), radiation chimeras were produced by transplanting transgenic bone marrow into lethally irradiated wild-type mice. Treatment of chimeras with DT resulted in depletion of over 90% of splenic DCs as confirmed by flow cytometry and immunohistochemistry (Fig. 4B and C). CD11c-DTR chimeric mice bearing

either the B- or T-cell lymphoma developed tumors with kinetics similar to wild-type BALB/c and C57BL/6 mice (Fig. 3C vs. 4D for BALB/c and Fig. 3D vs. 4E for C57BL/6). As the BCL<sub>1</sub> tumor is maintained *in vivo*, tumors were passaged through transgenic animals at least twice before use, to eliminate any contaminating nontransgenic DC. In models of both B- and T-cell lymphoma our results showed that long-term survival following combination therapy with RT plus CD40 mAb is dependent on the activity of CD11c<sup>+</sup> DCs, with the frequency of LTS reducing from 80% to 0% in BCL<sub>1</sub> bearing mice (Fig. 4D) and from ~70% to 0% in EL4 bearing mice (Fig. 4E) (both  $P < 0.05$  Log-rank; Mantel-Cox test). Using pentamers specific for the immunodominant class I MHC-restricted ovalbumin epitope SIINFEKL, we found a strong trend of reduced tumor antigen-specific CTLs in the peripheral blood of DC depleted vs. non depleted EG7 tumor bearing mice treated with RT/CD40 mAb combination therapy ( $P=0.075$  two-tailed Student's *t* test) (Supplementary Figure 2). DC-depleted EL4 tumor-bearing mice had an increased incidence of lymphatic metastasis following RT plus CD40 mAb therapy when compared to nondepleted cohorts (~30% to greater than 80% incidence in nondepleted vs. depleted mice treated with RT plus CD40 mAb) (Fig. 4F).

Previous studies have demonstrated that in addition to depletion of CD11c<sup>+</sup> cells, administration of DT to CD11c-DTR mice also depletes CD169<sup>+</sup> MØs, which can contribute to cross-presentation of dead cell-associated antigens (23). Therefore, to delineate the possible role of CD169<sup>+</sup> MØs in T-cell priming we used CD169-DTR mice, which following administration of DT are specifically depleted of this MØ population. Our data showed that specific depletion of CD169<sup>+</sup> MØs had no effect on the efficacy of combination RT plus CD40 mAb (Supplemental Fig. S3).

**Dendritic cells critical for therapeutic effect of RT and TLR7 agonist.** We have previously demonstrated that combination therapy with low-dose fractionated RT and the systemically administered TLR7 agonist, R848, leads to durable protective CD8<sup>+</sup> T-cell responses in models of B- and T-cell lymphoma (3) (and Supplemental Fig. S4). Like CD40 mAb therapy, administration of TLR7 agonists can activate multiple APC populations. To determine whether T-cell priming in this context was also DC-dependent, we next evaluated how depletion of APC populations impacted the generation of tumor antigen-specific CD8<sup>+</sup> T-cell responses following combination RT and R848. In EL4 lymphoma bearing mice, depletion of DC, but not B cells (Fig. 5A and 5B) or MØs (Fig. 5C), abrogated the generation of protective immune responses following combined therapy. In keeping with the RT plus CD40 mAb data we again observed loss of both local and distal tumor control following depletion of DC (Fig. 5A). Together, these data confirm the requirement of DC for the priming of anti-lymphoma responses following treatment with RT in combination with a range of immunotherapies.

## **Discussion**

We have previously shown that treatment of syngeneic murine lymphoma models with the combination of RT and either CD40 mAb or TLR7 agonists can induce protective CD8<sup>+</sup> T-cell responses (3, 7, 8). However, the importance of different APC subpopulations to priming therapeutic responses following combination approaches remained unknown. We utilized models that enable the depletion of key APC populations, namely B cells, MØs, and DCs to evaluate their relative contribution, and demonstrate that the therapeutic efficacy of RT delivered in combination with immunomodulatory therapy is primarily dependent on DCs, as depletion of

this population markedly decreases the therapeutic effect on long-term tumor free survival. In contrast, therapeutic responses appear to be independent of MØs and B-cell-mediated priming.

As pan-depletion of MØs did not impact the induction of long-term protective responses after combination RT plus CD40 mAb or TLR7 agonist therapy, this suggested that MØ populations were not the primary APC eliciting therapeutic outcome. This was despite the fact that MØs are known to promote antiproliferative and tumor-static activity. MØs activated by CD40 ligation or TLR7 activation produce pro-inflammatory cytokines, upregulate MHC II and costimulatory molecules, and produce NO, resulting in suppression of tumor growth, induction of tumor apoptosis, and direct killing (24, 25). Moreover, MØs can prime naïve CD8<sup>+</sup> T-cell effector function and memory cell differentiation *in vivo* (16). However, our data suggests that in the context of combination treatment with RT and either  $\alpha$ CD40 or TLR7, MØs are not a major contributing APC for generating host immune responses against tumor and subsequent therapeutic outcome. This may in part be due to the effects that uptake of dying tumor cells have on MØs, such as modulation of their phenotype, function, and antigen-presenting capacity, which may limit their potential to effectively prime T cells (26, 27). Furthermore, MØs display broad heterogeneity, plasticity, and range of activation states. The liposomal approach we used does not allow us to determine the relative contribution of particular MØ subtypes, which may vary between either CD40 or TLR7 monotherapy and RT combination treatment strategies. However, as protective responses remained intact in MØ depleted mice, our data suggest that MØs were redundant in priming effector T-cell responses in these lymphoma models.

Similarly, our data indicate that B cells may be dispensable for priming of protective responses generated by RT and immunomodulatory agent combinations. Normal B cells can cross-prime naïve CD8<sup>+</sup> T cells in vivo (28) and CD40 activation enhances antigen presentation, endowing both normal and malignant B cells with the capacity to stimulate CD4<sup>+</sup> and CD8<sup>+</sup> T-cells responses (29, 30). Likewise, ligation of TLR7 on B cells promotes maturation, accompanied by upregulation of costimulatory markers and MHC molecules as well as Fc receptors (31, 32). However, depletion of B cells using CD20 mAb had no significant impact on the therapeutic response in our T-cell lymphoma model suggesting that, even if B cells are activated, their contribution to T-cell priming was minimal. Indeed, tumor growth in B-cell depleted mice was slightly reduced by combination therapy, possibly because CD40 mAb in depleted mice is not sequestered by nonmalignant B cells, potentially increasing the ligation of CD40 on DC. This observation cannot be confirmed in the B-cell lymphoma model; we could not selectively deplete normal B cells without having a direct impact on the tumor. Treatment with agonistic CD40 mAb as a monotherapy has been shown to be critically dependent on the ability to cross-link FcγRIIB, which can be improved by isotype switching to enhance antibody effector function (33, 34). In the BCL<sub>1</sub> model, FcγRIIB expression on the lymphoma cells themselves was sufficient to cross-link mAb bound to CD40, resulting in CD8<sup>+</sup> T-cell dependent efficacy (4, 35). Thus, it is possible that following CD40 mAb therapy, the B-cell lymphoma cells could directly stimulate T-cell responses. However, as depletion of DCs completely abrogates the long-term therapeutic effect of RT and CD40 mAb combination therapy, it is clear that alternative priming pathways were activated. In this scenario, RT may alter the response due to the potent cytotoxic effects on lymphocytes, leading to significant BCL<sub>1</sub> tumor reduction (7). Thus, for agonistic CD40 mAb given in combination with RT, the dependency on FcγR cross-linking may be minimized due to

tumor depletion, with a concomitant increase in dying tumor cells skewing the response towards uptake and subsequent antigen presentation by CD40-activated DCs. The requirement of Fc $\gamma$ R for B-cell activation after agonistic CD40 mAb therapy is not obligate, as activation of B cells using the clinical CD40 mAb, CP-870, 393 is independent of Fc $\gamma$ R cross-linking (36). For such mAb, for which isotype switching to enhance efficacy may be less relevant, combination with RT may provide an alternative means of enhancing therapeutic efficacy.

In contrast to M $\emptyset$  or B-cell depletion, conditional ablation of DCs using a CD11c-DTR transgenic model completely abrogated long-term protection following both CD40 mAb and TLR7 agonist therapy when delivered in combination with RT. However, survival in DC-depleted animals was still enhanced over controls suggesting that either residual DCs remaining after DT-mediated depletion are sufficient to prime a partially protective response or other APCs, including B-cell tumors themselves in the case of the BCL<sub>1</sub> model, are able to cooperate in priming a sub-optimal T-cell response.

This DC-dependency may represent the confluence of several immunostimulatory signals arising from both RT and immunotherapy that could potentially act in concert to enhance priming of a tumor-specific T-cell response. Direct effects of radiation on enhancing DC antigen processing, presentation, and priming are dose-dependent and remain controversial (37). However, a wealth of evidence now suggests that cytotoxic therapies such as RT can induce the release of damage-associated molecular patterns (DAMPs) from dying tumor cells, which function as danger signals, rendering death more immunogenic by promoting the effective acquisition and processing of tumor antigen by DC (38-41). Localized DAMP release also contributes to the intra-tumoral recruitment of DC required for priming CD8<sup>+</sup> responses (41). Thus, RT may serve



to create a pool of dying tumor cells that act as a source of antigen for uptake and presentation by DCs, a process which is directly enhanced by RT-related modulation of DC activity. However, our data demonstrate that in the context of an established tumor microenvironment, RT alone is insufficient to generate protective T-cell responses (3, 7). Therefore, combination approaches that aim to target dominant immune-suppressive pathways or co-activating pathways may cooperate with RT-mediated effects to facilitate the induction of durable therapeutic anti-cancer immune responses. In this respect, ligation of CD40 by agonistic mAb acts as an effective substitute for binding by the natural ligand, CD154, expressed on cognate CD4<sup>+</sup> T cells, to deliver DC maturation and activation signals, licensing DC for T-cell priming (42-44). These data are consistent with studies using human DC and the CD40 agonist CP-870,893, which demonstrated the DC requirement for antitumor responses *in vivo* (45). Thus, when used in combination, the co-activating signals delivered by CD40 mAb may work in concert with RT to enhance DC functionality at a number of levels and contribute to more effective licensing of an antitumor CD8<sup>+</sup> T-cell response.

Similar to our observations with CD40 mAb we determined that the significantly improved efficacy of RT in combination with systemically administered TLR7 selective agonist was also DC dependent. Activation of TLR7 leads to the production of type I and II IFN, which facilitates DC activation and the robust stimulation of CD8<sup>+</sup> and NK effector responses (5, 46, 47). Recent studies also demonstrate that the immunogenicity of RT lies in its ability to generate intratumoral expression of type I IFN, which facilitates antitumor CD8<sup>+</sup> responses (48). Thus, signaling pathways converge after both RT and TLR7 agonist therapy at the level of the DC, which may further explain the critical contribution of this APC to therapeutic outcome in this treatment setting.

In summary, we have determined the differential role played by MØs, B cells, and DCs in promoting antitumor immunity in response to combination RT and immunotherapy in syngeneic murine models of B- and T-cell lymphoma. Our data demonstrates that while MØs and B cells were redundant, DCs appear to be critical for the induction of long-term protection, with survival significantly reduced in mice ablated of DCs. A number of CD40 mAbs (including CP-870,893, dacetuzumab, lucatumumab, and chiLOB7/4) and TLR7 agonists (imiquimod, resiquimod, and TMX-101) are being tested in clinical trials (49, 50). Evidence arising from the present study indicates that the efficacy of these therapies may be further improved by combination with RT, with DCs as the critical mediator driving tumor-specific CD8<sup>+</sup> T-cell responses. It is likely that successful immunotherapy of cancer will require tiered combinations targeting multiple co-activating and co-inhibitory pathways used alongside established effective cytoreductive anticancer therapy. Our data demonstrate that strategies which co-operate to augment DC priming of anti-cancer CD8<sup>+</sup> T-cell responses are likely to improve therapeutic outcome.

### **Acknowledgements**

We are grateful for the help of members of the Cancer Research UK Manchester Institute BRU and Flow Cytometry Core Facilities. We also thank all members of the Targeted Therapy Group for helpful discussion and critical review of this manuscript. This work was supported by grants from Leukaemia & Lymphoma Research and Cancer Research UK.

### **Authorship**

S.J.D. and J.H. designed and conducted research, analyzed and interpreted data, and wrote the manuscript; G.L.B., L.M., E.J.C., conducted and analyzed research; M.J.G. provided reagents and edited manuscript; S.A.B. conducted and analyzed research, and edited the manuscript; T.M.I. designed research, analyzed and interpreted data, and wrote the manuscript. No conflicts of interest are declared.

## References

1. Brahmer JR, Tykodi SS, Chow LQ, Hwu WJ, Topalian SL, Hwu P, et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N Engl J Med*. 2012;366:2455-65.
2. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med*. 2010;363:711-23.
3. Dovedi SJ, Melis MH, Wilkinson RW, Adlard AL, Stratford IJ, Honeychurch J, et al. Systemic delivery of a TLR7 agonist in combination with radiation primes durable antitumor immune responses in mouse models of lymphoma. *Blood*. 2013;121:251-9.
4. French RR, Chan HT, Tutt AL, Glennie MJ. CD40 antibody evokes a cytotoxic T-cell response that eradicates lymphoma and bypasses T-cell help. *Nat Med*. 1999;5:548-53.
5. Koga-Yamakawa E, Dovedi SJ, Murata M, Matsui H, Leishman AJ, Bell J, et al. Intratracheal and oral administration of SM-276001: a selective TLR7 agonist, leads to antitumor efficacy in primary and metastatic models of cancer. *Int J Cancer*. 2013;132:580-90.
6. Vonderheide RH, Glennie MJ. Agonistic CD40 antibodies and cancer therapy. *Clin Cancer Res*. 2013;19:1035-43.
7. Honeychurch J, Glennie MJ, Johnson PW, Illidge TM. Anti-CD40 monoclonal antibody therapy in combination with irradiation results in a CD8 T-cell-dependent immunity to B-cell lymphoma. *Blood*. 2003;102:1449-57.
8. Adlard AL, Dovedi SJ, Telfer BA, Koga-Yamakawa E, Pollard C, Honeychurch J, et al. A novel systemically administered Toll-like receptor 7 agonist potentiates the effect of ionizing radiation in murine solid tumor models. *Int J Cancer*. 2014;135:820-9.

9. Honeychurch J, Glennie MJ, Illidge TM. Cyclophosphamide inhibition of anti-CD40 monoclonal antibody-based therapy of B cell lymphoma is dependent on CD11b<sup>+</sup> cells. *Cancer Res.* 2005;65:7493-501.
10. Kroemer G, Galluzzi L, Kepp O, Zitvogel L. Immunogenic cell death in cancer therapy. *Annu Rev Immunol.* 2013;31:51-72.
11. Reits EA, Hodge JW, Herberts CA, Groothuis TA, Chakraborty M, Wansley EK, et al. Radiation modulates the peptide repertoire, enhances MHC class I expression, and induces successful antitumor immunotherapy. *J Exp Med.* 2006;203:1259-71.
12. Sistigu A, Yamazaki T, Vacchelli E, Chaba K, Enot DP, Adam J, et al. Cancer cell-autonomous contribution of type I interferon signaling to the efficacy of chemotherapy. *Nat Med.* 2014;20:1301-9.
13. Surace L, Lysenko V, Fontana AO, Cecconi V, Janssen H, Bievic A, et al. Complement is a central mediator of radiotherapy-induced tumor-specific immunity and clinical response. *Immunity.* 2015;42:767-77.
14. Heath WR, Carbone FR. Cross-presentation, dendritic cells, tolerance and immunity. *Annu Rev Immunol.* 2001;19:47-64.
15. den Haan JM, Lehar SM, Bevan MJ. CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. *J Exp Med.* 2000;192:1685-96.
16. Pozzi LA, Maciaszek JW, Rock KL. Both dendritic cells and macrophages can stimulate naive CD8 T cells in vivo to proliferate, develop effector function, and differentiate into memory cells. *J Immunol.* 2005;175:2071-81.
17. Norian LA, Rodriguez PC, O'Mara LA, Zabaleta J, Ochoa AC, Cella M, et al. Tumor-infiltrating regulatory dendritic cells inhibit CD8<sup>+</sup> T cell function via L-arginine metabolism. *Cancer Res.* 2009;69:3086-94.
18. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol.* 2002;23:549-55.
19. Schultze JL, Cardoso AA, Freeman GJ, Seamon MJ, Daley J, Pinkus GS, et al. Follicular lymphomas can be induced to present alloantigen efficiently: a conceptual model to improve their tumor immunogenicity. *Proc Natl Acad Sci U S A.* 1995;92:8200-4.

20. Van Rooijen N, Sanders A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J Immunol Methods*. 1994;174:83-93.
21. Schulz O, Reis e Sousa C. Cross-presentation of cell-associated antigens by CD8 $\alpha$ <sup>+</sup> dendritic cells is attributable to their ability to internalize dead cells. *Immunology*. 2002;107:183-9.
22. Jung S, Unutmaz D, Wong P, Sano G, De los Santos K, Sparwasser T, et al. In vivo depletion of CD11c(+) dendritic cells abrogates priming of CD8(+) T cells by exogenous cell-associated antigens. *Immunity*. 2002;17:211-20.
23. Miyake Y, Asano K, Kaise H, Uemura M, Nakayama M, Tanaka M. Critical role of macrophages in the marginal zone in the suppression of immune responses to apoptotic cell-associated antigens. *J Clin Invest*. 2007;117:2268-78.
24. Lum HD, Buhtoiarov IN, Schmidt BE, Berke G, Paulnock DM, Sondel PM, et al. Tumoristatic effects of anti-CD40 mAb-activated macrophages involve nitric oxide and tumour necrosis factor-alpha. *Immunology*. 2006;118:261-70.
25. Makela SM, Strengell M, Pietila TE, Osterlund P, Julkunen I. Multiple signaling pathways contribute to synergistic TLR ligand-dependent cytokine gene expression in human monocyte-derived macrophages and dendritic cells. *J Leukoc Biol*. 2009;85:664-72.
26. Delamarre L, Pack M, Chang H, Mellman I, Trombetta ES. Differential lysosomal proteolysis in antigen-presenting cells determines antigen fate. *Science*. 2005;307:1630-4.
27. Huynh ML, Fadok VA, Henson PM. Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF-beta1 secretion and the resolution of inflammation. *J Clin Invest*. 2002;109:41-50.
28. Heit A, Huster KM, Schmitz F, Schiemann M, Busch DH, Wagner H. CpG-DNA aided cross-priming by cross-presenting B cells. *J Immunol*. 2004;172:1501-7.
29. Lapointe R, Bellemare-Pelletier A, Housseau F, Thibodeau J, Hwu P. CD40-stimulated B lymphocytes pulsed with tumor antigens are effective antigen-presenting cells that can generate specific T cells. *Cancer Res*. 2003;63:2836-43.
30. Wennhold K, Shimabukuro-Vornhagen A, Theurich S, von Bergwelt-Baildon M. CD40-activated B cells as antigen-presenting cells: the final sprint toward clinical application. *Expert Rev Vaccines*. 2013;12:631-7.

31. Barr TA, Brown S, Ryan G, Zhao J, Gray D. TLR-mediated stimulation of APC: Distinct cytokine responses of B cells and dendritic cells. *Eur J Immunol*. 2007;37:3040-53.
32. Hanten JA, Vasilakos JP, Riter CL, Neys L, Lipson KE, Alkan SS, et al. Comparison of human B cell activation by TLR7 and TLR9 agonists. *BMC Immunol*. 2008;9:39.
33. Li F, Ravetch JV. Inhibitory Fcγ receptor engagement drives adjuvant and anti-tumor activities of agonistic CD40 antibodies. *Science*. 2011;333:1030-4.
34. White AL, Chan HT, Roghanian A, French RR, Mockridge CI, Tutt AL, et al. Interaction with FcγRIIB is critical for the agonistic activity of anti-CD40 monoclonal antibody. *J Immunol*. 2011;187:1754-63.
35. White AL, Dou L, Chan HT, Field VL, Mockridge CI, Moss K, et al. Fcγ receptor dependency of agonistic CD40 antibody in lymphoma therapy can be overcome through antibody multimerization. *J Immunol*. 2014;193:1828-35.
36. Richman LP, Vonderheide RH. Role of crosslinking for agonistic CD40 monoclonal antibodies as immune therapy of cancer. *Cancer Immunol Res*. 2014;2:19-26.
37. Manda K, Glasow A, Paape D, Hildebrandt G. Effects of ionizing radiation on the immune system with special emphasis on the interaction of dendritic and T cells. *Front Oncol*. 2012;2:102.
38. Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A, et al. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. *Nat Med*. 2007;13:1050-9.
39. Honeychurch J, Melis MH, Dovedi SJ, Mu L, Illidge TM. Immunogenic potential of irradiated lymphoma cells is enhanced by adjuvant immunotherapy and modulation of local macrophage populations. *Leuk Lymphoma*. 2013;54:2008-15.
40. Obeid M, Tesniere A, Ghiringhelli F, Fimia GM, Apetoh L, Perfettini JL, et al. Calreticulin exposure dictates the immunogenicity of cancer cell death. *Nat Med*. 2007;13:54-61.
41. Ma Y, Adjemian S, Mattarollo SR, Yamazaki T, Aymeric L, Yang H, et al. Anticancer chemotherapy-induced intratumoral recruitment and differentiation of antigen-presenting cells. *Immunity*. 2013;38:729-41.
42. Schoenberger SP, Toes RE, van der Voort EI, Offringa R, Melief CJ. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature*. 1998;393:480-3.

43. Bennett SR, Carbone FR, Karamalis F, Flavell RA, Miller JF, Heath WR. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature*. 1998;393:478-80.
44. Ridge JP, Di Rosa F, Matzinger P. A conditioned dendritic cell can be a temporal bridge between a CD4<sup>+</sup> T-helper and a T-killer cell. *Nature*. 1998;393:474-8.
45. Gladue RP, Paradis T, Cole SH, Donovan C, Nelson R, Alpert R, et al. The CD40 agonist antibody CP-870,893 enhances dendritic cell and B-cell activity and promotes anti-tumor efficacy in SCID-hu mice. *Cancer Immunol Immunother*. 2011;60:1009-17.
46. Boonstra A, Asselin-Paturel C, Gilliet M, Crain C, Trinchieri G, Liu YJ, et al. Flexibility of mouse classical and plasmacytoid-derived dendritic cells in directing T helper type 1 and 2 cell development: dependency on antigen dose and differential toll-like receptor ligation. *J Exp Med*. 2003;197:101-9.
47. Qi H, Denning TL, Soong L. Differential induction of interleukin-10 and interleukin-12 in dendritic cells by microbial toll-like receptor activators and skewing of T-cell cytokine profiles. *Infect Immun*. 2003;71:3337-42.
48. Burnette BC, Liang H, Lee Y, Chlewicki L, Khodarev NN, Weichselbaum RR, et al. The efficacy of radiotherapy relies upon induction of type I interferon-dependent innate and adaptive immunity. *Cancer Res*. 2011;71:2488-96.
49. Galluzzi L, Vacchelli E, Eggermont A, Fridman WH, Galon J, Sautes-Fridman C, et al. Trial Watch: Experimental Toll-like receptor agonists for cancer therapy. *Oncoimmunology*. 2012;1:699-716.
50. Honeychurch J, Cheadle EJ, Dovedi SJ, Illidge TM. Immuno-regulatory antibodies for the treatment of cancer. *Expert Opin Biol Ther*. 2015;15:787-801.

## Figure Legends

**Figure 1. Combination therapy with RT and CD40 mAb led to the generation of durable antitumor immune responses capable of preventing disease recurrence in murine lymphoma models.** A, Survival curve of EG7 tumor bearing mice following treatment with a



single 10 Gy dose of RT either alone or in combination with CD40 mAb. \*  $P < 0.05$  compared to control mice (log-rank; Mantel-Cox test). B and C, survival curve following contralateral rechallenge of LTS mice with EG7 (B) and tumor growth curve for rechallenge with EL4 (C) cells. D and E, representative dot blot of IFN $\gamma$  production (D) and frequency of IFN $\gamma^+$  CD8 $^+$  T cells (E) isolated from either tumor naïve or LTS mice originally treated with RT and CD40 mAb following coculture with 25 Gy irradiated EL4 cells for 5 days, followed by priming with 25 Gy irradiated EL4 cells. \*\*  $P < 0.01$  (Mann-Whitney test). F, representative density plots of peripheral blood confirming lymphocyte depletion. G, Survival curve of mice bearing established BCL $_1$  tumors following treatment with a single dose of 5 Gy RT in combination with CD40 mAb. Lymphocytes (CD4 $^+$  or CD8 $^+$ ) were depleted 1 day prior to therapy with depletion maintained for up to 2 weeks. \*\*\*,  $P > 0.001$ , \*,  $P > 0.01$ , \*,  $P > 0.05$ , Mann-Whitney test. Experimental groups contained at least 5 mice and are representative of at least 2 independent experiments.

**Figure 2. Depletion of B cells does not impact the efficacy of combination therapy with RT and CD40 mAb in models of T cell lymphoma.** A and B, Survival curve of EL4 (A) and tumor volumes of EG7 (B) bearing mice following combined therapy (10 Gy RT in combination with CD40 mAb). Not significant (n/s),  $P > 0.05$  compared to control mice (log-rank; Mantel-Cox test). n/s,  $P < 0.05$ , Mann-Whitney test. Depletion of B cells achieved by i.p. administration of CD20 mAb 1 day prior to therapy. C, representative dot plots of peripheral blood from control or CD20 mAb-treated mice to confirm depletion. Experimental groups contained at least 5 mice and are representative of 2 independent experiments.



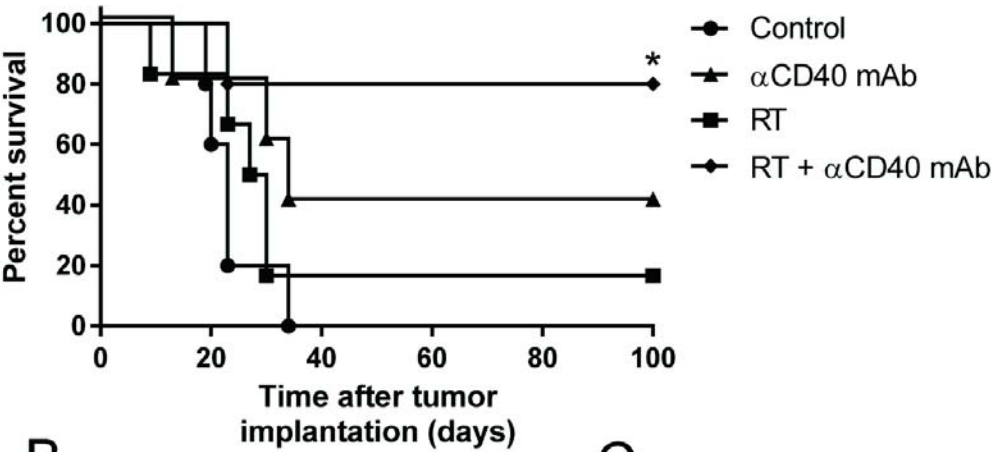
**Figure 3. Macrophages are dispensable for efficacy following combination therapy with RT and CD40 mAb.** A and B) BCL<sub>1</sub> lymphoma cells labeled with PKH-26 were injected i.p in to BALB/c mice and treated with 5 Gy RT. Phagocytosis of irradiated tumor cells by peritoneal MØs was assessed after 24 h by fluorescence microscopy (A) or flow cytometry (B). MØs were labeled with alexa fluor 488-phalloidin or F4/80-FITC respectively. C and D, Survival curves of BCL<sub>1</sub> (C) and EL4 (D) tumor bearing mice following treatment with a single 5 Gy (C) or 10 Gy (D) dose of RT in combination with CD40 mAb. Mice received either PBS or clodronate encapsulated liposomes (PBS-lip and Clod-lip, respectively) on day -1 day +2 and +4 with respect to therapy. n/s,  $P > 0.05$  compared to controls (log-rank; Mantel-Cox test). Experimental groups contained at least 5 mice and are representative of at least 2 independent experiments.

**Figure 4. Dendritic cell depletion abrogates the therapeutic effect of combination therapy.** A, Splenic CD11c<sup>+</sup> DC were isolated and co-incubated with PKH-26–labeled irradiated BCL<sub>1</sub> lymphoma cells at a ratio of 20:1. Uptake was assessed by flow cytometry after 3 h. \*\*\*,  $P < 0.001$  vs. control). B and C, CD11c-DTR chimeric mice received DT (100 ng i.p) and spleen cells analysed for presence of CD11c<sup>+</sup> cells after 24 hours in BALB/c mice by flow cytometry (B) or by immunohistochemistry in C57Bl/6 mice (C). D and E, mice were inoculated with either BCL<sub>1</sub> (D) or EL4 (E) tumor cells and received combined therapy (RT; either 5 Gy or 10 Gy respectively, in combination with CD40 mAb). For DC depletion, animals received 100 ng DT i.p. 3 times per week for up to 2 weeks i.p (D). F, Incidence of metastatic disease present in the lymph nodes in mice following treatment outlined in (E). \*  $P < 0.05$  compared to combination therapy (log-rank; Mantel-Cox test). Experimental groups contained at least 5 mice and are representative of at least 2 independent experiments.

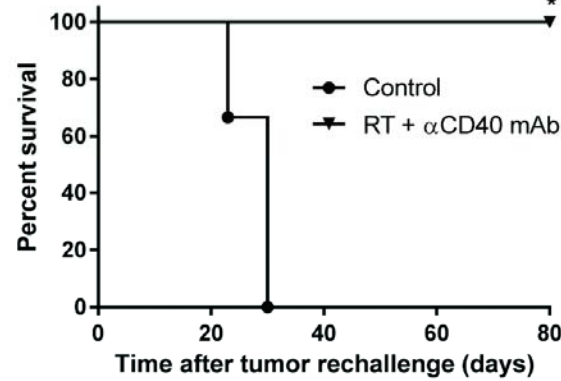
**Figure 5. Dendritic cells are critical for the therapeutic effect of combination therapy with RT and a systemically administered TLR7 agonist.** A and B, Growth (A) and survival (B) curves of EL4 tumor bearing mice following combination therapy with 10 Gy RT and R848. Frequency of systemic disease in cohort expressed as fraction relative to population size (A). Depletion of B cells was achieved by i.p. administration of CD20 mAb 1 day prior to therapy. For DC depletion, animals received 100 ng DT i.p. 3 times per week for up to 2 weeks i.p. *n/s*, *P* > 0.05 compared to controls (log-rank; Mantel-Cox test). C, Mice received either PBS or clodronate encapsulated liposomes (PBS-lip and Clod-lip, respectively) on day -1 day +2 and +4 with respect to therapy with 10 Gy RT and R848. Experimental groups contained at least 5 mice and are representative of at least 2 independent experiments.

Figure 1

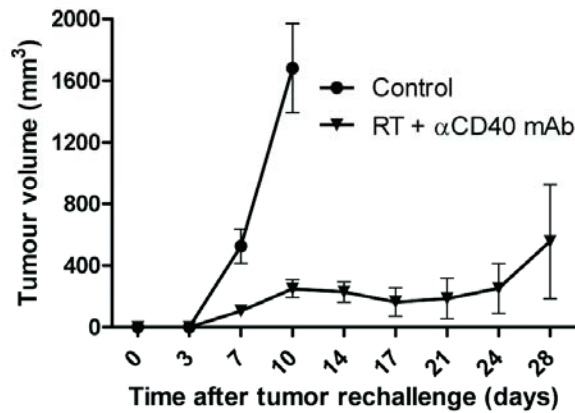
A



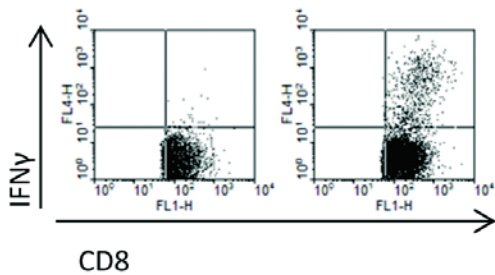
B



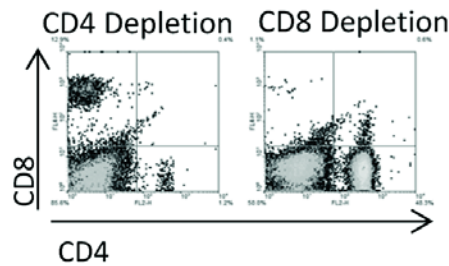
C



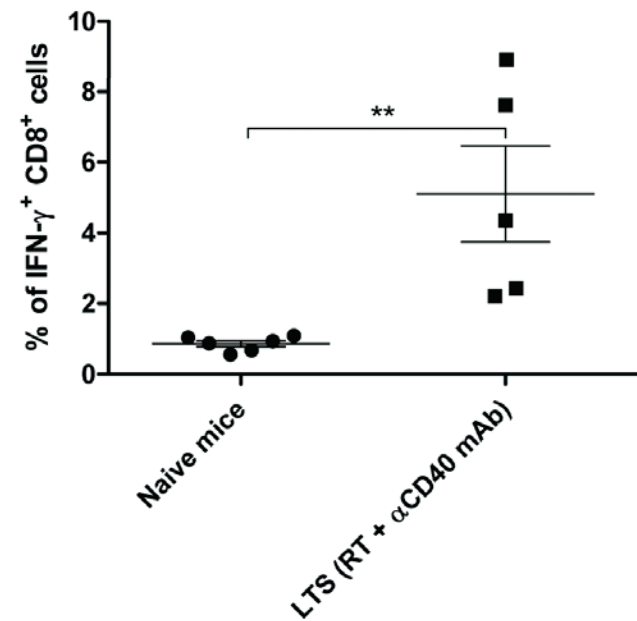
D



F



E



G

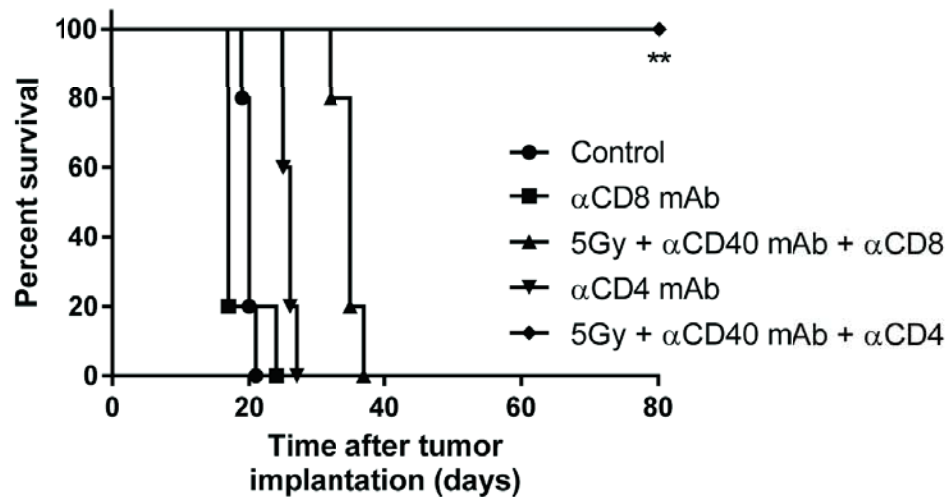
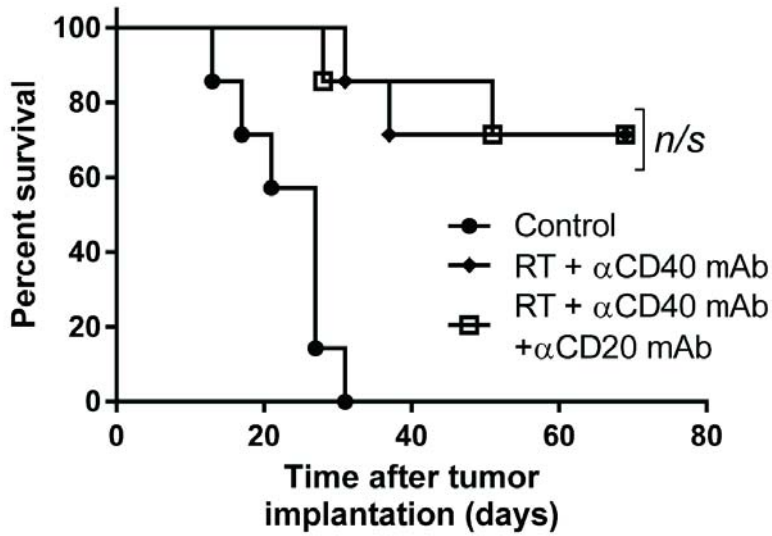
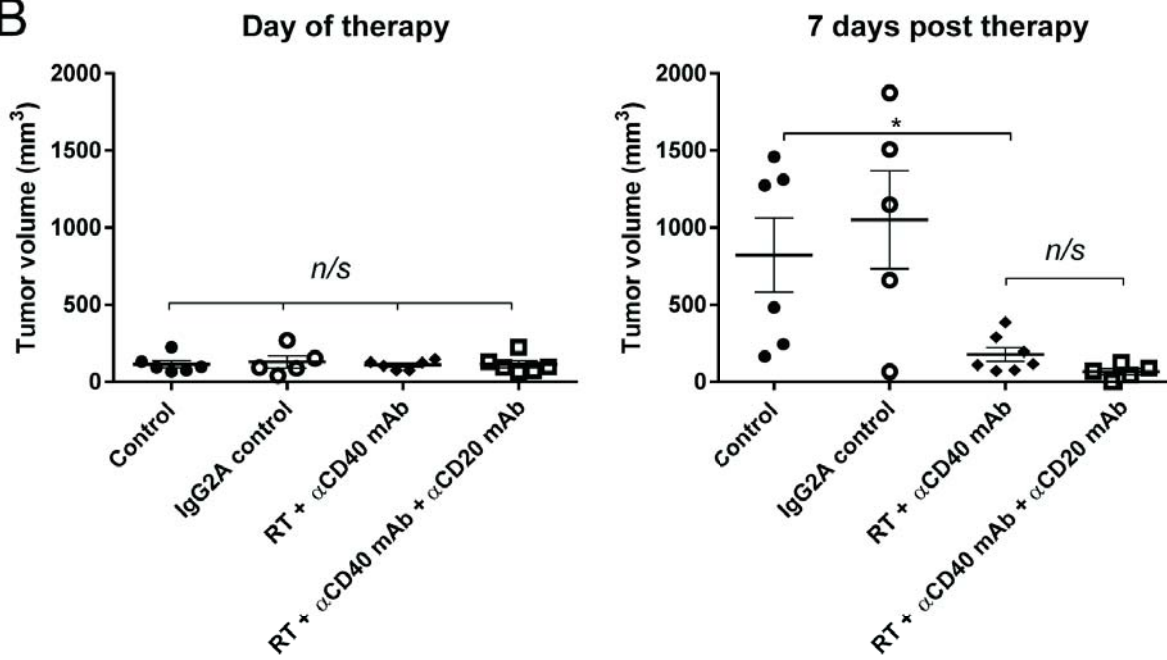


Figure 2

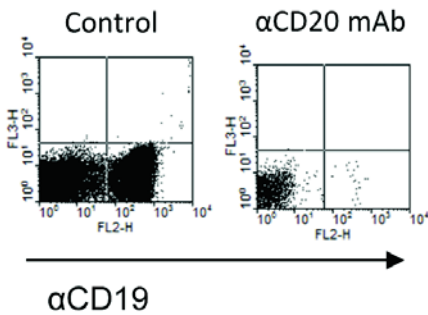
A



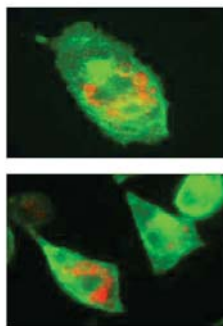
B



C



A



B

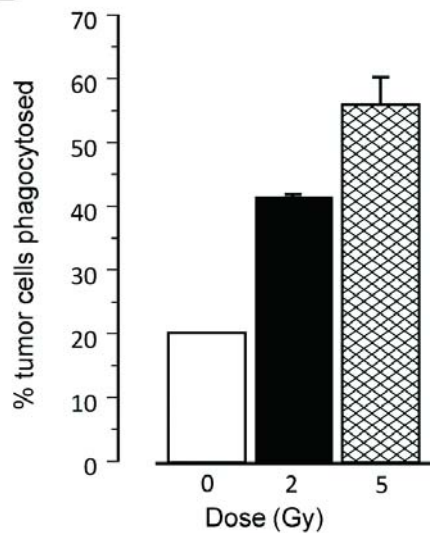
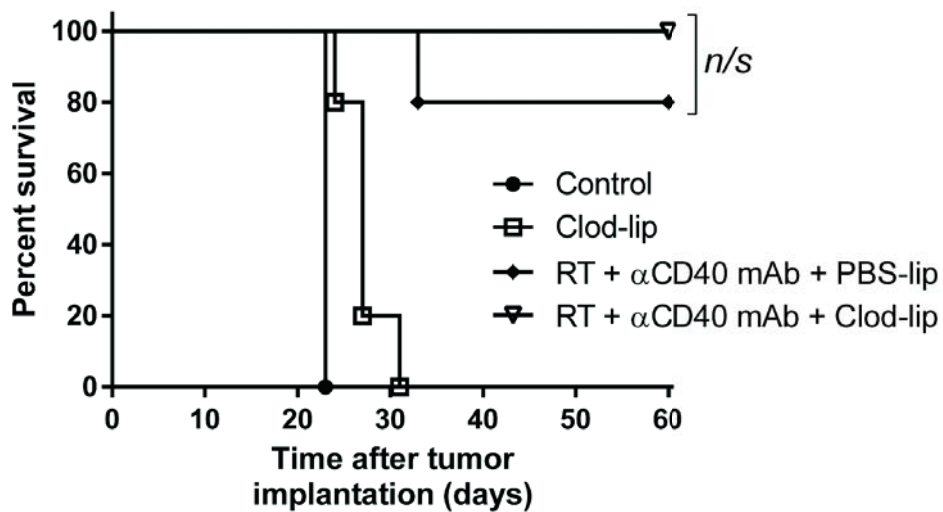
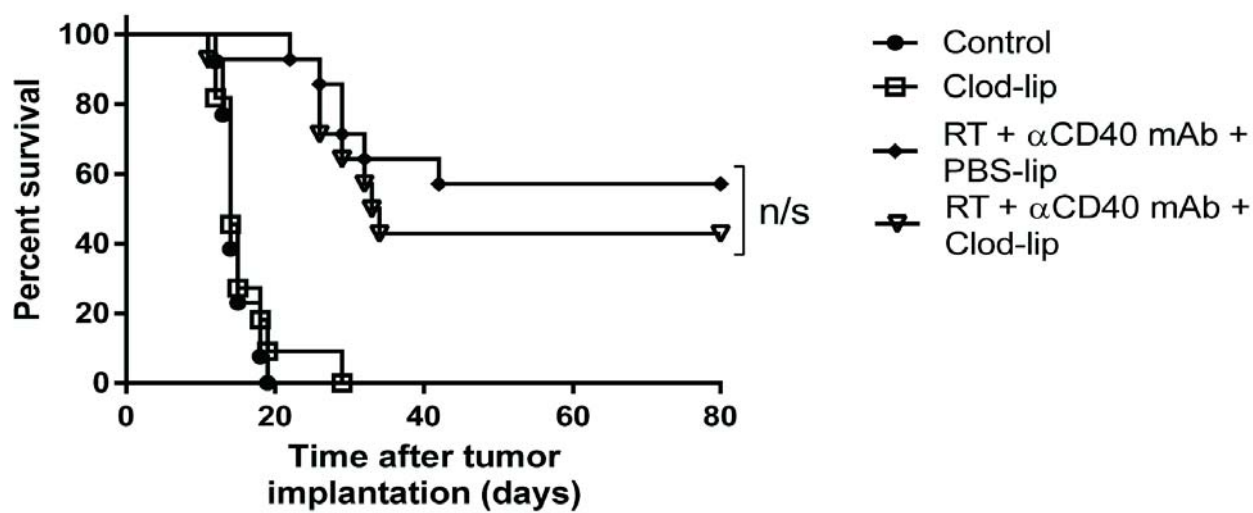


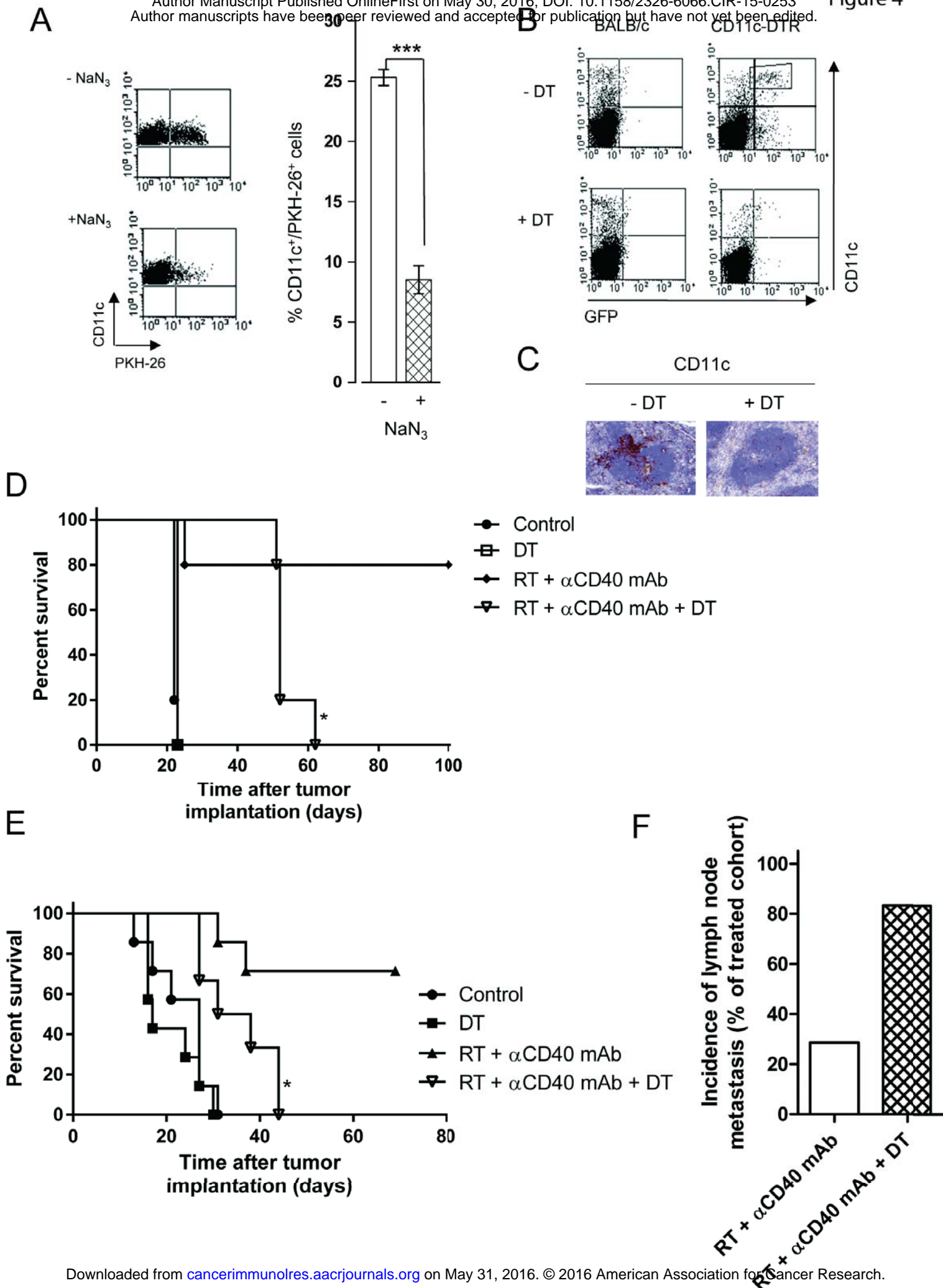
Figure 3

C

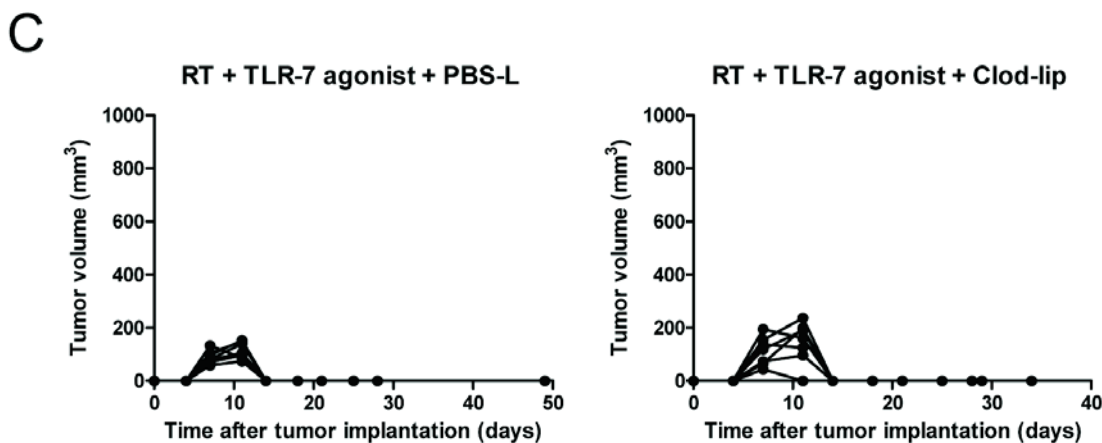
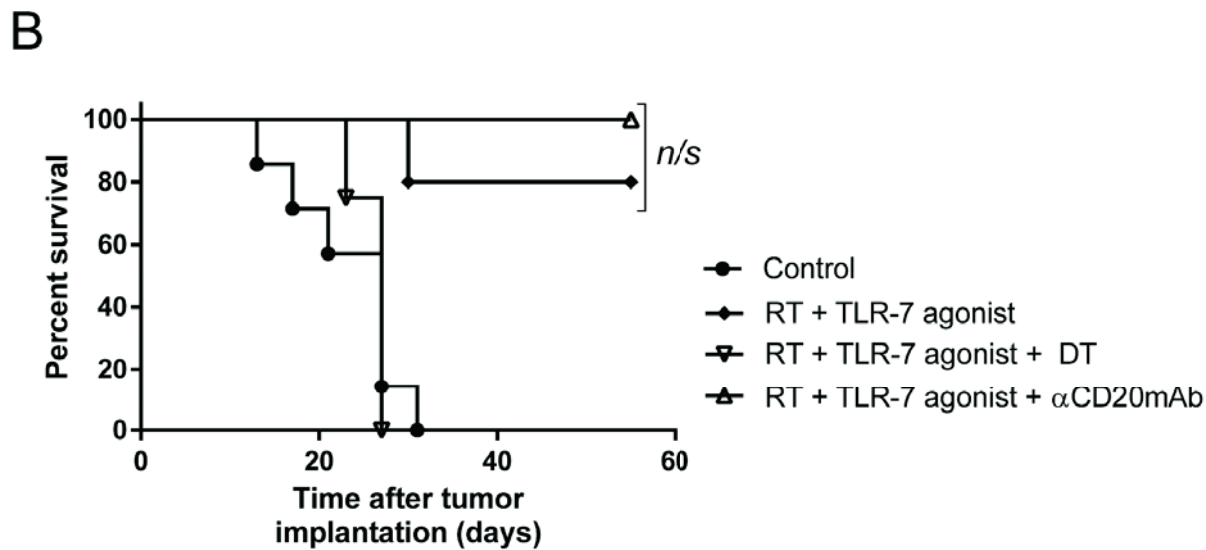
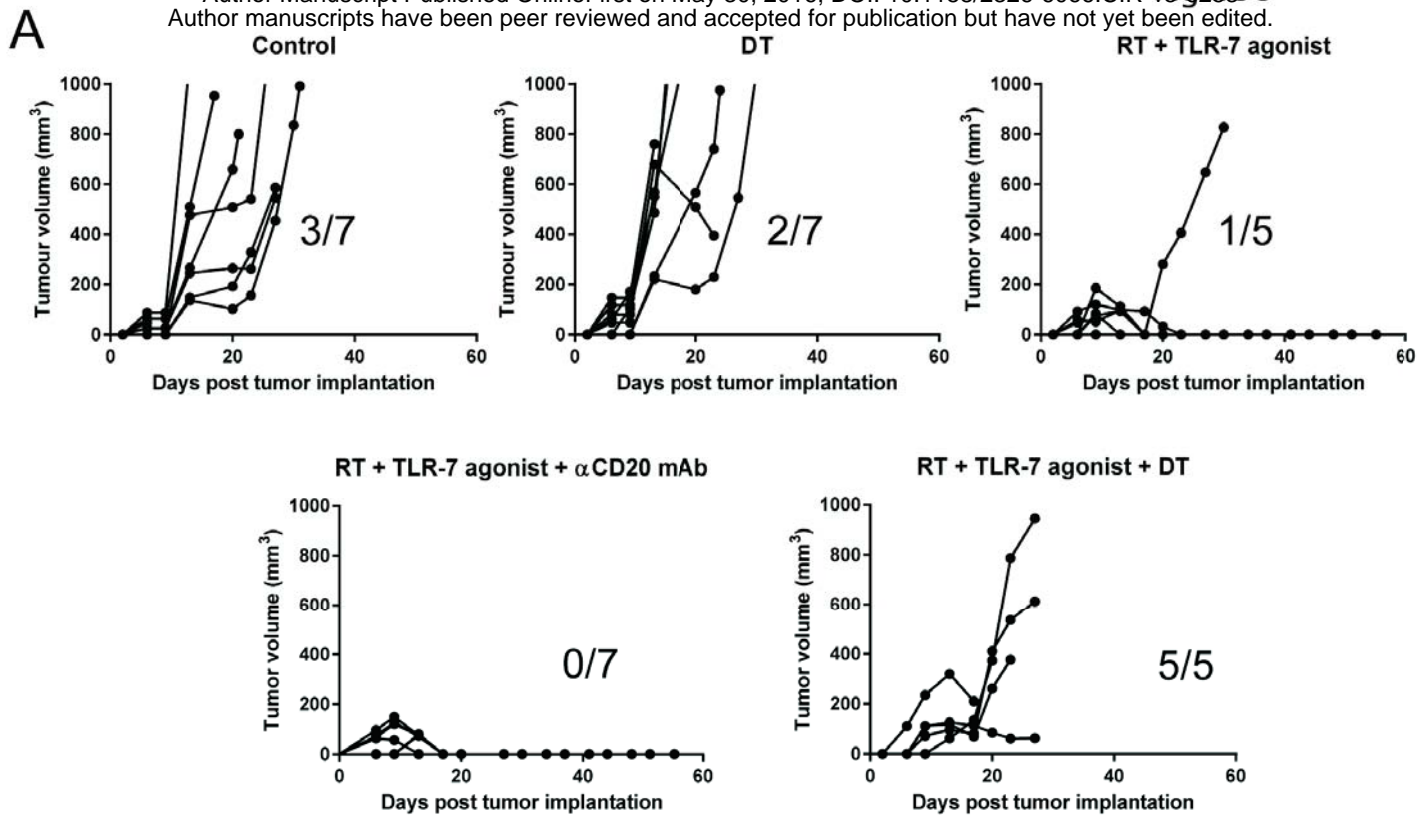


D









# Cancer Immunology Research

## Anti-tumor efficacy of radiation and immunotherapy combinations is dependent on dendritic cell activation of effector CD8<sup>+</sup> T-cells

Simon J Dovedi, Grazyna L Lipowska-Bhalla, Stephen A Beers, et al.

*Cancer Immunol Res* Published OnlineFirst May 30, 2016.

<b>Updated version</b>	Access the most recent version of this article at: doi: <a href="https://doi.org/10.1158/2326-6066.CIR-15-0253">10.1158/2326-6066.CIR-15-0253</a>
<b>Supplementary Material</b>	Access the most recent supplemental material at: <a href="http://cancerimmunolres.aacrjournals.org/content/suppl/2016/05/28/2326-6066.CIR-15-0253.DC1.html">http://cancerimmunolres.aacrjournals.org/content/suppl/2016/05/28/2326-6066.CIR-15-0253.DC1.html</a>
<b>Author Manuscript</b>	Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

<b>E-mail alerts</b>	<a href="#">Sign up to receive free email-alerts</a> related to this article or journal.
<b>Reprints and Subscriptions</b>	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at <a href="mailto:pubs@aacr.org">pubs@aacr.org</a> .
<b>Permissions</b>	To request permission to re-use all or part of this article, contact the AACR Publications Department at <a href="mailto:permissions@aacr.org">permissions@aacr.org</a> .