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

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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.

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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.

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Keywords

Lymphoma; CD40; TLR7; macrophage; radiation therapy; dendritic cell; immunotherapy

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 $CD8^+$ 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 $CD8^+DEC^-205^+$ DCs (15), although MØs are also capable of priming naïve $CD8^+$ T cells after antigen capture (16). Conversely, tumor-associated DCs can function to impair $CD8^+$ 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 TGFβ, and are phenotypically polarized towards immune suppression within the tumor microenvironment (18). Malignant B cells can present antigen to both $CD4^+$ and $CD8^+$ 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 $CD8^+$ 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, MØs 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

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immune system and DC populations to the generation of durable therapeutic antitumor $CD8^+$ T-cell responses that lead to long-term clearance of tumors.

Materials and methods

Animals and cell lines

C57B1/6 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 C57B1/6 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_1 lymphoma (and πBCL_1 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 x 10^6 EG7, 1 x 10^5 EL4 (both s.c.) or 1 x 10^6 BCL_1 cells (i.v.). For the s.c. models, local tumor irradiation was performed 7 days after inoculation (when tumors were approximately 100 mm^3) as previously described (3). For the BCL_1 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 μg, BCL_1 model) or s.c. (500 μg, 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 μL/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 μg, mouse IgG2a clone 18B12, a gift from Robert Dunn, Biogen-Idec, U.S.) 1 day prior to tumor cell inoculation. For MØ depletion, liposomes encapsulating dichloromethylene-bisphosphonate (Cl_2MBP; clodronate) were prepared according to the method of Van Rooijen et al. (20). PBS-liposomes were used as control. MØ depletion was achieved using serial injections of clodronate-liposomes (400 μL followed by up to 2 doses of 200 μL either i.v. or i.p.) 48 h apart. Peritoneal MØ depletion was achieved by a single 250 μL injection, i.p. For CD11c

depletion, 8 week old BALB/c or C57B1/6 mice were irradiated (10 Gy low-dose rate ionising radiation) and reconstituted with 2 x 10^6 CD11c-DTR/GFP bone marrow cells i.v. After 6 weeks chimerised mice were randomised and used in therapeutic studies. DC or $CD169^+$ MØ 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 $CD8^+$ or $CD4^+$ 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 $CD8^+$ 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 x 10^6 BCL_1 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, $CD11c^+$ 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 $F(ab)_2$ (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 $CD8^+$ 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 $CD8^+$ T-cells in LTS mice compared to tumor naïve control mice (Fig.1D and E). Enhanced efficacy after combination treatment was $CD8^+$ T-cell–dependent, as observed in the BCL_1 model of established systemic lymphoma, with survival abrogated when $CD8^+$, but not $CD4^+$ 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 $CD8^+$ T-cell responses capable of both improving survival and preventing recurrence of T- and B-cell lymphoma.

B cells do not contribute to antitumor $CD8^+$ T-cell responses

Given the capacity of B cells to prime $CD8^+$ 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_1 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_1 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_1 (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 $CD8^+$ 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 $CD11c^+$ 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_1 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 $CD11c^+$ DCs, with the frequency of LTS reducing from 80% to 0% in BCL_1 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 $CD11c^+$ cells, administration of DT to CD11c-DTR mice also depletes $CD169^+$ MØs, which can contribute to cross-presentation of dead cell–associated antigens (23). Therefore, to delineate the possible role of $CD169^+$ 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 $CD169^+$ 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 $CD8^+$ 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 $CD8^+$ 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 $CD8^+$ 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 priming naïve $CD8^+$ 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 αCD40 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 $CD8^+$ T cells in vivo (28) and CD40 activation enhances antigen presentation, endowing both normal and malignant B cells with the capacity to stimulate $CD4^+$ and $CD8^+$ 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_1 model, FcγRIIB expression on the lymphoma cells themselves was sufficient to cross-link mAb bound to CD40, resulting in $CD8^+$ 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_1 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 FcγR 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 FcγR 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 MØ 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_1 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 $CD8^+$ 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, CDl54, expressed on cognate $CD4^+$ 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 $CD8^+$ 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 $CD8^+$ 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 $CD8^+$ 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 $CD8^+$ 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 $CD8^+$ T-cell responses are likely to improve therapeutic outcome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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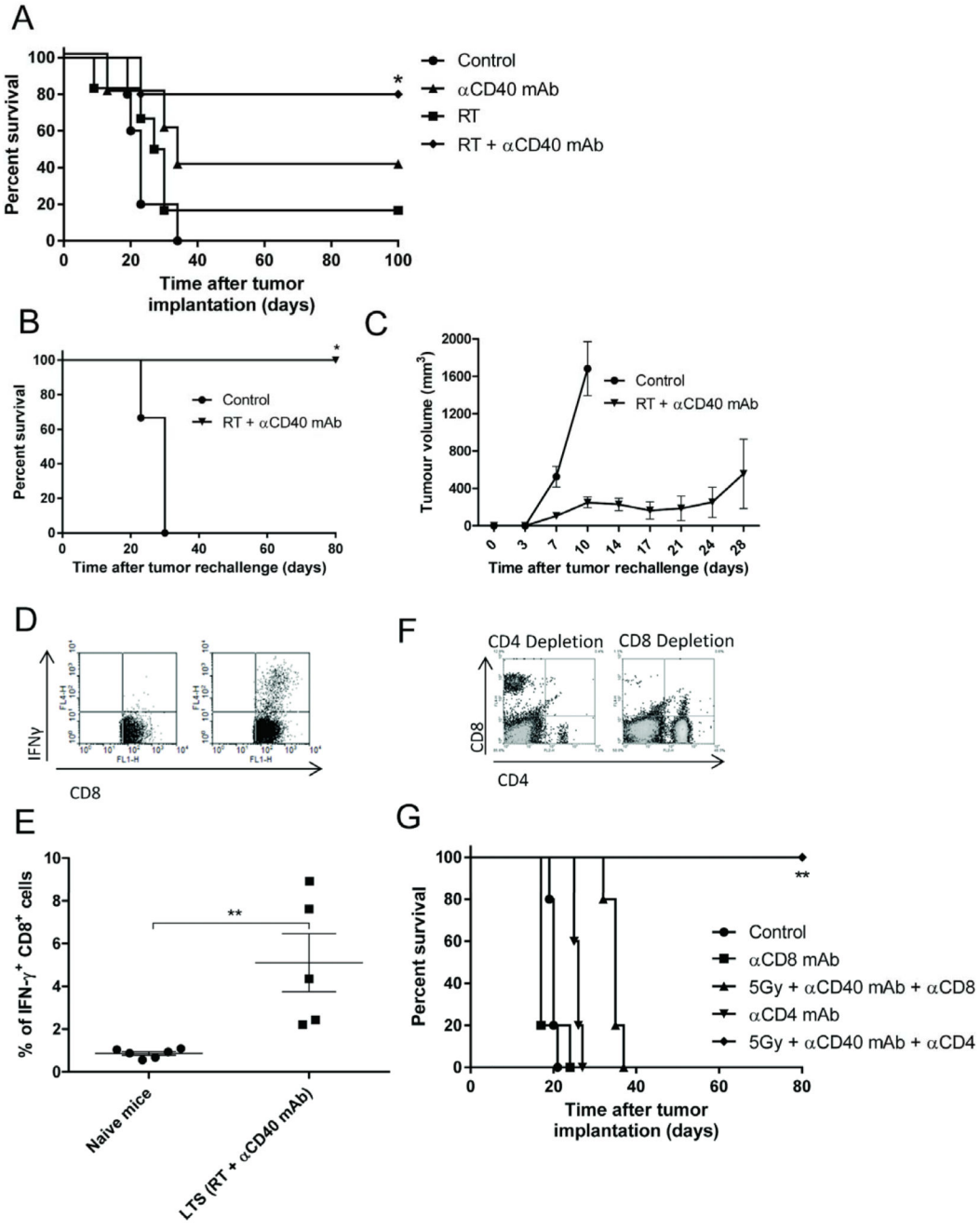

Figure 1. Combination therapy with RT and CD40 mAb led to the generation of durable antitumor immune responses capable of preventing disease recurrence in murine lymphoma models.

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 IFNγ production (D) and frequency of IFNγ$^+$ 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. ** $P < 0.0l$ (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. ***, $P > 0.00l$, *, $P > 0.0l$, *, $P > 0.05$, Mann-Whitney test. Experimental groups contained at least 5 mice and are representative of at least 2 independent experiments.

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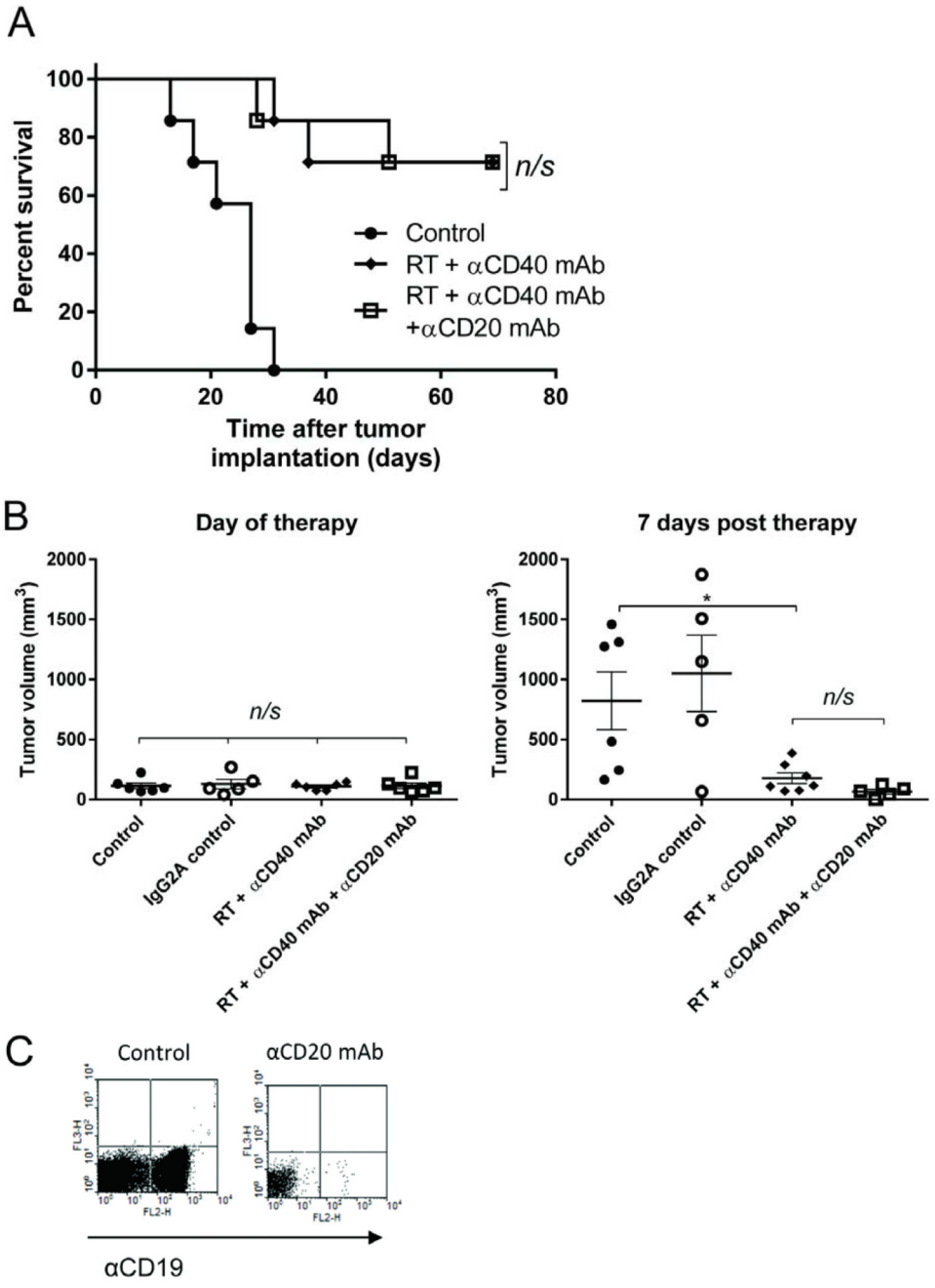

Figure 2. Depletion of B cells does not impact the efficacy of combination therapy with RT and CD40 mAb in models of T cell lymphoma.

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.

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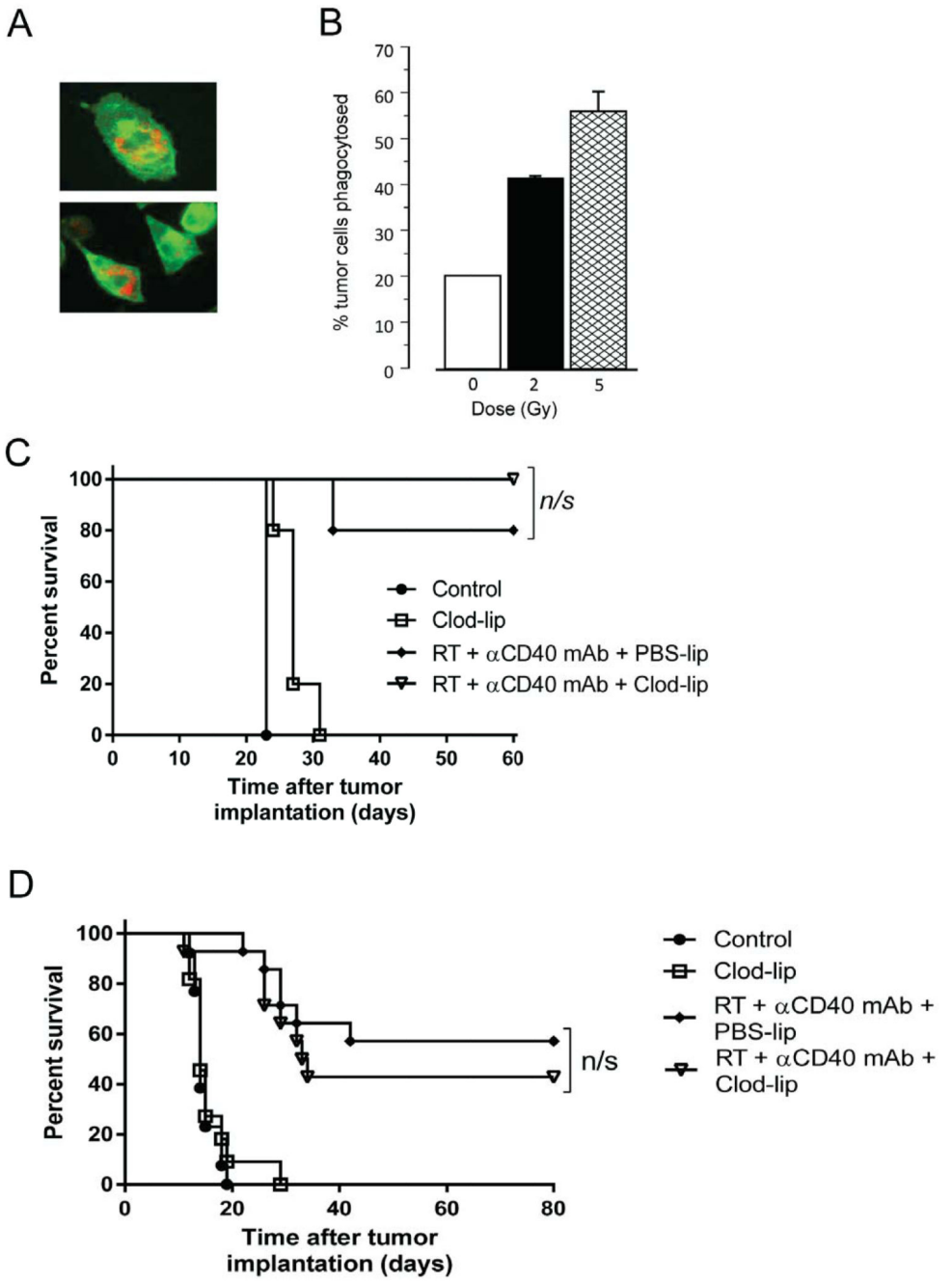

Figure 3. Macrophages are dispensable for efficacy following combination therapy with RT and CD40 mAb.

A and B) BCL_1 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_1 (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.

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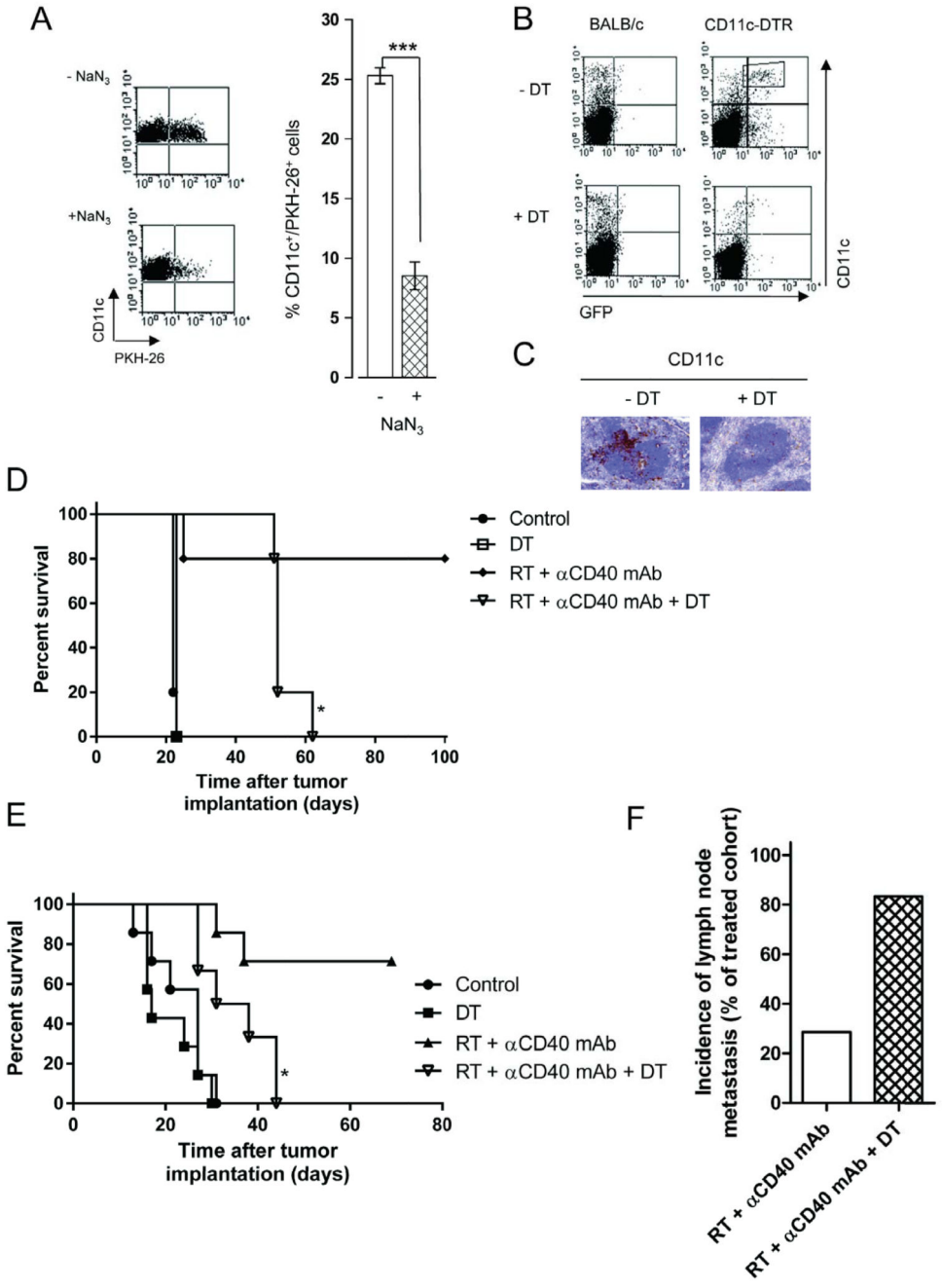

Figure 4. Dendritic cell depletion abrogates the therapeutic effect of combination therapy.

A, Splenic $CD11c^+$ DC were isolated and co-incubated with PKH-26–labeled irradiated BCL1 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 $CD11c^+$ cells after 24 hours in BALB/c mice by flow cytometry (B) or by immunohistochemistry in C57B1/6 mice (C). D and E, mice were inoculated with either BCL_1 (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.

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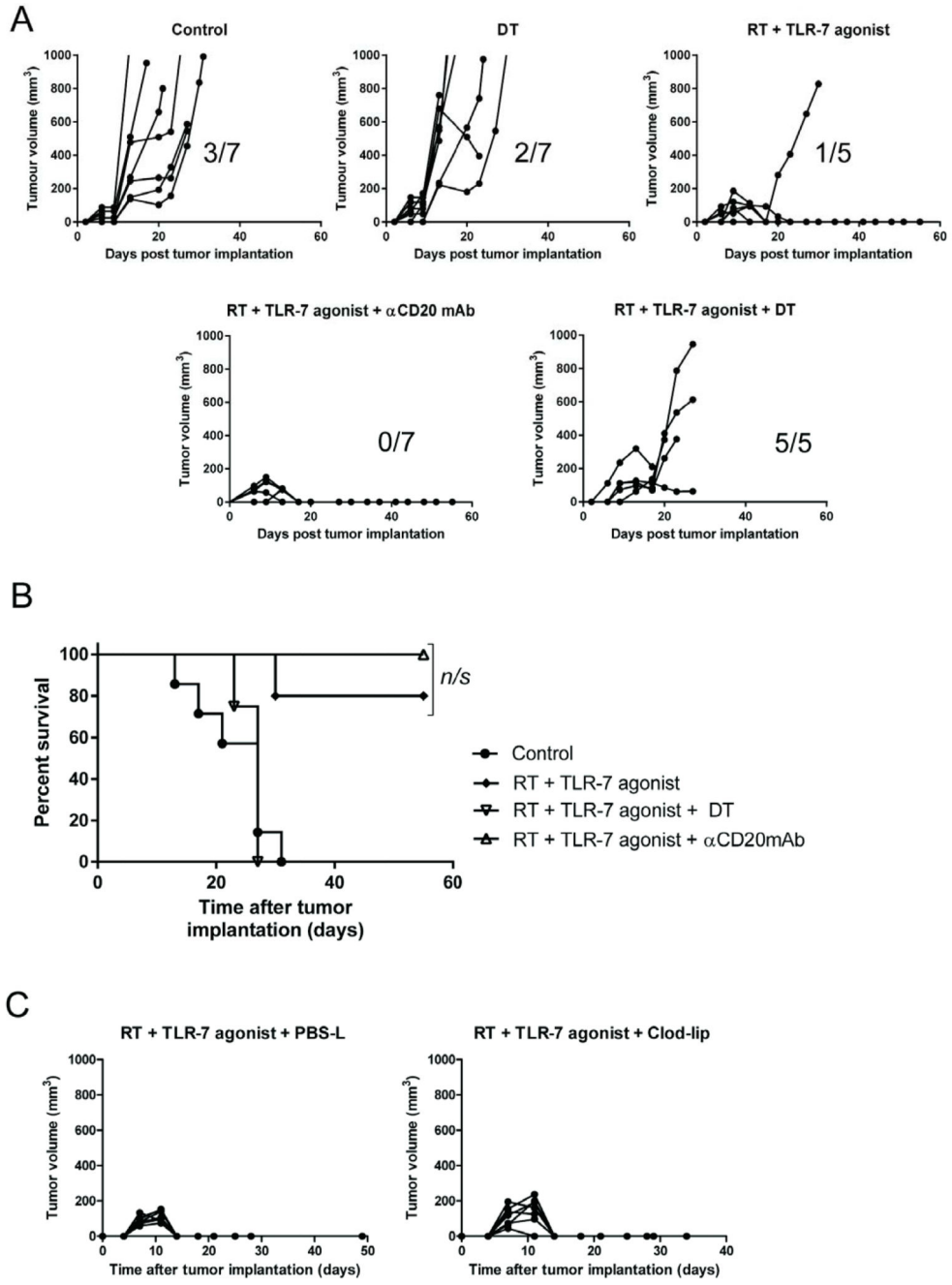

Figure 5. Dendritic cells are critical for the therapeutic effect of combination therapy with RT and a systemically administered TLR7 agonist.

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.

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