Antitumor Efficacy of Radiation plus Immunotherapy Depends upon Dendritic Cell Activation of Effecter CD8\(^{+}\) 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 (APC) 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 affect this antitumor immune response. Using APC depletion models, we now show that dendritic cells (DC), but not macrophages or B cells, were responsible for the generation of long-term immunologic 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 is 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 APCs 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 (MØ), and dendritic cells (DC), 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 TGF\(_\beta\), and are phenotypically polarized toward 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 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 immune system and DC populations to the generation of durable therapeutic antitumor CD8\textsuperscript+ 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. 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\textsubscript{1}, lymphoma (and pBCL\textsubscript{1}, variant) were provided by M. Glennie, University of Southampton, UK, 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 (catalog number TIR-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\textsubscript{1}, cells (i.v.). For the s.c. models, local tumor irradiation was performed 7 days after inoculation (when tumors were approximately 100 mm\textsuperscript{3}) as previously described (3). For the BCL\textsubscript{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 acidic water (pH 2.5; 1N HCl) supplemented with neomycin sulfate (2 g/L Sigma Aldrich), starting 1 week prior to TBI and continuing for 4 weeks afterward. Mice were treated with CD40 mAb either i.v. (100 μg, BCL\textsubscript{1}, model) or s.c. (500 μg, EL4 and EG7 models) 4 hours 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 contralaterally 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 of at least two independent experiments.

Immune cell depletion studies

For B-cell depletion, mice received CD20 mAb (250 μg, mouse IgG2a clone 188B12, a gift from Robert Dunn, Biogen-Idec) 1 day prior to tumor cell inoculation. For MØ depletion, liposomes encapsulating dichloromethylene-bisphosphonate (CL\textsubscript{3}MBP, clodronate) were prepared according to the method of Van Rooijen and colleagues (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 hours apart. Peritoneal MØ depletion was achieved by a single 250-μL injection, i.p. For CD11c depletion, 8-week-old BALB/c or C57Bl/6 mice were irradiated (10 Gy low-dose rate ionizing radiation) and reconstituted with 2 \times 10^6 CD11c-DTR/GFP bone marrow cells i.v. After 6 weeks chimerized mice were randomized and used in therapeutic studies. DC or CD169\textsuperscript+ MØ depletion was achieved with i.p. injection of 100-ng diptheria toxin (DT) up to 3 times per week starting 1 day before tumor cell implantation (Sigma). For CD8\textsuperscript+ or CD4\textsuperscript+ 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 of at least two independent experiments.

Cytokine produced by CD8\textsuperscript+ T cells from LTS 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 to 5 mice and are representative of two independent experiments.

Phagocytosis assays

Mice were inoculated i.p. with 5 \times 10^6 BCL\textsubscript{1} cells labeled with PKH-26 (Sigma Aldrich) and treated with external-beam irradiation; 24 hours later MØs were isolated from peritoneal lavage, sedimented on to glass coverslips, counterstained with Alexa Fluor phalloidin, and phagocytosis was visualized by fluorescence microscopy. Uptake was quantified by flow cytometry following counterstaining with F4/80 Ab (AbD Serotec). For DC uptake, CD11c\textsuperscript+ cells were isolated from the spleens of BALB/c mice using MACS as described (21). DCs (10^6) were cocultured with PKH-26-labeled irradiated lymphoma cells (10 Gy; +72 hours) at an E:T ratio of 20:1 and uptake assessed after 3 hours by flow cytometry using a FACScan (BD Biosciences). 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) used a goat anti-hamster biotin-conjugated F(ab)_2 (Jackson ImmunoResearch) with amplification by the ABC method (Vector Lab) and diaminobenzidine (DAB; Sigma) visualization.

Results

RT plus CD40 mAb generates durable CD8\textsuperscript+ T-cell immunity

RT modulates the immunogenicity of tumor cells but is rarely able to initiate systemic antitumor responses. In models of T-cell 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 LTS mice 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 approximately 6-fold more tumor antigen–specific CD8+ T cells in LTS mice compared with tumor-naive 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 BCL1 tumors following treatment with a single 5-Gy dose of 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 two independent experiments. aCD4, CD4 mAb; aCD8, CD8 mAb; aCD40, CD40 mAb.

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, in terms of either overall survival or tumor volume, of combination therapy in mice bearing established T-cell lymphoma (EL4, Fig. 2A; EG7, Fig. 2B). Depletion of B cells was >95% as confirmed by flow cytometry (Fig. 2C). Given the expression of CD20 on the BCL1 cells, we were unable to selectively deplete B cells in mice bearing this tumor model. These data showed that B cells do not influence the generation of antitumor immune responses after RT plus CD40 mAb therapy.

Macrophages do not affect the efficacy of combination RT plus CD40 mAb therapy

To determine the level of uptake of irradiated tumor cells by M0s in vivo, PKH-26–labeled BCL1 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 hours later by lavage and uptake by M\text subtiles by microscopy (Fig. 3A). M\text subtiles (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 M\text subtiles after 5 Gy, compared with 40% after 2 Gy RT (Fig. 3B). A similar RT dose–dependent increase in uptake by peritoneal M\text subtiles was observed when tumor cells were irradiated in vitro and injected into mice (data not shown).

To assess the influence of M\text subtiles on therapeutic outcome following RT plus CD40 mAb therapy, clodronate-encapsulated liposomes (Clod-lip) were used to deplete M\text subtiles in mice bearing established B-cell and T-cell lymphomas. Initially we confirmed in vitro that treatment with Clod-lip was not directly affecting tumor cell viability (Supplementary Fig. S1A) and that treatment in vivo led to successful depletion of M\text subtiles (confirmed in parallel cohorts sacrificed after treatment; Supplementary Fig. S1B). Depletion of M\text subtiles had no effect on the combination RT plus CD40 mAb in either the BCL1 (Fig. 3C; frequency of LTS: 100% vs. 80%; combined therapy + Clod-lip vs. combined therapy + PBS-lip; \( P = 0.44 \)) or EL4 models (Fig. 3D; frequency of LTS: 43% vs. 57%; combined therapy + Clod-lip vs. combined therapy + PBS-lip; \( P = 0.31 \)). In addition, M\text subtiles depletion had no significant effect on therapeutic outcome after treatment with RT alone (data not shown). These observations demonstrate that M\text subtiles were dispensable for T-cell priming in response to combination therapy with RT and CD40 mAb.

**DC 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, DCs are an attractive candidate for being the principal antigen cross-presenting cells involved in generating the CD8\text sup+ T-cell response following combination therapy, particularly as both B cells and M\text subtiles appear to be dispensable (Figs. 2 and 3, respectively). Using a phagocytosis assay whereby CD11c\text sup+ DCs isolated from the spleen were cocultured with PKH-26–labeled tumor cells, we showed 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 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-cell or T-cell lymphoma developed tumors with kinetics similar to those in 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 BCL1 tumor is maintained in vivo, tumors were passaged through transgenic animals at least twice before use, to eliminate any contaminating nontransgenic DCs. In models of both B-cell 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\textsuperscript{+} DCs, with the frequency of LTS reducing from 80% to 0% in BCL1-bearing mice (Fig. 4D) and from approximately 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 versus nondepleted EG7 tumor–bearing mice treated with RT plus CD40 mAb combination therapy (\(P = 0.075\) two-tailed Student \(t\) test; Supplementary Fig. S2). DC-depleted EL4 tumor–bearing mice had an increased incidence of lymphatic metastasis following RT plus CD40 mAb therapy when compared with nondepleted cohorts (approximately 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\textsuperscript{+} cells, administration of DT to CD11c-DTR mice also depletes CD169\textsuperscript{+} M\textsubscript{0}s, which can contribute to cross-presentation of dead cell–associated antigens (23). Therefore, to delineate the possible role of CD169\textsuperscript{+} M\textsubscript{0}s in T-cell priming we used CD169-DTR mice, which, following administration of DT are specifically depleted of this M\textsubscript{0} population. Our data showed that specific depletion of CD169\textsuperscript{+} M\textsubscript{0}s had no effect on the efficacy of combination RT plus CD40 mAb (Supplementary Fig. S3).
Figure 4. DC depletion abrogates the therapeutic effect of combination therapy. A, splenic CD11c^+ DCs were isolated and coincubated with PKH-26–labeled irradiated BCL1 lymphoma cells at a ratio of 20:1. Uptake was assessed by flow cytometry after 3 hours. *** P < 0.001 vs. control. B and C, CD11c-DTR chimeric mice received DT (100 ng i.p.) and spleen cells analyzed for the presence of CD11c^+ 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 BCL1 (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 as shown in E. *, P < 0.05 compared with combination therapy (log-rank; Mantel-Cox test). Experimental groups contained at least 5 mice and are representative of at least two independent experiments. αCD40, CD40 mAb.
DCs critical for therapeutic effect of RT and TLR7 agonist

We have previously demonstrated that combination therapy with low-dose fractionated RT and a systemically administered TLR7 agonist R848 leads to durable protective CD8+ T-cell responses in models of B-cell and T-cell lymphoma (ref. 3; and Supplementary 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 affected the generation of tumor antigen–specific CD8+ T-cell responses following combination therapy with 10 Gy RT and R848. In EL4 lymphoma–bearing mice, depletion of DCs, but not B cells or MØs, 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 DCs 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 used 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
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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 affect 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 proinflammatory 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⁺ T-cell effector function and memory cell differentiation in vivo (16). However, our data suggest that, in the context of combination treatment with RT and either 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⁺ 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-cell 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 DCs. However, 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 BCL1 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 BCL1 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 toward 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-870,893 is independent of FcγR cross-linking (36). For such a 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 with MØ or B-cell depletion, conditional ablation of DCs using a CD11c-DTR transgenic model completely abrogated long-term protective responses following both CD40 mAb and TLR7 agonist therapy when delivered in combination with RT. However, survival in DC-depleted animals was still enhanced over that of controls, suggesting that either residual DCs remaining after DT-mediated depletion are sufficient to prime a partially protective response or that other APCs, including B-cell tumors themselves in the case of the BCL1 model, are able to cooperate in priming a suboptimal 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 presentation, uptake and 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 (DAMP) from dying tumor cells, which function as danger signals, rendering death more immunogenic by promoting the effective acquisition and processing of tumor antigen by DCs (38–41). Localized DAMP release also contributes to the intratumoral recruitment of DCs 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 that 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 immunosuppressive pathways or coactivating pathways may cooperate with RT-mediated effects to facilitate the induction of durable therapeutic anticancer 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⁺ T cells, to deliver DC maturation and activation signals, licensing DCs for T-cell priming (42–44). These data are consistent with studies using human DCs and the CD40 agonist CP-870,893, which demonstrated the DC requirement for antitumor responses in vivo (45). Thus, when used in combination, the coactivating 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 have shown 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-cell and T-cell lymphoma. Our data show 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 coactivating and coinhibitory pathways used alongside established effective cytoreductive anticancer therapy. Our data show that strategies that cooperate to augment DC priming of anticancer CD8+ T-cell responses are likely to improve therapeutic outcome.

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

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