OPEN ACCESS
CellPress

Cell Reports
Article

Complement-Dependent Modulation of Antitumor Immunity following Radiation Therapy

Michelle Elvington,[1,4] Melissa Scheiber,[1,4] Xiaofeng Yang,[1] Katherine Lyons,[2] Dustin Jacqmin,[2] Casey Wadsworth,[1] David Marshall,[2] Kenneth Vanek,[2] and Stephen Tomlinson[1,3,*]

[1]Department of Microbiology and Immunology, Medical University of South Carolina, Charleston, SC 29425, USA
[2]Department of Radiation Oncology, Medical University of South Carolina, Charleston, SC 29425, USA
[3]Ralph H. Johnson Veterans Affairs Medical Center, Charleston, SC 29401, USA
[4]Co-first author
*Correspondence: tomlinss@musc.edu
http://dx.doi.org/10.1016/j.celrep.2014.06.051

SUMMARY

Complement is traditionally thought of as a proinflammatory effector mechanism of antitumor immunity. However, complement is also important for effective clearance of apoptotic cells, which can be an anti-inflammatory and tolerogenic process. We show that localized fractionated radiation therapy (RT) of subcutaneous murine lymphoma results in tumor cell apoptosis and local complement activation. Cotreatment of mice with tumor-targeted complement inhibition markedly improved therapeutic outcome of RT, an effect linked to early increases in apoptotic cell numbers and increased inflammation. Improved outcome was dependent on an early neutrophil influx and was characterized by increased numbers of mature dendritic cells and the subsequent modulation of T cell immunity. Appropriate complement inhibition may be a promising strategy to enhance a mainstay of treatment for cancer.

INTRODUCTION

Radiation therapy (RT) is a mainstay of treatment for many malignancies and is frequently used as primary or adjuvant therapy, often in combination with surgery or chemotherapy or both. Whereas RT causes direct tumor cell death, an emerging paradigm is that the antitumor effects of RT also depend to a varying extent on the immune system, with RT able to modulate the immune response via its effect on the release of damage-associated molecular patterns (DAMPs). Nevertheless, the mechanisms by which RT modulates either an innate or an adaptive antitumor immune response remain largely unknown, and it is likely that different mechanisms operate depending on the type of cancer and RT dose.

Direct killing of tumor cells by RT is due to irreversible DNA damage, which leads to the induction of cellular senescence, mitotic catastrophe, necrosis, and/or apoptosis. Apoptosis represents a major form of radiation-induced cell death, especially for some types of cancer such as lymphoma, but in terms of an RT-induced immune response, apoptosis is generally considered noninflammatory and is physiologically designed to preserve immunological tolerance. In the context of cancer, however, a general immunotherapeutic goal is to break tolerance to tumor-associated antigens. Although apoptotic cell death can be inflammatory depending on how it is activated and in what cell types, necrotic cell death results in a more sustained and powerful inflammatory response, with increased production of DAMPs and a skewing of proinflammatory cytokines and chemokines released by stimulated phagocytes. Impaired apoptotic cell clearance can lead to secondary necrosis, and the recognition and clearance of necrotic cells is a highly immunogenic process. There is strong evidence indicating that uncleared apoptotic cells are a source of immunogenic self-antigens and can lead to autoimmunization.

The complement system is a key mediator of inflammation, but it also plays an important role in promoting the clearance of apoptotic cells, which can be an anti-inflammatory and tolerogenic process. Complement activation can occur via the classical, lectin, or alternative pathways, all of which converge at the cleavage of C3 and the subsequent generation of various biologically active fragments. Complement activation by any pathway leads to opsonization of target cells with the C3 activation products iC3b and C3d, which have been shown to promote C3 receptor-dependent phagocytic clearance of apoptotic cells (Mevorach et al., 1998). In addition, C1q and MBL not only initiate the classical and lectin pathways, respectively (resulting in C3 opsonization), but also function directly as serum opsonins for phagocytosis (Ogden et al., 2001; Tenner, 1998). C1q can bind directly (albeit weakly) to apoptotic cell membranes (Korb and Ahearn, 1997; Navratil et al., 2001), but both C1q and MBL bind natural immunoglobulin M (IgM) antibodies that recognize neoepitopes exposed on apoptotic cells (Chen et al., 2009; Silverman et al., 2009). Properdin can also bind apoptotic cells and initiate the alternative pathway to promote C3-dependent phagocytosis (Kemper et al., 2008), and C-reactive protein can bind to apoptotic cells and activate the classical pathway (Gershov et al., 2000).

Traditional lines of study with regard to cancer and complement have focused on strategies to enhance complement activation on cancer cells. However, we hypothesized that in the context of RT, inhibiting complement activation will improve

therapeutic outcome by interfering with the phagocytic uptake of apoptotic cells, leading to increased necrotic burden and the formation of a more immunogenic tumor environment. We investigated this hypothesis using a mouse model of lymphoma, a generally radiosensitive type of cancer in which tumor cell apoptosis is known to occur following fractionated RT. To inhibit complement, we utilized CR2-Crry, a targeted inhibitor that blocks all complement pathways at the C3 activation step. The CR2 moiety of the fusion protein binds to deposited C3 cleavage products and thus targets the construct to sites of complement activation (Song et al., 2003), such as the surface of apoptotic cells. The benefits of CR2-mediated targeted complement inhibition versus systemic complement inhibition have been shown previously and include improved bioavailability and efficacy and maintenance of host immunity to infection (Atkinson et al., 2005). A CR2 targeting strategy for complement inhibition is currently in clinical trials for paroxysmal nocturnal hemoglobinuria (http://www.clinicaltrials.gov/ct2/results?term=tt30).

RESULTS

Targeted Complement Inhibition Enhances the Therapeutic Outcome of RT

The effect of CR2-Crry on the therapeutic outcome of RT was investigated using a subcutaneous mouse model of lymphoma (EL4). Starting on day 12 after tumor challenge, mice received 1.5 Gy to a depth of 10 mm per treatment for a total of ten treatments given over 14 days (15 Gy total). Although lymphoma would typically be treated with a higher total dose, the fractionated dose is in line with common clinical fractionation schemes, and a pilot dose-escalation study demonstrated it was the optimum dose for retardation of tumor growth with no overt signs of toxicity in this model. CR2-Crry treatment was initiated 24 hr after the first fraction of RT and every 3 days thereafter for the duration of RT (Figure S1). Tumor size and survival were followed as markers of treatment outcome. Surviving mice were sacrificed at the end of the treatment schedule (day 14). Tumor growth rate was significantly reduced in mice receiving localized RT compared to nonirradiated mice, but cotreatment with RT plus CR2-Crry (RT+CR2-Crry) further reduced the tumor growth rate (Figure 1A), with tumor burden at death or sacrifice significantly lower in mice receiving RT+CR2-Crry compared to RT alone (Figure 1B). Furthermore, compared to mice that received RT alone, combined RT+CR2-Crry treatment significant improved survival at 14 days posttreatment initiation (Figure 1C). Treatment of mice with CR2-Crry in the absence of RT had no significant effect on tumor growth rate, tumor burden, or survival compared to untreated mice, demonstrating a therapeutic benefit of CR2-Crry only in the context of RT. Consequently, a CR2-Crry alone treatment group was not included in subsequent experiments.

CR2-Crry Targets to Irradiated Tumors and Inhibits Complement

We have previously demonstrated that CR2-Crry targets and specifically binds the C3d complement activation product in vitro and in vivo (Atkinson et al., 2005). To investigate C3d deposition and the targeting and retention of CR2-Crry in tumors, we performed in vivo whole-animal fluorescence imaging. Tumor-bearing mice that had received either RT for 4 days at 1.5 Gy/day or no RT were injected with fluorescently labeled CR2-Crry via the tail vein, and animals were imaged over a period of 48 hr. Labeled CR2-Crry localized to tumors in both groups of mice, but the fluorescent signal was significantly higher in irradiated tumors compared to nonirradiated tumors at all time points (1, 5, 24, and 48 hr) (Figures 2A and 2B). CR2-Crry deposition in tumors peaked at 24 hr in mice receiving RT and remained high at 48 hr, whereas CR2-Crry peaked at 5 hr in nonirradiated mice and was almost undetectable by 48 hr. The changing fluorescent signal over time likely reflects a dynamic of ongoing complement activation and binding of CR2-Crry from the circulation (CR2-Crry has a circulatory half-life of 8.7 hr; Atkinson et al., 2005). These data indicate that localized RT induces complement activation within tumors, with the subsequent targeting and localization of systemically injected CR2-Crry. At earlier time points, some fluorescent signal was seen in the location of the liver in irradiated and nonirradiated mice, and this may be associated with the nonspecific clearance of the protein and possibly of complement opsonized tumor cell debris. Increased complement activation in irradiated tumors compared to nonirradiated tumors was also confirmed by immunohistochemical staining for C3d and analysis of the downstream activation product C5a in tumor homogenates. Anti-C3d staining also demonstrated complement activation in irradiated tumors and that CR2-Crry inhibited RT-induced complement activation (Figures 2C and 2D). It is not clear why we did not detect increased C5a levels in tumors from irradiated versus nonirradiated mice (Figure 2E), but CR2-Crry treatment significantly reduced C5a levels, in line with reduced C3d deposition.

Targeted Complement Inhibition Promotes Inflammation within Irradiated Tumors

Complement activation is normally associated with inflammation, and complement inhibition is normally anti-inflammatory, which is not a characteristic generally associated with an antitumor response. We therefore investigated whether targeted complement inhibition modulates the inflammatory environment within tumors after localized RT. We first investigated the profile of select cytokines 7 days after initiation of our treatment protocol. Compared to tumors from nonirradiated mice, low-dose fractionated RT had no significant effect on tumor levels of interferon-γ (IFN-γ), interleukin-6 (IL-6), IL-17, or IL-10. However, levels of IFN-γ, IL-6, and IL-17 were significantly higher in tumors from mice receiving combined RT+CR2-Crry compared to mice receiving RT alone (Figure 3A). IL-10 levels were not significantly different between any of the treatment groups.

Tumor-infiltrating macrophages (total, M1, and M2), myeloid-derived suppressor cells (MDSCs) (granulocytic and myeloid), natural killer (NK) cells, $CD4^+$ T cells, $CD8^+$ T cells, T regulatory cells (Tregs), and neutrophils were also assessed at 7 days after initiation of our treatment protocol. Except for neutrophils, there were no differences in the numbers of infiltrating cells in tumors isolated from mice receiving either RT or RT+CR2-Crry, and only MDSCs showed an increase in irradiated compared to nonirradiated tumors (data not shown). $CD8^+$ T cells and Tregs were essentially absent in tumors from all animal groups. Neutrophil infiltration, however, was significantly increased in tumors from

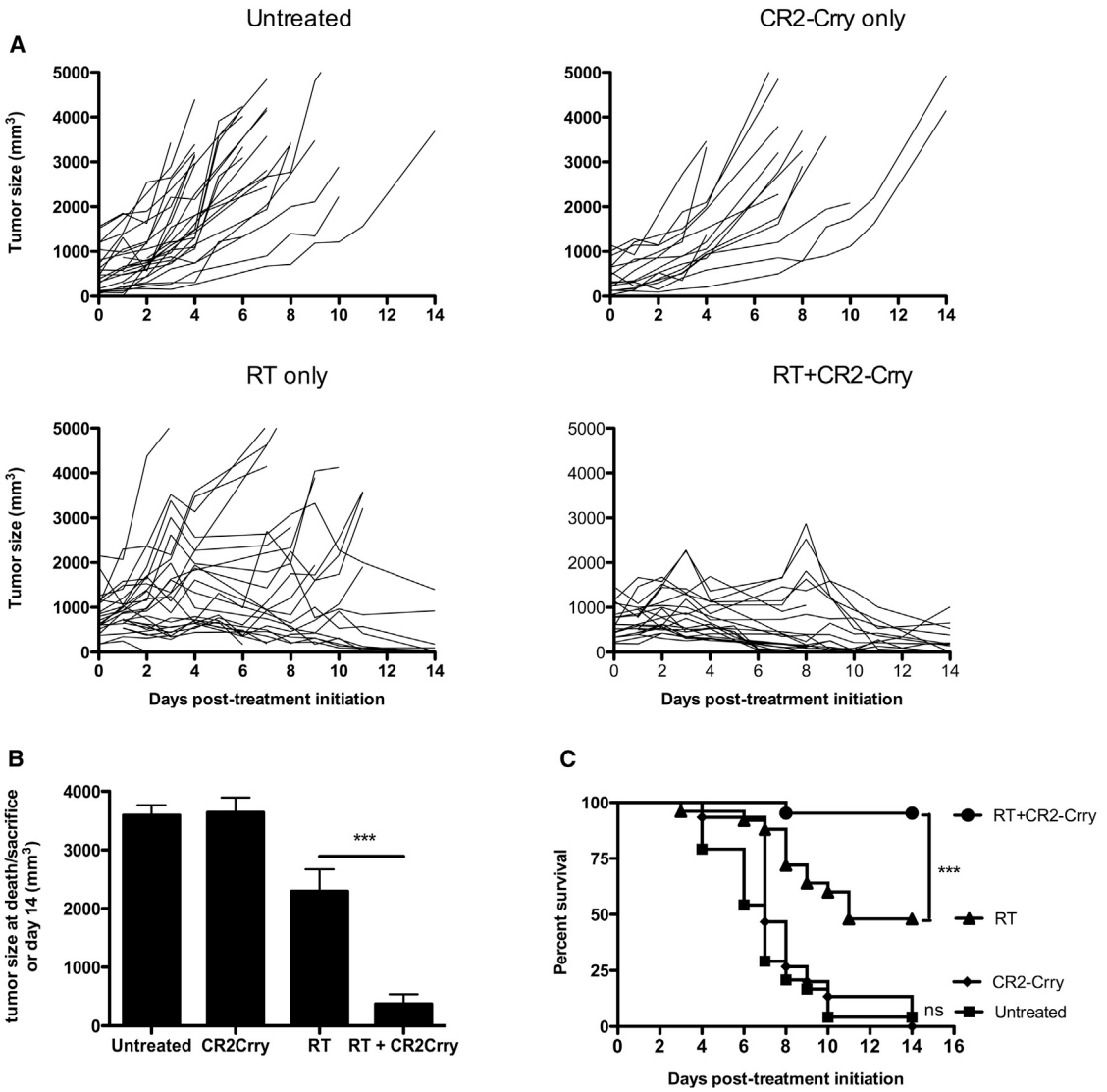

Figure 1. Complement Inhibition with CR2-Crry Enhances the Therapeutic Effect of RT

Subcutaneous EL4 tumors were left untreated or were treated with RT alone, CR2-Crry alone, or with RT+CR2-Crry according to our treatment protocol (refer to Figure S1).

(A) Growth of EL4 tumors over the course of 14 days from initiation of treatment protocol (individual replicates; n = 15–26).

(B) Tumor size (mm^3) at time of death or sacrifice on day 14 (mean ± SEM; n = 12–26; ***$p < 0.001$).

(C) Survival of mice over a 14-day period from initiation of treatment protocol (n = 12–26; ***$p < 0.001$). Data are combined from three independent experiments, except for Crry-alone data, which are combined from two independent experiments. The separate experiments all yielded similar results.

mice receiving RT+CR2-Crry compared to RT alone, as measured by both immunohistochemistry and myeloperoxidase (MPO) activity (Figures 3B and 3C). Together, the above data indicate that although complement inhibition is normally anti-inflammatory, in the context of RT, targeted complement inhibition promotes a proinflammatory tumor environment.

The Effect of Complement Inhibition on Apoptotic Tumor Cell Numbers after RT

Complement opsonization promotes the phagocytic uptake of apoptotic cells, and the failure to clear apoptotic cells is associated with secondary necrosis and inflammation (Kepp et al., 2009). Since apoptosis is a major form of radiation-induced cell death, we investigated the effect of CR2-Crry on apoptotic cell numbers within irradiated tumors. TUNEL staining of sections from tumors isolated 7 days after initiation of our treatment protocol revealed a significantly higher percentage of apoptotic cells in tumors from mice receiving RT+CR2-Crry compared to tumors from nonirradiated mice and mice receiving RT alone (Figure 3D). There was no difference in apoptotic cell numbers between nonirradiated mice and mice receiving only RT. These data are consistent with impaired apoptotic cell clearance in irradiated tumors from complement-inhibited mice.

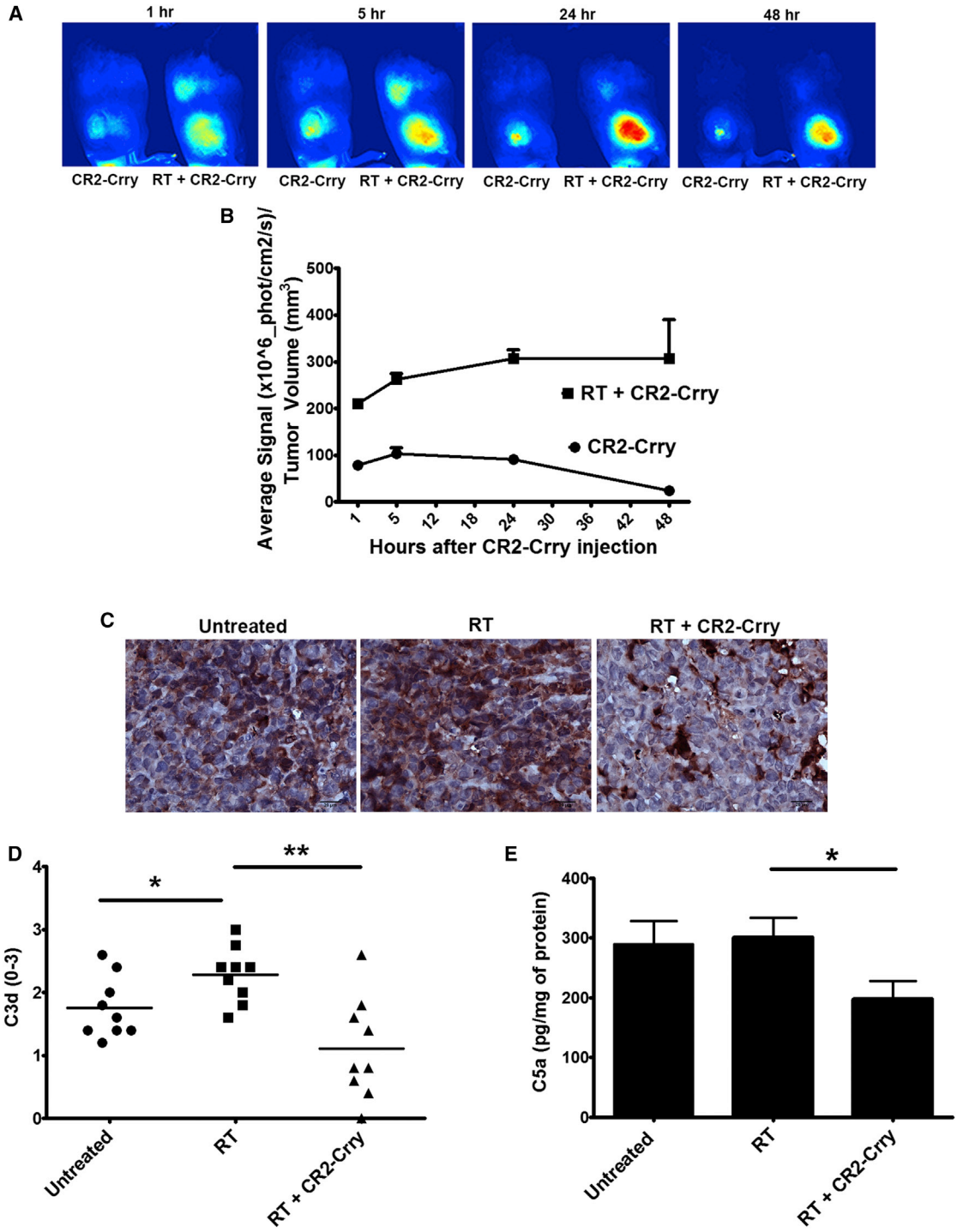

Figure 2. CR2-Crry Localization and Complement Activation in Irradiated Tumors

Mice with subcutaneous EL4 tumors were treated with localized RT for 4 days or left untreated and then injected with fluorescently labeled CR2-Crry. Targeting of CR2-Crry to irradiated and nonirradiated tumors was visualized by in vivo fluorescence tomography.

(A) Representative images (n = 3).

(B) Quantification of tumor fluorescence (mean ± SEM; n = 3; *p < 0.05 at all time points).

Tumors were removed from untreated mice 48 hr after initiation of treatment protocol (24 hr after first CR2-Crry treatment) and sections stained for C3d.

(C) Representative images (n = 6).

(D) Quantification of positive cells on scale of 0 to 3 in five high-powered fields (n = 6; *p < 0.05).

(E) C5a levels in tumors removed from mice 48 hr after initiation of treatment protocol (mean ± SEM; n = 9; *p < 0.05).

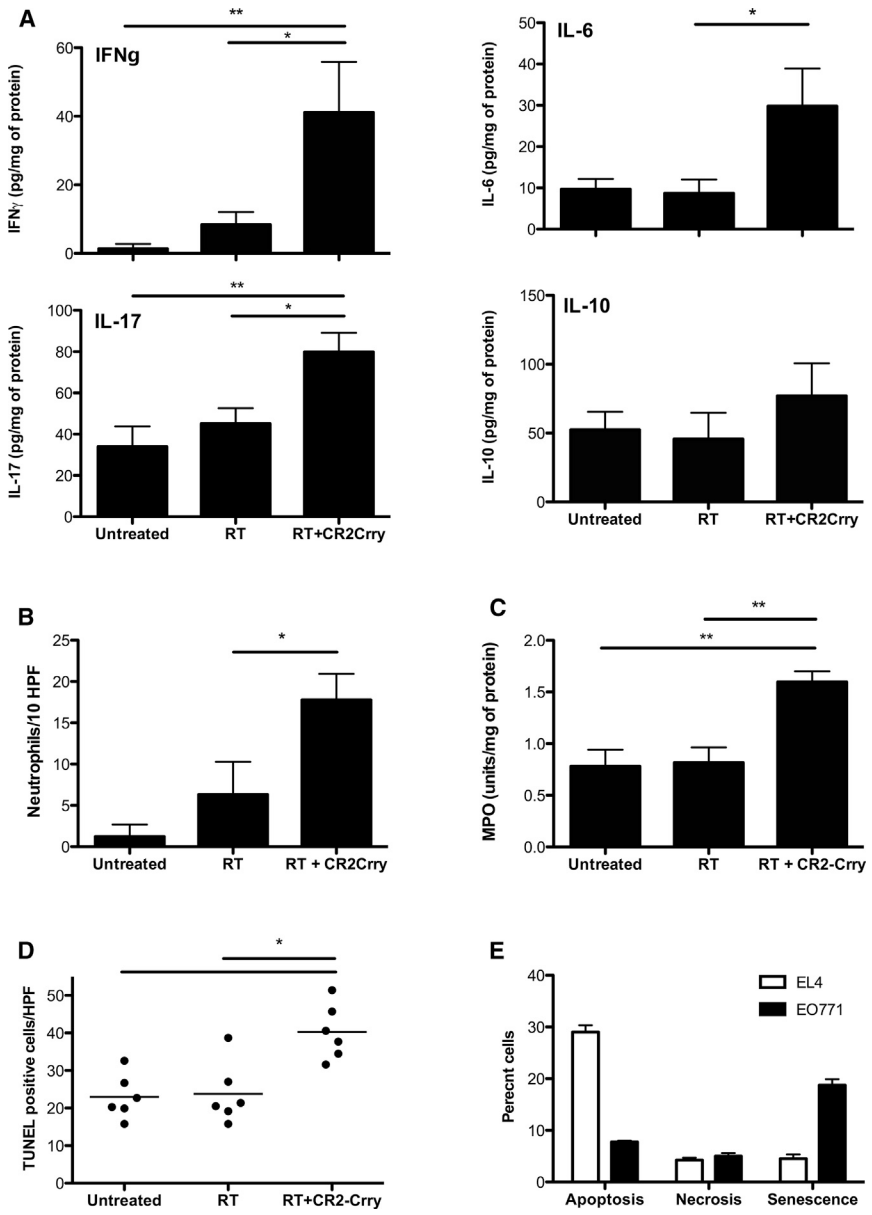

Figure 3. The Effect of Complement Inhibition on the Early Inflammatory Response within Tumors and Apoptotic Cell Numbers after RT

(A) Levels of IFN-γ, IL-6, IL-17, and IL-10 in tumor homogenates 7 days posttreatment initiation. Cytokine levels were measured by ELISA (mean ± SEM; n = 9–10).

(B) Neutrophil infiltration determined by immunostaining of tumor sections. $Gr1^+$ cells in ten randomly selected high-power fields (HPFs) per section (mean ± SEM; n = 4–5).

(C) Neutrophil infiltration determined by MPO activity in tumor homogenates (mean ± SEM; n = 7). For all data (A–C), *p < 0.05, **p < 0.01.

(D) Apoptotic cell numbers within tumors in sections from tumors isolated 7 days after treatment initiation. Quantification of TUNEL-positive cells in HPFs of tumor section is shown (n = 6; *p < 0.05).

(E) In vitro analysis of EL4 and EO771 cell death and senescence after exposure of cells to 4 × 2 Gy/day (mean ± SEM; n = 3).

The Effect of CR2-Crry on the Outcome of RT Is Neutrophil Dependent

Based on the above data showing that CR2-Crry significantly increased the number of tumor-infiltrating neutrophils after RT, we investigated whether the effect of CR2-Crry on the outcome of RT was neutrophil dependent. Following challenge with EL4, mice were depleted of neutrophils by injection of anti-Ly6G monoclonal antibody (mAb) (IA8) prior to initiation of our treatment protocol and again during the treatment protocol to sustain depletion. Depletion was confirmed by flow cytometry (not shown). Neutrophil depletion had no discernible effect on tumor growth in nonirradiated mice and in mice that received RT alone, and tumor burden at death or day 15 after initiation of the treatment protocol was not significantly different between the RT and RT+IA8 groups. On the other hand, neutrophil depletion completely abrogated the effect of CR2-Crry on enhancing the outcome of RT (Figure 4A), and there was no significant difference in tumor burden between the RT+IA8 and RT+CR2-Crry+IA8 groups (Figure 4B). Together with the above data on neutrophil infiltration, these data indicate that the enhancing effect of CR2-Crry on RT is mediated via the induction of an early influx of neutrophils into the tumor.

Although apoptosis is a major type of cell death induced by radiation, especially for lymphoma, alternative cell death modalities or growth arrest can be significant after radiation (Golden et al., 2012; Kepp et al., 2009). We therefore additionally performed an in vitro analysis of RT-induced effects. EL4 cells, and for comparison murine mammary carcinoma EO771 cells, were exposed to 2 Gy/day for 4 days. Analysis of cell death revealed that about 30% of EL4 cells underwent apoptosis, with less than 5% of cells becoming necrotic or senescent. EO771 cells were more resistant to RT-induced apoptosis at this dose, with a higher proportion of cells becoming senescent (Figure 3E). These data confirm that a low-dose RT fractionation scheme results in predominantly apoptotic cell death of EL4 cells and support our interpretation of in vivo data.

Complement Inhibition Modulates Later Immune Cell Infiltration after RT

Infiltrating immune cells, including cells associated with adaptive immunity, were analyzed 14 days after initiation of our treatment protocol. At this time point, only a comparison between RT- and

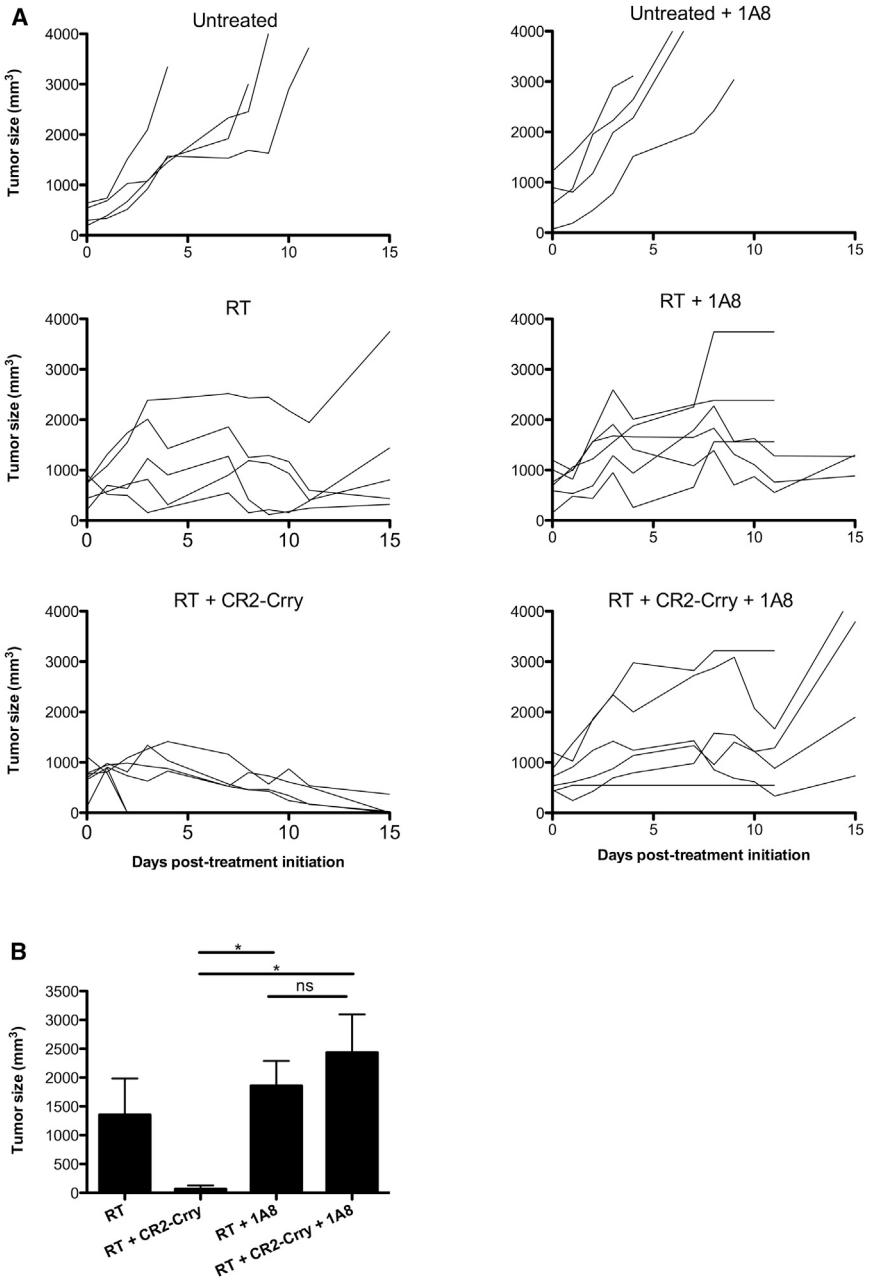

Figure 4. Neutrophil Depletion Abrogates the Enhancing Effect of Complement Inhibition on the Outcome of RT

Tumor-bearing mice were depleted of neutrophils using anti-Ly6G mAb IA8 prior to initiation of therapeutic protocol.

(A) Growth of EL4 tumors over the course of 15 days from initiation of treatment protocol (individual replicates; n = 4–6).

(B) Tumor size at day 15 posttreatment initiation (mean ± SEM; n = 4–6; *p < 0.05).

RT+CR2-Crry-treated mice was possible due to survival. Similar to the 7-day analyses reported above, at 14 days posttreatment initiation, RT+CR2-Crry did not alter numbers of tumor-infiltrating MDSCs or NK cells compared to RT alone (data not shown). In contrast to the 7-day data, however, there was a significant relative increase in the number of F4/80$^+$ macrophages with an M1 phenotype (F4/80$^+$, CD11c$^+$, CD206$^-$) in tumors from complement-inhibited mice, a phenotype generally associated with an antitumor response (Figures 5A and S2). There was no difference in relative numbers of macrophages with an M2 phenotype (F4/80$^+$, CD11c$^-$, CD206$^+$) between the two groups. Compared to RT alone, RT+CR2-Crry treatment also resulted in significantly higher numbers of infiltrating mature DCs (CD11c$^+$, CD86$^+$, and CD80$^+$) and CD8$^+$ T cells (CD3$^+$ and CD8$^+$) (Figures 5B and 5C). It is important to note that these differences in infiltrating immune cells were significant even though not all mice receiving RT alone survived to 14 days, and these data thus represent an analysis of tumors responding best to RT alone. Furthermore, not all mice receiving RT+CR2-Crry had detectable tumors at day 14, and thus analysis of tumors from this group represents the poorest responders. There were no differences between the two groups in the numbers of CD4$^+$ or CD4$^+$, CD25$^+$, FoxP3$^+$ cells in tumors (data not shown). Taken together with the above data, these results indicate that targeted complement inhibition is modifying the effect of RT by inducing a proinflammatory and immune-stimulatory tumor environment.

The Effect of CR2-Crry on the Outcome of RT Is Dependent on Adaptive Immunity

To investigate whether an adaptive immune response is necessary for the effect of complement inhibition on the outcome of RT, we repeated our treatment protocol in Rag1$^{-/-}$ mice challenged with EL4. Tumor growth rates in mice receiving RT alone and RT+CR2-Crry were similar, and CR2-Crry did not reduce the tumor burden in RT-treated mice as measured on day 14 after initiation of treatment (Figure 6). Thus, since CR2-Crry did not enhance the therapeutic effect of RT in Rag1$^{-/-}$ mice, the effect of complement inhibition on outcome after RT is T and/or B cell dependent. Also, tumor burden following RT alone was similar in wild-type (WT) and Rag1$^{-/-}$ mice, indicating that T and B cells are not necessary for the therapeutic response to RT alone.

We also investigated whether RT or RT+CR2-Crry may be modulating an antitumor antibody response, but we found no evidence of an anti-EL4 IgG response in any treatment group in WT

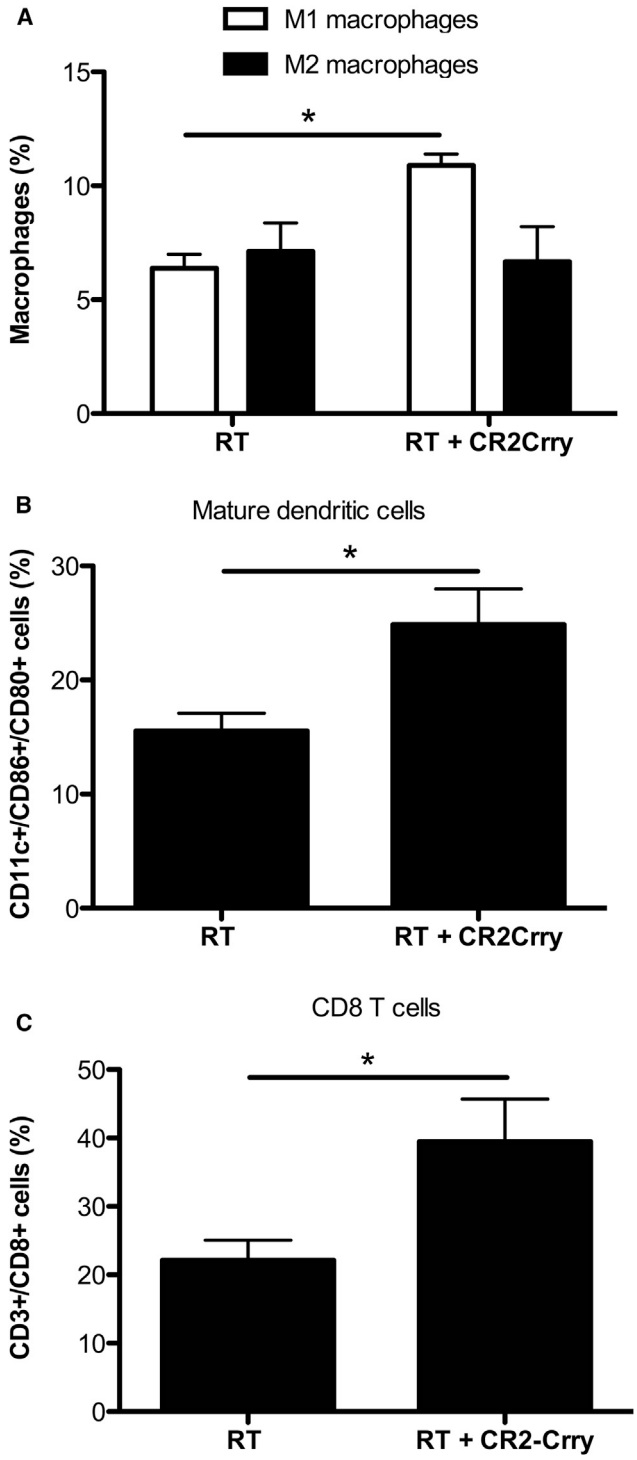

Figure 5. Complement Inhibition with CR2-Crry Alters the Profile of Late Tumor-Infiltrating Immune Cells after RT

Determined by flow cytometry 14 days after initiation of treatment protocol. (A) Percentage of F4/80 positive M1 ($F4/80^+$, $CDllc^+$, $CD206^-$) and M2 ($F4/80^+$, $CDllc^-$, $CD206^+$) macrophages (mean ± SEM; n = 3).

(B) Percentage of mature DCs ($CDllc^+/CD80^+/CD86^+$) (mean ± SEM; n = 3–5).

(C) Percentage of $CD8^+$ T cells ($CD3^+/CD8^+$) (mean ± SEM; n = 5–9). For all analyses, *$p < 0.05$.

mice when analyzed 18 days after treatment initiation (data not shown). We have shown previously that naive C57BL/6 mice contain anti-EL4 IgM and that this IgM does not affect EL4 tumor growth (Elvington et al., 2012).

To investigate whether complement inhibition may be enhancing the outcome of RT by promoting a cellular antitumor immune response, splenocytes were isolated from mice 18 days after treatment initiation and plated with EL4 cells as targets. Assay of culture supernatants revealed a significant increase in IFN-γ, IL-10, and IL-6 production from splenocytes isolated from mice treated with RT+CR2-Crry compared to RT alone (Figure 7A). Together with the data above showing an increase in tumor infiltration of $CD8^+$ T cells in mice receiving RT+CR2-Crry compared to RT alone, the increase in IFN-γ levels suggest that the combined treatment is promoting an antitumor $CD8^+$ T cell response. Although NK T cells can also be a source of IFN-γ, we did not see any difference in tumor-infiltrating NK cells between the RT+CR2-Crry and RT groups. IL-10 is generally considered to be immunosuppressive, but this cytokine has also been reported to have an antitumor role, particularly in preventing metastasis (Huang et al., 1996; Kundu et al., 1996; Kundu and Fulton, 1997) and in enhancing $CD8^+$ T cell responses (Emmerich et al., 2012). IL-6 promotes T cell and B cell expansion and T cell activation and has been shown to have antitumor properties indirectly through modulation of immune cells (Mulé et al., 1990). Pooled splenocytes from tumor-bearing mice from all treatment groups incubated in the absence of EL4 cells ruled out increased background cytokine production from lymphocytes with tumors, and analysis of EL4 culture supernatants ruled out the possibility that the tumor cells were producing any of the cytokines analyzed. These data indicate that complement inhibition is enhancing the therapeutic effect of RT by promoting a systemic antitumor T cell response.

Complement Inhibition Prolongs Survival after RT

Based on evidence of a systemic antitumor immune response, we investigated the effect of complement inhibition on long-term outcome after RT. Following administration of our therapeutic protocol, outcome was measured in terms of survival, tumor size on day 11 (the last day of treatment), and at time of death or sacrifice (due to tumor size). The experiment was terminated on day 42 after treatment initiation. The median survival of mice receiving RT+CR2-Crry was significantly extended compared to mice receiving RT alone (28 versus 17 days), although only one mouse receiving combined treatment survived long-term (>42 days) (Figure 7B). Mean tumor size was also significantly reduced at both day 11 and at death/sacrifice for mice receiving RT+CR2-Crry compared to RT alone (Figure 7C). Notably, six out of seven mice receiving RT alone had to be sacrificed because of tumor size, whereas 8 out of 11 mice receiving combined treatment had no detectable primary tumor at time of death. Necropsy revealed lymph node metastasis in most animals in both treatment groups. As expected from the above data, no untreated mice survived beyond 8 days (data not shown). Thus, in the context of RT, CR2-Crry significantly extended survival time and protected against growth/recurrence of the primary tumor.

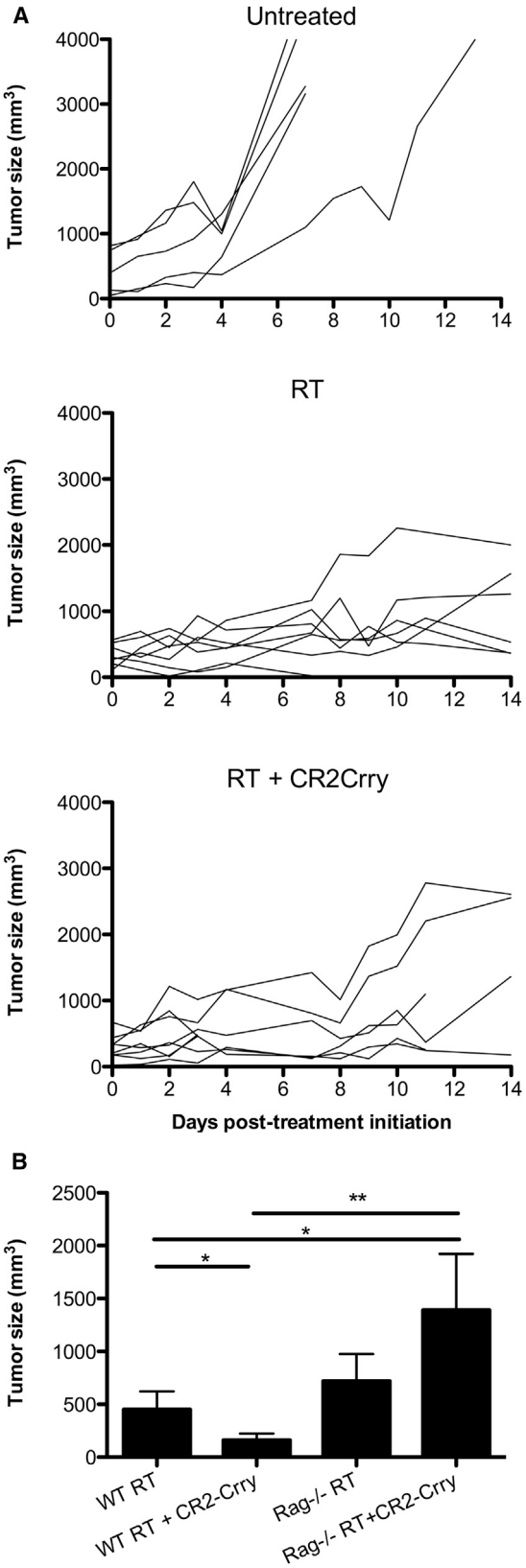

Figure 6. An Adaptive Immune System Is Required for the Enhancing Effect of Complement Inhibition on the Outcome of RT

(A) Growth of EL4 tumors in Rag1$^{-/-}$ mice over the course of 14 days from initiation of treatment protocol (individual replicates; n = 5–8).

(B) Tumor size (mm^3) in WT or Rag1$^{-/-}$ mice at sacrifice on day 14 (mean ± SEM; n = 5–8; *p < 0.05, **p < 0.01).

Complement Inhibition Improves Therapeutic Outcome in a Second Cancer Model

All of the above in vivo studies were performed using the EL4 lymphoma model, and to determine whether our findings may be more generalizable, we also investigated the therapeutic effect of RT and complement inhibition in a subcutaneous mouse model of breast cancer. The treatment schedule for RT and CR2-Crry treatment was similar to that for EL4, except that a higher fractionated RT dose of 5 Gy was delivered. This higher dose was used based on above in vitro data showing that EO771 cells are relatively resistant to low-dose RT-induced apoptosis (see above), together with additional in vitro data showing that a single high dose (30 Gy) resulted in predominantly apoptotic death of EO771 cells (data not shown). The fractionated dose would be relevant to whole breast hypofractionation schemes. As above, tumor size and survival were followed as markers of treatment outcome. EO771 tumor growth rate was reduced in mice receiving localized RT compared to nonirradiated mice, but cotreatment with RT+CR2-Crry further reduced tumor growth rate and significantly prolonged survival (Figure S3).

DISCUSSION

With regard to cancer, complement is traditionally thought of as an effector system of antitumor immunity. The vast majority of studies involving therapeutic modulation of the complement system in cancer are directed at strategies to increase complement activation by tumor cells. Highlighting this, many antitumor mAbs used clinically are known to utilize complement as an effector mechanism, and new generations of mAbs are being designed to increase their complement-activating properties (Kubota et al., 2009). Indeed, we have also previously demonstrated that CR2-Fc, a construct that utilizes the same targeting strategy used in the current study but that delivers a complement activator (Fc domain) instead of an inhibitor, significantly improves the outcome of mAb therapy in an EL4 model of metastatic lymphoma (Elvington et al., 2012). Nevertheless, in something of a paradigm shift, recent studies have shown that complement activation can also promote carcinogenesis. It has been shown that the complement activation product C5a can promote tumor growth by suppressing an antitumor T cell response via the recruitment of Tregs and MDSCs (Corrales et al., 2012; Markiewski et al., 2008) and that complement deficiency or inhibition can impair tumor growth in the absence of any other treatment, at least in some mouse models of cancer (Nunez-Cruz et al., 2012). Other potential protumorigenic mechanisms of complement include promoting tumor cell proliferation, invasiveness, and metastasis and enhancing angiogenesis (Pio et al., 2014). In the current study, complement inhibition alone had no effect on tumor growth. However, when coadministered with RT, complement inhibition significantly improved the therapeutic

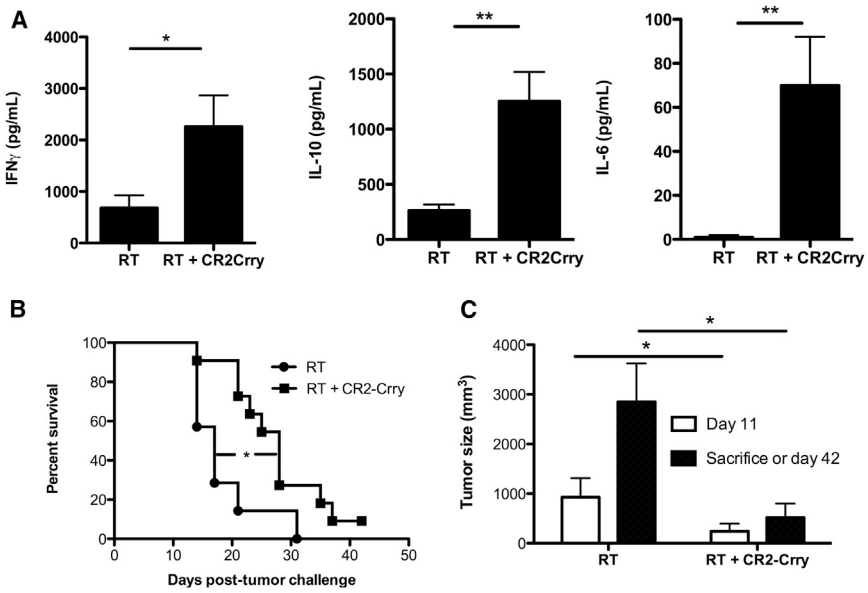

Figure 7. Complement Inhibition Induces a Systemic Antitumor Response, Extends Median Survival Time, and Reduces Primary Tumor Size/Recurrence after RT

(A) Cytokine production was measured from splenocytes isolated from RT- and RT+CR2-Crry-treated groups and incubated with EL4 target cells. Also shown is cytokine production from splenocytes from RT- and RT+CR2-Crry-treated tumor-bearing mice cultured in the absence of EL4 cells (unstimulated) and from EL4 cells cultured alone (EL4). Splenocytes were isolated 18 days after initiation of treatment protocol and cytokines assayed by ELISA (mean ± SEM; n = 5–6; *p < 0.05, **p < 0.01).

(B) Survival of mice after treatment protocol (n = 7–11; *p < 0.05).

(C) Tumor size (mm^3) on the last day of treatment (day 11) and at death or sacrifice (due to tumor size) (mean ± SEM; n = 7–11; *p < 0.05).

outcome of RT. Although complement inhibition is normally anti-inflammatory, in the context of localized RT, complement inhibition was found to promote inflammation within the tumor, induce an early influx of neutrophils, and promote a systemic antitumor immune response. We focused on a lymphoma model, since lymphoma represents a generally radiosensitive type of cancer, with induction of apoptosis a known mechanism of tumor cell killing (Ganem et al., 2010). Localized fractionated RT was shown to result in complement activation within the tumor environment, and the subsequent targeted inhibition of complement in the tumor resulted in an early increase in the level of apoptotic tumor cells after initiation of treatment. These data, together with previous data demonstrating an important role for C3 opsonins in the phagocytosis of apoptotic cells (Mevorach et al., 1998), indicate a therapeutic mechanism involving reduced complement-dependent clearance of apoptotic cells. Failure to clear apoptotic cells is associated with inflammation and the generation of an immunostimulatory environment, with a reservoir of modified immunological self-antigens that can provide a challenge to tolerance (Fadok et al., 1998; Gallucci et al., 1999; Peng et al., 2007).

The increase in early neutrophil recruitment into tumors was essential for the enhanced therapeutic effect of CR2-Crry treatment on RT, since neutrophil depletion ablated the effect of CR2-Crry. Although neutrophils are a hallmark of acute inflammation and provide an important host defense mechanism, they are not generally considered to be potent antitumor effector cells. They have, nevertheless, been shown to directly kill tumor cells by oxidative damage, induction of apoptosis via Fas ligand, and by antibody-dependent cellular cytotoxicity (Fridlender and Albelda, 2012). However, in the current study, a functional adaptive immune system was also required for the enhanced therapeutic effect of CR2-Crry, as demonstrated by experiments performed in $Rag1^{-/-}$ mice that lack T and B cells. Interestingly, one of the most successful antitumor immunotherapies to date, *Mycobacterium bovis Bacillus Calmette-Guerin* (BCG), also depends on early neutrophil infiltration that directs the subsequent cellular-mediated antitumor immune response (Suttmann et al., 2006). Neutrophils express the complement receptors C3aR and C5aR, and complement activation can have a direct effect on neutrophil recruitment through C3a/C5a mobilization, with C5a-mediated recruitment of neutrophils demonstrated in several disease models (Godau et al., 2004; Mueller et al., 2013). However, since CR2-Crry inhibits complement activation and C5a generation in the tumor after RT, the increased infiltration of neutrophils is unlikely to be due to C5a-medited recruitment and is thus likely due to cytokine/chemokine-mediated migration. Indeed, the tumor environment early after combined RT+CR2-Crry treatment was characterized by significantly increased levels of IFN-γ, IL-6, and IL-17 compared to other treatment groups. IL-17 recruits neutrophils to sites of inflammation through induced production of IL-6 as well as other neutrophil chemoattractants (Laan et al., 1999; Miyamoto et al., 2003). Nevertheless, the data do not rule out the possibility that the increased neutrophil numbers in RT+CR2-Crry-treated mice are the source of the increased cytokines, rather than the cytokines mediating neutrophil recruitment. Tumor-infiltrating neutrophils were analyzed by anti-GR1 immunohistochemistry, MPO activity, and functionally by depletion with anti-Ly6G, and we note that these analyses cannot definitively rule out granulocytic MDSCs, although Ly6G is expressed only by a subset of MDSCs. Definitive differentiation would require functional analysis, but in this context, increased infiltration is associated with an antitumor response, not immunosuppression, and depletion of $Ly6G^+$ cells ablates the antitumor response.

Although radiation alone can significantly modify the tumor environment, radiation alone is not usually able to induce an effective antitumor immune response. However, clinical and experimental evidence suggests that RT can function as an immune adjuvant, and increased T cell infiltration and abscopal

effects indicative of systemic immunity have been observed when RT is combined with some immunotherapies (Demaria and Formenti, 2012; Dewan et al., 2009; Dovedi et al., 2013; Hodge et al., 2008; Lee et al., 2009; Lugade et al., 2005; Postow et al., 2012). T cells have been shown to play a key role in antitumor immune responses induced by RT, and targeting T cell costimulatory (e.g., CD137, CD40) or coinhibitory (e.g., CTLA-4, PD-1) receptors in combination with RT has been shown to produce durable antitumor immune responses (reviewed in Demaria and Formenti, 2012; Hodge et al., 2008). Another approach that has been used to modify RT-induced antitumor immunity is the stimulation of dendritic cells (DCs), and activated DCs are important to support antitumor $CD8^+$ T cells following RT (Finkelstein and Fishman, 2012). Here, we demonstrate that compared to localized RT alone, combination treatment with a targeted complement inhibitor increases DC maturation and $CD8^+$ T cell infiltration into tumors. In addition to increased $CD8^+$ T cell infiltration, splenocytes isolated from mice receiving the combined treatment generate increased IFN-γ when stimulated with tumor cells. These data indicate immune response modification by RT+CR2-Crry, with the promotion of a systemic antitumor response characterized by DC maturation that supports the induction of anti-EL4-specific $CD8^+$ T cells.

With regard to the use of CR2-Crry, the safety and pharmacokinetics of this inhibitor have been characterized previously in a model of inflammatory disease (Atkinson et al., 2005). Unlike complement inhibition with an untargeted counterpart, the targeting of CR2-Crry to sites of complement activation obviates the need for systemic inhibition and does not impact host susceptibility to infection; a potentially important consideration for patients receiving RT for lymphoma. Contributing to this safety aspect is the short circulatory half-life of CR2-Crry (8.7 hr) (Atkinson et al., 2005) with retention at sites of complement activation (Atkinson et al., 2008). Humanized counterparts of CR2-Crry are under clinical development. The rationale for using a Crry-based inhibitor is that Crry functions at the C3 activation step of all three complement-activation pathways and inhibits the generation of C3 opsonins that have been shown to be involved in apoptotic cell uptake by phagocytes. Crry is a structural and functional analog of human CR1. Other complement inhibitors that are specific for a certain activation pathway are likely to provide less complete protection against C3 opsonization, and inhibitors that function later in the pathway, such as C5 mAb or a C5b-9 inhibitor, would not be expected to inhibit C3 opsonization.

Although combined treatment significantly extended survival and improved tumor burden compared to RT alone, it did not provide long-term protection. It is interesting, however, that mice treated with RT alone had recurrence of the primary tumor following cessation of treatment, whereas mice cotreated with CR2-Crry were protected from primary tumor relapse, even though they eventually succumbed to disease. It is likely that alternate dosing strategies will differentially modulate antitumor immunity and a potential abscopal effect. Here, we used low-dose fractionated RT, a conventional clinical approach for lymphoma, although in some cancer models, there is evidence that high single dose of ablative radiation is more immunostimulatory and produces a more robust T cell response, whereas fractionated RT is immunosuppressive (Burnette et al., 2011; Lee et al., 2009). In contrast, however, there is also evidence that fractionated RT is superior to single high-dose RT at enhancing antitumor immunity in combination with antibody therapy (Dewan et al., 2009; Dovedi et al., 2013). Regardless, here we present the use of a targeted complement inhibitor to promote, rather than suppress, inflammation and demonstrate that complement inhibition modulates antitumor immunity and enhances outcome after RT. Even though combined treatment did not provide complete long-term protection, controlling local disease and accelerating or completing the clearance of a tumor that is being irradiated, as we show here, has the potential for major clinical impact. How different RT dosing strategies, as well as different apoptosis-based therapies, such as chemotherapy, respond to complement inhibition deserves further study.

EXPERIMENTAL PROCEDURES

Cell Line

The EL4 mouse lymphoma cell line was purchased from ATCC and grown in RPMI 1640 with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO_2.

Mice

Wild-type C57BL/6 mice and $Rag^{-/-}$ mice on C57BL/6 background were purchased from The Jackson Laboratory. Male mice between the ages of 8 and 10 weeks were used for all experiments. The Medical University of South Carolina Institutional Animal Care and Use Committee, in accordance with the NIH *Guide for the Care and Use of Laboratory Animals*, approved all animal procedures.

Subcutaneous Tumor Models and Treatment Protocol

Mice were injected subcutaneously on the right flank with 5×10^5 EL4 cells. Twelve days after tumor challenge, localized RT was initiated. Mice received a total dose of 15 Gy in 1.5 Gy fractions (see below). The recombinant protein CR2-Crry was prepared and purified as described previously (Atkinson et al., 2005), and mice were treated with 0.25 mg of CR2-Crry 24 hr after the first fraction of RT and every 72 hr thereafter for the duration of treatment (see schematic, Figure S1). Depending on the experiment, surviving mice were sacrificed at different time points after treatment initiation or when tumor size exceeded 4,000 mm^3 or when mice showed signs of severe stress. In a second model, mice were injected subcutaneously on the right flank with 5×10^5 EO771 cells (C57BL/6 breast carcinoma cell line) and treated in a similar protocol, except that 5 Gy fractions were delivered.

Irradiation Technique and Radiation Dosimetry

The mice received localized RT using the following irradiation technique. The mice were placed in a pie-shaped holder that held eight mice in equally sized sectors. A 6-mm-thick lead shield with eight 1.75-cm-diameter holes was placed on top of the holder. The mice were aligned such that only their flank tumors were exposed through the holes. A slab of tissue-equivalent material with a thickness of 1 cm was placed on top of the lead before irradiation. The mice were irradiated with a Varian 21eX clinical linear accelerator using a 9 MeV electron beam and a 25×25 cm^2 open applicator. The mouse holder was centered under the applicator at a source-to-surface distance of 100 cm, measured to the top of the lead shield. The prescribed dose was 1.5 Gy per fraction for EL4 and 5 Gy per fraction for EO771 at a depth of 10 mm below the surface of the tumor. Dosimetry was performed with radiochromic film to determine the number of monitor units (MU) needed to deliver the prescribed dose.

In Vivo Imaging

The NIR dye XenoLight CF 750 (PerkinElmer) was used to label CR2-Crry per the manufacturer's instructions. Fluorescent multispectral images were

obtained using the Maestro *In-Vivo* Imaging System (PerkinElmer). Spectral libraries were manually computed using the Maestro software, with each tissue used as its own background control.

The fluorescence images of the EL4 tumor bearing mice, with or without RT (4 doses of 1.5 Gy) were acquired 1 hr, 5 hr, 24 hr, and 48 hr postinjection of fluorescently labeled CR2-Crry (0.25 mg). To evaluate signal intensities, tumor regions were selected and the total fluorescence signal from those areas determined. The average signal in the selected region, defined in photons measured at the surface of the animal, was divided by the area (in pixels) as well as the exposure time (in seconds). The average signal ($\times 10^6$ photons/cm^2/s) of CR2-Crry within the tumors was then divided by the size of the tumor (mm^3). The final average signal ($\times 10^6$ photons/cm^2/s)/mm^3 of the CR2-Crry within the tumors was compared to between the two groups (CR2-Crry only versus RT+CR2-Crry). Using the Compare Images tool on the Maestro software, grayscale images collected over the five time periods were compared, accounting for differences in intensity due to bit depth, exposure time, and binning. Pseudocolor was applied to the grayscale images using the "Jet" color map.

Analysis of Complement Activation and Apoptosis

For analysis of C3d deposition, tumors were removed 24 hr after first CR2-Crry treatment. Tumors were formalin fixed and paraffin embedded, and 5 μm tissue sections cut. Sections were analyzed by immunohistochemistry with anti-C3d (R&D Systems) as previously described (Atkinson et al., 2009). Slides were analyzed by light microscopy and a blinded observer quantified positive cells on a scale of 0 to 3 in five high-powered fields per slide. C5a levels in tumor homogenates were quantified by a mouse C5a ELISA according to the manufacturer's instructions (BD Pharmingen).

For analysis of apoptotic tumor cells, mice were sacrificed and tumors removed 7 days posttreatment initiation. Paraffin-embedded sections were analyzed for apoptotic cell number by TUNEL staining using the Apoptag kit from Millipore per the manufacturer's instructions. Slides were analyzed by light microscopy and a blinded observer quantified positive cells in ten high-powered fields per slide. For in vitro analysis of cell death after radiation exposure, early log-phase EL4 or EO771 (mouse mammary carcinoma) cells, at 1×10^6 cells/ml in RPMI 1640, 10% FBS, were exposed to 2 Gy/day for 4 days. Twenty-four hours after last exposure, cells were stained using the FITC Annexin V Apoptosis Detection kit (BD Pharmingen) and propidium iodide to determine apoptosis and necrosis according to the manufacturer's instructions. Senescence was determined using cytospin slides with staining for β-galactosidase using a kit from Cell Signaling Technology.

Neutrophil Depletion

Neutrophils were specifically depleted by an anti-Ly6G mAb, IA8, which preserves other cell populations expressing $Gr1^+$ (Daley et al., 2008). IA8 (500 μg 100 μl intraperitoneally) was injected 24 hr prior to the first irradiation dose and 24 hr and 5 days after the first irradiation dose. Depletion was confirmed over the course of treatment by assaying $Gr1^+$ cells in the blood by flow cytometry (anti-Gr1 mAb, BD Biosciences).

Analysis of Neutrophil Infiltration

Immunohistochemistry staining for $Gr1^+$ cells (anti-Gr1, BD Biosciences) was performed on paraffin-embedded tumor sections. Positive cells were visualized by light microscopy and quantified by a blinded observer. Levels of MPO were determined in tumor homogenates using a MPO activity assay from Northwest Life Science Specialties according to the manufacturer's instructions.

Cytokines and Analysis of Immune Cell Populations

Levels of IFN-γ, IL-10, IL-6, and IL-17 in homogenates of tumors isolated 7 days after treatment initiation were assayed by ELISA (BD Biosciences; except IL-17 [R&D Systems]). Immune cell populations within the tumor were analyzed at 7 or 14 days posttreatment initiation by flow cytometry. Staining for specific cell types were performed using the following antibodies: for $CD8^+$ T cells, anti-CD3e and anti-CD8; for mature DCs, anti-CD11c, anti-CD80, and anti-CD86; for M1/M2 macrophages, anti-F4/80 (total), anti-CD11c (M1), and anti-CD206 (M2). M1 macrophages were classified as ($F4/80^+$, $CD11c^+$, $CD206^-$) and M2 macrophages as ($F4/80^+$, $CD11c^-$, $CD206^+$); for MDSCs, anti CD11b, anti-Gr1, with granulocytic and myeloid cells distinguished by side scatter. All antibodies for flow cytometry were purchased from BD Biosciences with the exception of anti-CD206 (BioLegend). Samples were analyzed on a BD LSRFortessa flow cytometer (BD Biosciences) and analyzed with FlowJo 9.3.3 software (TreeStar).

Analysis of Antitumor Cellular Immune Response

Total splenocytes were isolated from mice 18 days after treatment initiation and plated either without EL4 cells or with EL4 tumor cells as targets at a 10:1 ratio. Coculture supernatant was collected 72 hr later and assayed for levels of IFN-γ, IL-10, and IL-6 by ELISA (BD Biosciences). Culture supernatant from cultured EL4 cells only was also analyzed to rule out the possibility that the cancer cells were producing any of the cytokines analyzed.

Statistical Analysis

GraphPad Prism version 5 was used for statistical analysis. Analysis of survival curves was done by the log-rank test. Parametrical analysis was done using a one-way ANOVA with the Bonferroni multiple comparison test. Nonparametric analysis (histology) was done using one-way ANOVA with Dunn's multiple comparison test. Comparisons between two groups over time were performed by two-way ANOVA. Comparisons between two groups were performed by the Student's t test (parametric) or Mann-Whitney U test (nonparametric). A p value < 0.05 was considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.06.051.

AUTHOR CONTRIBUTIONS

M.E. and M.S. designed and performed experiments, analyzed data, and wrote the manuscript. X.Y. prepared and characterized essential reagents. K.L., D.J., D.M., and K.V. performed experiments, provided expertise in radiation therapy, and interpreted data. C.W. performed experiments and analyzed data. S.T. designed experiments, interpreted data, and wrote the manuscript. All coauthors reviewed the manuscript prior to submission.

ACKNOWLEDGMENTS

This work was supported by a grant from the NIH (RO1CA158179). We thank Ms. Emily Paulling for expert technical assistance. S.T. holds licensed patents for CR2-targeted complement inhibitors.

Received: March 20, 2014
Revised: April 17, 2014
Accepted: June 25, 2014
Published: July 24, 2014

REFERENCES

Atkinson, C., Song, H., Lu, B., Qiao, F., Burns, T.A., Holers, V.M., Tsokos, G.C., and Tomlinson, S. (2005). Targeted complement inhibition by C3d recognition ameliorates tissue injury without apparent increase in susceptibility to infection. J. Clin. Invest. *115*, 2444–2453.

Atkinson, C., Qiao, F., Song, H., Gilkeson, G.S., and Tomlinson, S. (2008). Low-dose targeted complement inhibition protects against renal disease and other manifestations of autoimmune disease in MRL/lpr mice. J. Immunol. *180*, 1231–1238.

Atkinson, C., Varela, J.C., and Tomlinson, S. (2009). Complement-dependent inflammation and injury in a murine model of brain dead donor hearts. Circ. Res. *105*, 1094–1101.

Burnette, B.C., Liang, H., Lee, Y., Chlewicki, L., Khodarev, N.N., Weichselbaum, R.R., Fu, Y.X., and Auh, S.L. (2011). The efficacy of radiotherapy relies

upon induction of type i interferon-dependent innate and adaptive immunity. Cancer Res. *71*, 2488–2496.

Chen, Y., Park, Y.B., Patel, E., and Silverman, G.J. (2009). IgM antibodies to apoptosis-associated determinants recruit C1q and enhance dendritic cell phagocytosis of apoptotic cells. J. Immunol. *182*, 6031–6043.

Corrales, L., Ajona, D., Rafail, S., Lasarte, J.J., Riezu-Boj, J.I., Lambris, J.D., Rouzaut, A., Pajares, M.J., Montuenga, L.M., and Pio, R. (2012). Anaphylatoxin C5a creates a favorable microenvironment for lung cancer progression. J. Immunol. *189*, 4674–4683.

Daley, J.M., Thomay, A.A., Connolly, M.D., Reichner, J.S., and Albina, J.E. (2008). Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice. J. Leukoc. Biol. *83*, 64–70.

Demaria, S., and Formenti, S.C. (2012). Role of T lymphocytes in tumor response to radiotherapy. Front Oncol *2*, 95.

Dewan, M.Z., Galloway, A.E., Kawashima, N., Dewyngaert, J.K., Babb, J.S., Formenti, S.C., and Demaria, S. (2009). Fractionated but not single-dose radiotherapy induces an immune-mediated abscopal effect when combined with anti-CTLA-4 antibody. Clin. Cancer Res. *15*, 5379–5388.

Dovedi, S.J., Melis, M.H., Wilkinson, R.W., Adlard, A.L., Stratford, I.J., Honeychurch, J., and Illidge, T.M. (2013). Systemic delivery of a TLR7 agonist in combination with radiation primes durable antitumor immune responses in mouse models of lymphoma. Blood *121*, 251–259.

Elvington, M., Huang, Y., Morgan, B.P., Qiao, F., van Rooijen, N., Atkinson, C., and Tomlinson, S. (2012). A targeted complement-dependent strategy to improve the outcome of mAb therapy, and characterization in a murine model of metastatic cancer. Blood *119*, 6043–6051.

Emmerich, J., Mumm, J.B., Chan, I.H., LaFace, D., Truong, H., McClanahan, T., Gorman, D.M., and Oft, M. (2012). IL-10 directly activates and expands tumor-resident CD8(+) T cells without de novo infiltration from secondary lymphoid organs. Cancer Res. *72*, 3570–3581.

Fadok, V.A., Bratton, D.L., Konowal, A., Freed, P.W., Westcott, J.Y., and Henson, P.M. (1998). Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. J. Clin. Invest. *101*, 890–898.

Finkelstein, S.E., and Fishman, M. (2012). Clinical opportunities in combining immunotherapy with radiation therapy. Front Oncol *2*, 169.

Fridlender, Z.G., and Albelda, S.M. (2012). Tumor-associated neutrophils: friend or foe? Carcinogenesis *33*, 949–955.

Gallucci, S., Lolkema, M., and Matzinger, P. (1999). Natural adjuvants: endogenous activators of dendritic cells. Nat. Med. *5*, 1249–1255.

Ganem, G., Cartron, G., Girinsky, T., Haas, R.L., Cosset, J.M., and Solal-Celigny, P. (2010). Localized low-dose radiotherapy for follicular lymphoma: history, clinical results, mechanisms of action, and future outlooks. Int. J. Radiat. Oncol. Biol. Phys. *78*, 975–982.

Gershov, D., Kim, S., Brot, N., and Elkon, K.B. (2000). C-Reactive protein binds to apoptotic cells, protects the cells from assembly of the terminal complement components, and sustains an antiinflammatory innate immune response: implications for systemic autoimmunity. J. Exp. Med. *192*, 1353–1364.

Godau, J., Heller, T., Hawlisch, H., Trappe, M., Howells, E., Best, J., Zwirner, J., Verbeek, J.S., Hogarth, P.M., Gerard, C., et al. (2004). C5a initiates the inflammatory cascade in immune complex peritonitis. J. Immunol. *173*, 3437–3445.

Golden, E.B., Pellicciotta, I., Demaria, S., Barcellos-Hoff, M.H., and Formenti, S.C. (2012). The convergence of radiation and immunogenic cell death signaling pathways. Front Oncol *2*, 88.

Hodge, J.W., Guha, C., Neefjes, J., and Gulley, J.L. (2008). Synergizing radiation therapy and immunotherapy for curing incurable cancers. Opportunities and challenges. Oncology (Williston Park) *22*, 1064–1070, discussion 1075, 1080–1081, 1084.

Huang, S., Xie, K., Bucana, C.D., Ullrich, S.E., and Bar-Eli, M. (1996). Interleukin 10 suppresses tumor growth and metastasis of human melanoma cells: potential inhibition of angiogenesis. Clin. Cancer Res. *2*, 1969–1979.

Kemper, C., Mitchell, L.M., Zhang, L., and Hourcade, D.E. (2008). The complement protein properdin binds apoptotic T cells and promotes complement activation and phagocytosis. Proc. Natl. Acad. Sci. USA *105*, 9023–9028.

Kepp, O., Tesniere, A., Schlemmer, F., Michaud, M., Senovilla, L., Zitvogel, L., and Kroemer, G. (2009). Immunogenic cell death modalities and their impact on cancer treatment. Apoptosis *14*, 364–375.

Korb, L.C., and Ahearn, J.M. (1997). C1q binds directly and specifically to surface blebs of apoptotic human keratinocytes: complement deficiency and systemic lupus erythematosus revisited. J. Immunol. *158*, 4525–4528.

Kubota, T., Niwa, R., Satoh, M., Akinaga, S., Shitara, K., and Hanai, N. (2009). Engineered therapeutic antibodies with improved effector functions. Cancer Sci. *100*, 1566–1572.

Kundu, N., and Fulton, A.M. (1997). Interleukin-10 inhibits tumor metastasis, downregulates MHC class I, and enhances NK lysis. Cell. Immunol. *180*, 55–61.

Kundu, N., Beaty, T.L., Jackson, M.J., and Fulton, A.M. (1996). Antimetastatic and antitumor activities of interleukin 10 in a murine model of breast cancer. J. Natl. Cancer Inst. *88*, 536–541.

Laan, M., Cui, Z.H., Hoshino, H., Lötvall, J., Sjöstrand, M., Gruenert, D.C., Skoogh, B.E., and Lindén, A. (1999). Neutrophil recruitment by human IL-17 via C-X-C chemokine release in the airways. J. Immunol. *162*, 2347–2352.

Lee, Y., Auh, S.L., Wang, Y., Burnette, B., Wang, Y., Meng, Y., Beckett, M., Sharma, R., Chin, R., Tu, T., et al. (2009). Therapeutic effects of ablative radiation on local tumor require CD8+ T cells: changing strategies for cancer treatment. Blood *114*, 589–595.

Lugade, A.A., Moran, J.P., Gerber, S.A., Rose, R.C., Frelinger, J.G., and Lord, E.M. (2005). Local radiation therapy of B16 melanoma tumors increases the generation of tumor antigen-specific effector cells that traffic to the tumor. J. Immunol. *174*, 7516–7523.

Markiewski, M.M., DeAngelis, R.A., Benencia, F., Ricklin-Lichtsteiner, S.K., Koutoulaki, A., Gerard, C., Coukos, G., and Lambris, J.D. (2008). Modulation of the antitumor immune response by complement. Nat. Immunol. *9*, 1225–1235.

Mevorach, D., Mascarenhas, J.O., Gershov, D., and Elkon, K.B. (1998). Complement-dependent clearance of apoptotic cells by human macrophages. J. Exp. Med. *188*, 2313–2320.

Miyamoto, M., Prause, O., Sjöstrand, M., Laan, M., Lötvall, J., and Lindén, A. (2003). Endogenous IL-17 as a mediator of neutrophil recruitment caused by endotoxin exposure in mouse airways. J. Immunol. *170*, 4665–4672.

Mueller, M., Herzog, C., Larmann, J., Schmitz, M., Hilfiker-Kleiner, D., Gessner, J.E., and Theilmeier, G. (2013). The receptor for activated complement factor 5 (C5aR) conveys myocardial ischemic damage by mediating neutrophil transmigration. Immunobiology *218*, 1131–1138.

Mulé, J.J., McIntosh, J.K., Jablons, D.M., and Rosenberg, S.A. (1990). Antitumor activity of recombinant interleukin 6 in mice. J. Exp. Med. *171*, 629–636.

Navratil, J.S., Watkins, S.C., Wisnieski, J.J., and Ahearn, J.M. (2001). The globular heads of C1q specifically recognize surface blebs of apoptotic vascular endothelial cells. J. Immunol. *166*, 3231–3239.

Nunez-Cruz, S., Gimotty, P.A., Guerra, M.W., Connolly, D.C., Wu, Y.Q., DeAngelis, R.A., Lambris, J.D., Coukos, G., and Scholler, N. (2012). Genetic and pharmacologic inhibition of complement impairs endothelial cell function and ablates ovarian cancer neovascularization. Neoplasia *14*, 994–1004.

Ogden, C.A., deCathelineau, A., Hoffmann, P.R., Bratton, D., Ghebrehiwet, B., Fadok, V.A., and Henson, P.M. (2001). C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells. J. Exp. Med. *194*, 781–795.

Peng, Y., Martin, D.A., Kenkel, J., Zhang, K., Ogden, C.A., and Elkon, K.B. (2007). Innate and adaptive immune response to apoptotic cells. J. Autoimmun. *29*, 303–309.

Pio, R., Corrales, L., and Lambris, J.D. (2014). The role of complement in tumor growth. Adv. Exp. Med. Biol. *772*, 229–262.

Postow, M.A., Callahan, M.K., Barker, C.A., Yamada, Y., Yuan, J., Kitano, S., Mu, Z., Rasalan, T., Adamow, M., Ritter, E., et al. (2012). Immunologic correlates of the abscopal effect in a patient with melanoma. N. Engl. J. Med. *366*, 925–931.

Silverman, G.J., Grönwall, C., Vas, J., and Chen, Y. (2009). Natural autoantibodies to apoptotic cell membranes regulate fundamental innate immune functions and suppress inflammation. Discov. Med. *8*, 151–156.

Song, H., He, C., Knaak, C., Guthridge, J.M., Holers, V.M., and Tomlinson, S. (2003). Complement receptor 2-mediated targeting of complement inhibitors to sites of complement activation. J. Clin. Invest. *111*, 1875–1885.

Suttmann, H., Riemensberger, J., Bentien, G., Schmaltz, D., Stöckle, M., Jocham, D., Böhle, A., and Brandau, S. (2006). Neutrophil granulocytes are required for effective Bacillus Calmette-Guérin immunotherapy of bladder cancer and orchestrate local immune responses. Cancer Res. *66*, 8250–8257.

Tenner, A.J. (1998). C1q receptors: regulating specific functions of phagocytic cells. Immunobiology *199*, 250–264.