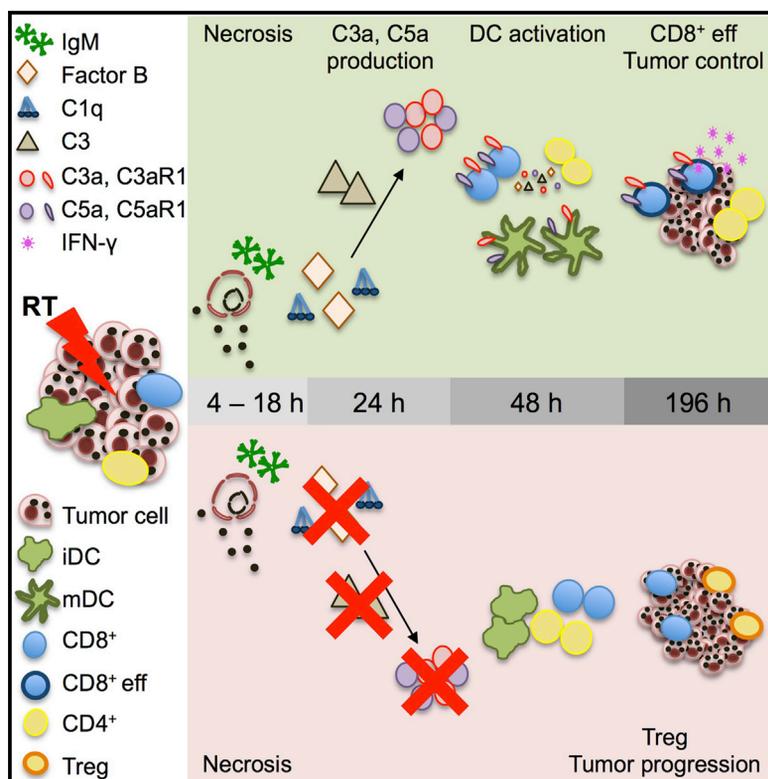


Immunity

Complement Is a Central Mediator of Radiotherapy-Induced Tumor-Specific Immunity and Clinical Response

Graphical Abstract



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In Brief

Anaphylatoxins are produced upon complement activation and are well-known pro-inflammatory molecules. van den Broek and colleagues demonstrate that anaphylatoxins are produced within a tumor after radiotherapy by immune cells, support tumor-specific immunity, and are essential to therapeutic efficacy.

Highlights

- RT induces local complement activation in mice and humans
- RT-mediated cell death and necrosis activates complement
- C3a and C5a accumulate in the tumor and promote tumor-specific immunity
- Dexamethasone inhibits complement activation and reduces efficacy of RT



Complement Is a Central Mediator of Radiotherapy-Induced Tumor-Specific Immunity and Clinical Response

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SUMMARY

Radiotherapy induces DNA damage and cell death, but recent data suggest that concomitant immune stimulation is an integral part of the therapeutic action of ionizing radiation. It is poorly understood how radiotherapy supports tumor-specific immunity. Here we report that radiotherapy induced tumor cell death and transiently activated complement both in murine and human tumors. The local production of pro-inflammatory anaphylatoxins C3a and C5a was crucial to the tumor response to radiotherapy and concomitant stimulation of tumor-specific immunity. Dexamethasone, a drug frequently given during radiotherapy, limited complement activation and the anti-tumor effects of the immune system. Overall, our findings indicate that anaphylatoxins are key players in radiotherapy-induced tumor-specific immunity and the ensuing clinical responses.

INTRODUCTION

It is now well accepted that the immune system can control tumors (Schreiber et al., 2011). For example, there is a positive correlation between tumor infiltration by effector T cells and survival (Fridman et al., 2011), and the risk to develop cancer is increased in immunosuppressed patients (Dunn et al., 2006), and dormant tumors are kept in check by the adaptive immune system (Koebel et al., 2007). Despite the presence of tumor-specific immunity in many cancer patients, complete rejection of clinically apparent tumors by the immune system is rare, presumably due to mechanisms that locally inhibit tumor-specific protective immunity (Schreiber et al., 2011). The clinical efficacy of so-called checkpoint blockade, antibodies that target co-inhibitory molecules such as CTLA-4 or PD-1, underscores the potential of tumor-specific immunity (McDermott and Atkins, 2013; Mellman et al., 2011).

Radiotherapy is a standard treatment for cancer that induces irreversible damage to DNA, thus targeting mainly rapidly dividing cells (Prise and O'Sullivan, 2009). Although radiotherapy was considered an immunosuppressive treatment (Merrick et al., 2005), there is accumulating evidence that it supports local tumor-specific immunity (Apetoh et al., 2007; Matsumura et al., 2008) and, in fact, that immune activation might be an integral part of radiotherapy (Formenti and Demaria, 2012; Gupta et al., 2012; Ma et al., 2010; Sharma et al., 2013). This is clinically relevant because tumor-specific immunity can target dormant lesions (Postow et al., 2012) that are presumably insensitive to radiotherapy. Several studies have addressed the question of how radiotherapy supports tumor-specific immunity, and various factors were suggested, including increased presence or function of tumor-infiltrating CD8⁺ T cells (Gupta et al., 2012; Lugade et al., 2005; Takeshima et al., 2010), type I interferon (IFN) resulting in enhanced antigen cross-presentation (Burnette et al., 2011; Fuertes et al., 2011), increased expression of major histocompatibility complex (MHC) class I glycoproteins and tumor-associated antigens (Reits et al., 2006), and maturation of tumor-associated dendritic cells (DCs) (Gupta et al., 2012), however, an initial event has not been identified.

We therefore performed an unbiased analysis of immune response-related transcripts after radiotherapy in a preclinical model and noted a strong and transient upregulation of genes associated with the complement pathway. This was unexpected as complement was described as tumor-promoting (Markiewski et al., 2008; Pio et al., 2014), although other studies have shown that complement supports adaptive immunity (Farrar and Sacks, 2014; Kopf et al., 2002; Lalli et al., 2007; Liszewski et al., 2013; Strainic et al., 2013). Because we observed similar changes in human tumor samples, we investigated the impact of complement on the anti-tumor immune response following radiotherapy.

RESULTS

Radiotherapy Induces Complement Activation

To identify the initial event in radiotherapy-induced tumor-specific immunity, we performed an unbiased analysis of immune

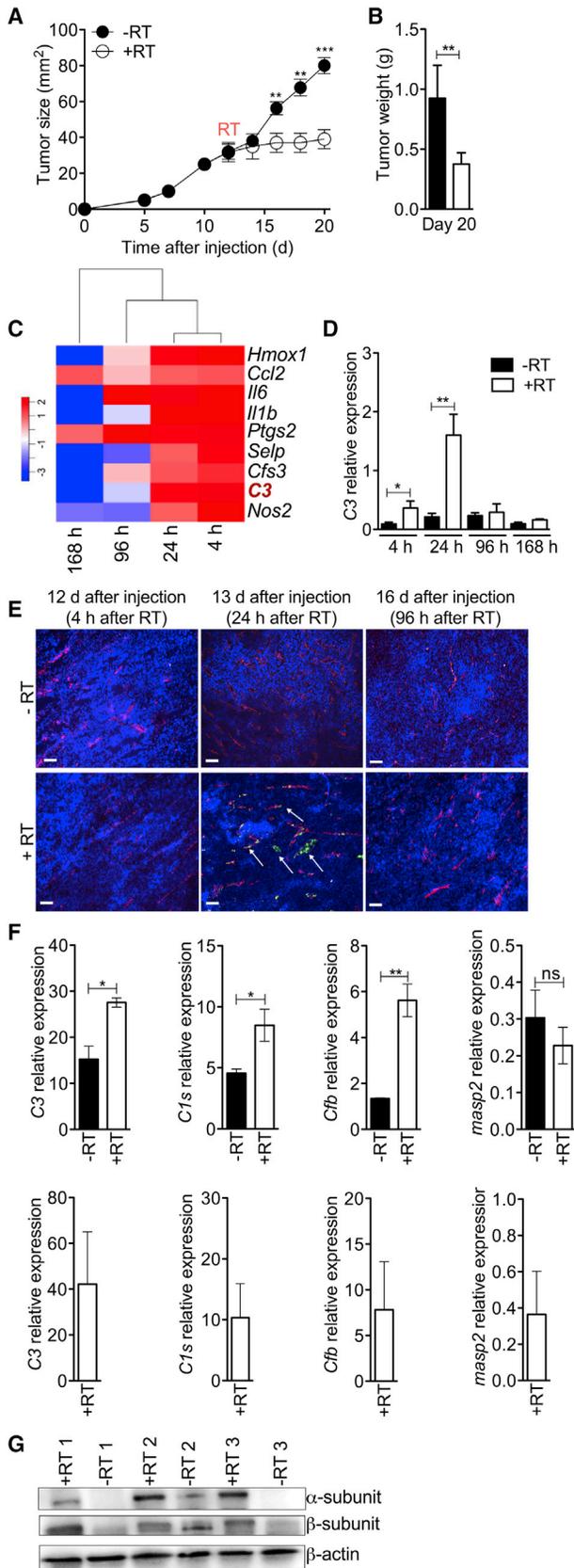


Figure 1. Radiotherapy Results in Transient Upregulation and Activation of Complement in Murine and Human Tumors

(A–E) Mice were injected with B16F10-OVA cells and received radiotherapy 12 days later. (A) Tumor growth curves, (n = 5 mice per group). Representative data from three independent experiments are shown. (B) Tumor weight (day 20). (C) Heatmaps of transcripts were created using the log₂ value of the fold increase of irradiated compared to untreated tumors at different time points after radiotherapy, (n = 6 mice per time point). Representative data from two independent experiments are shown. (D) Relative expression of C3 mRNA in tumors at different time points after radiotherapy. The data show the mean ± SD of triplicates from three independent analyses. Radiotherapy, RT. (E) Immunofluorescence of irradiated and untreated tumors. Sections were stained with an antibody recognizing C3b, iC3b, and C3c (green), CD31 for blood vessels (red), and DAPI (blue). Scale bars represent 100 μm (n = 6 mice per group). Representative images from two independent experiments are shown.

(F and G) Patient biopsies were collected before and 24–36 hr after radiotherapy (1.5–2 Gy; upper panel) or only after radiotherapy (lower panel). Quantification of complement transcripts by qPCR. Data show the mean ± SD of triplicates from two independent analyses (upper panel). (G) Quantification of C3-α and -β subunits by immuno-blot.

Data are shown as mean ± SD. *p < 0.05, **p < 0.005, ***p < 0.0005 by Student’s t test (A, B, and F) or two-way ANOVA with the Bonferroni correction (D). See also Figure S1.

response-related transcripts after radiotherapy. Local irradiation with a single dose of 20 Gy significantly reduced progression of B16F10-OVA tumors in C57BL/6 mice (Figures 1A and 1B). Because transplantable mouse tumors only allow for a short therapeutic window, radiotherapy must be applied as a single high dose (Lugade et al., 2005). To dissect which pathways are crucial to radiotherapy-induced stimulation of the immune response, we quantified immune response-related transcripts in tumors at different time points 4, 24, 96, and 168 hr after local irradiation (Figures 1C and S1A). We observed an upregulation of the complement system (represented in this panel only by C3) and the inflammation cascade at 4 and 24 hr, whereas both pathways were downregulated at 96 and 168 hr after irradiation (Figure 1C). Because 20 Gy might be of limited clinical relevance, we performed the same analysis 24 hr after irradiation with a single dose of 5 Gy and observed a similar transcriptional upregulation (Figure S1B).

C3 is the central protein of the complement cascade at which all three known pathways (classical, alternative, and lectin) converge and which gives rise to various bioactive components (Markiewski and Lambris, 2009). Because complement might be tumor-promoting (Markiewski et al., 2008; Markiewski and Lambris, 2009; Pio et al., 2014), we investigated whether radiotherapy-induced upregulation of complement supported or antagonized the efficacy of this treatment. We first quantified four different complement-related transcripts in response to radiotherapy: C3, C1s, Masp2 and Cfb. The classical and alternative pathways are the main pathways induced by radiotherapy on the transcriptional level (Figures 1D and S1C). Because NF-κB, JAK, and STAT transcriptional pathways (Chen et al., 2011; Fukuoka et al., 2013; Hasegawa et al., 2014; Huang et al., 2002), as well as S100 calcium-binding proteins A8 and A9 (S100A8, S100A9) (Schonthaler et al., 2013) are involved in the transcription of complement factors, we analyzed such pathways by immuno-blot and found an increased production of STAT 1, STAT 2, STAT 3, NF-κB, and JAK and increased phosphorylation of STAT 2, STAT3, and JAK (Figure S1D) 4 hr, but not

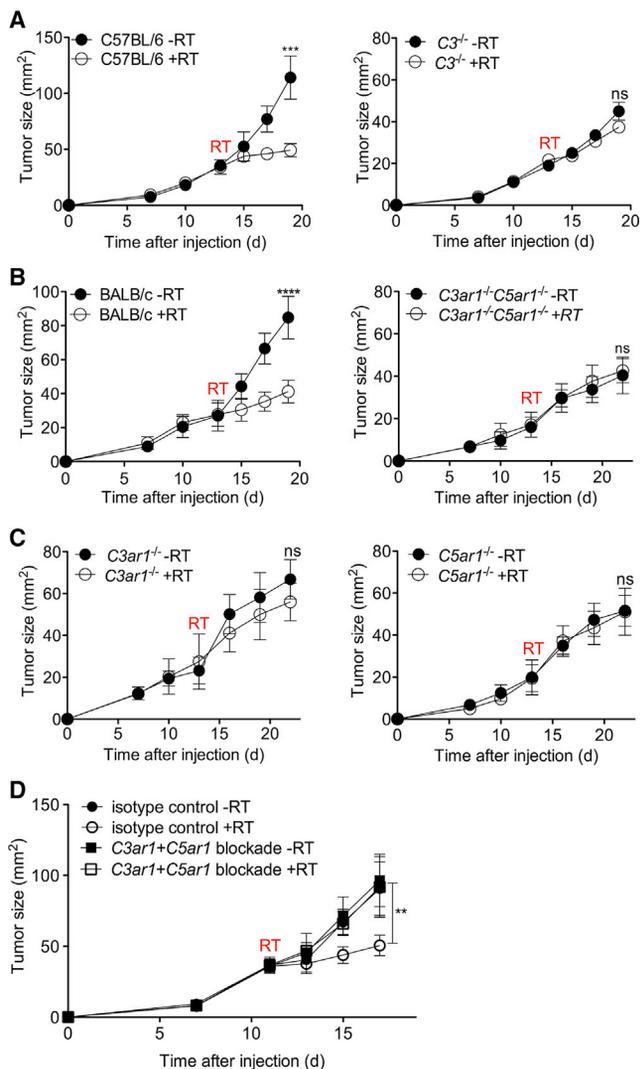


Figure 2. C3a and C5a Are Crucial to the Therapeutic Efficacy of Radiotherapy

(A) Control and $C3^{-/-}$ mice were injected with B16F10-OVA cells and received radiotherapy 13 days later. Growth curves, ($n = 5$ mice per group). Representative data from two independent experiments are shown. Radiotherapy, RT. (B) BALB/c, $C3ar1^{-/-}$, $C5ar1^{-/-}$, and $C3ar1^{-/-}C5ar1^{-/-}$ were injected with CT26 cells and received radiotherapy 13 days later. Tumor growth curves (BALB/c $n = 5$ mice per group; $C3ar1^{-/-}$, $C5ar1^{-/-}$, and $C3ar1^{-/-}C5ar1^{-/-}$ $n = 8$ mice per group). Representative data from two independent experiments are shown.

(C) Mice were injected with B16F10-OVA cells and received radiotherapy 12 days later. SB290157 was administered at 2 mg/kg to block C3aR1 and anti-C5aR1 mAb 20/70 or an isotype control at 0.6 mg/kg to block C5aR1 (administered every second day starting on day 12 until day 19). ($n = 5$ mice per group).

Data are shown as the mean \pm SD. ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.00005$ by two-way ANOVA with the Bonferroni correction. See also Figure S2.

24 hr, after radiotherapy. Transcripts of *S100a8* and *S100a9* were unaffected (data not shown).

To show that these translated in different complement factor amounts, we analyzed the tumors prior to and after radiotherapy by immunofluorescence (Figure S1E) and by immuno-blot (Fig-

ure S1F). Because C1q and Factor B are more abundant compared to mannose-binding lectin C (MBL-C), these results confirmed our qPCR data (Figure S1C). We analyzed the activation status of complement and found deposition of fragments derived from C3 cleavage (C3b, iC3b, and C3c) 24 hr after radiotherapy in the proximity of (green fluorescence) or associated with (yellow fluorescence) blood vessels (Figure 1E). Similarly to C3 cleavage products, we observed C1q and Factor B mainly associated to blood vessels, whereas MBL-C showed a markedly different pattern of deposition. We obtained similar results using B16F10 cells, both at the transcriptional (data not shown) and protein level (Figure S1G). We confirmed radiotherapy-induced complement activation by immuno-blotting using an antibody recognizing C3 plus its fragments C3b, C3c, and C3dg (Figure S1H). Because deposition of C3b, iC3b, and C3c was not detected at other time points besides 24 hr after radiotherapy, this suggests that radiotherapy-induced complement activation is a rapid and transient response.

We analyzed the expression of complement-related transcripts and proteins in paired human tumors that were collected before and 24–36 hr after a single, low dose of radiotherapy (1.5 or 2 Gy) and detected upregulated expression (Figure 1F) and activation of complement (Figure 1G). To exclude that taking the first biopsy prior to radiotherapy rather than radiotherapy was responsible for complement activation, we analyzed additional four biopsies that were taken 24–36 hr after irradiation with 1.5–2 Gy but without previous intervention. The amounts of complement transcripts were comparable to those after radiotherapy in the paired samples, excluding this option (Figure 1F).

Together, we found that radiotherapy induces transient, local production, and activation of the classical and alternative complement pathway in both human and murine tumors.

Therapeutic Efficacy of Radiotherapy Depends on C3a and C5a

To investigate the impact of complement activation on therapeutic efficacy, we applied radiotherapy to tumor-bearing $C3^{-/-}$ mice. Because tumors grew more slowly in $C3^{-/-}$ mice (Figures 2A and S2A) (Markiewski et al., 2008; Qing et al., 2012), $C3^{-/-}$ and C57BL/6 mice were irradiated at two different time points, i.e., 13 (Figure 2A) or 17 days (Figure S2B) after tumor injection. Comparison of C57BL/6 mice irradiated at day 13 (Figure 2A, left panel) with $C3^{-/-}$ mice irradiated at day 17 (Figure S2B, right panel) shows the response of tumors with a similar size (36–40 mm²) at the time point of irradiation. Radiotherapy was not efficient in $C3^{-/-}$ mice irrespective of the day of therapy (Figures 2A, S2A, and S2B), suggesting that complement activation is crucial to efficacy.

Anaphylatoxins (C3a and C5a) modulate adaptive immunity (Schmudde et al., 2013; Strainic et al., 2013). As we observed higher local amounts of C3a and C5a (Figure S2C) and their receptors (Figures S2D) upon radiotherapy, we investigated their role in the response to radiotherapy using $C3ar1^{-/-}$, $C5ar1^{-/-}$, and $C3ar1^{-/-}C5ar1^{-/-}$ mice. As these mice were only available on a BALB/c background, we confirmed that radiotherapy resulted in local complement activation (Figure S2E), showing that radiotherapy-induced complement activation is a general phenomenon independent of the strain or tumor cell line used. Similar to $C3^{-/-}$ mice, $C5ar1^{-/-}$,

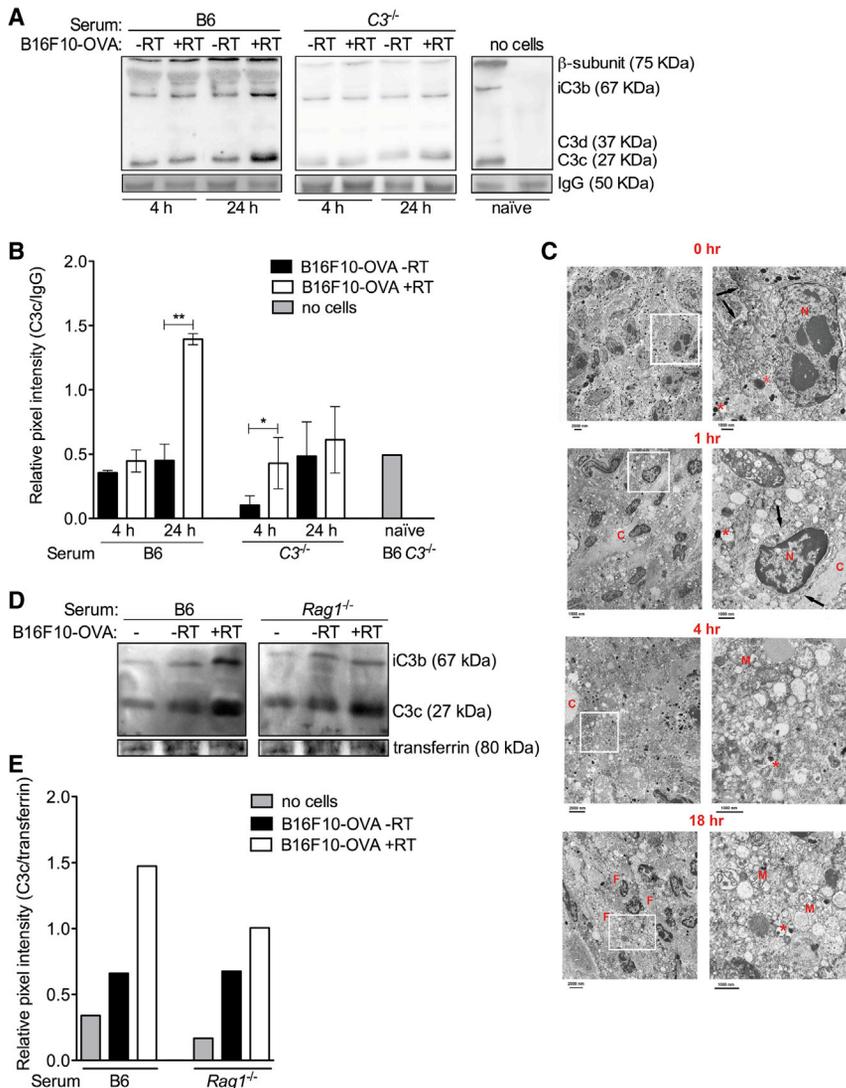


Figure 3. Radiotherapy-Induced Tumor Cells Death Activates Complement

(A and B) B16F10-OVA cells were irradiated in vitro with 20 Gy or left untreated. Serum from C57BL/6 or C3^{-/-} mice was added to the cultures immediately after irradiation and C3 cleavage products were analyzed 4 and 24 hr later in the supernatants by immuno-blot using a polyclonal anti-C3 antibody (n = 3 mice per group). Representative data of two independent experiments are shown. (B) Relative intensities were calculated using Bio1D software. Radiotherapy, RT.

(C) Ultrastructural analysis of tumor response to radiotherapy. B16F10-OVA tumors were isolated from mice prior to and different times after radiotherapy. Fixed tumor tissue was epon embedded and analyzed by EM. Shown are images of tumor tissues before (0 h), 1, 4, and 18 hr after radiotherapy. Left column, overview; Right column, zoom-in of region indicated in left image. Bar indicates magnification. N, nucleus; M, mitochondrion; black arrow, cell boundaries; F, fat bodies; C, collagen; *, melanosomes.

(D and E) B16F10-OVA cells were irradiated in vitro with 20 Gy or left untreated. Serum from Rag1^{-/-} mice was added to the cultures immediately after irradiation and C3 cleavage products were analyzed as described in (A), (n = mice 3 per group). (E) Relative intensities were calculated using Bio1D software.

Data in (B) are shown as the mean ± SD. *p < 0.05, **p < 0.005, by two-way ANOVA with the Bonferroni correction.

radiotherapy or a local tumor results in systemic activation of complement. Local radiotherapy results in apoptosis (Wang, 2008), mitotic catastrophe, and necrosis (Eriksson and Stigbrand, 2010), all of which can be potent activators of complement (Basu et al., 2000; Kemper et al., 2008; Markiewski and Lambris, 2007). We first evaluated the possibility of direct complement activation by radiation and exposed serum from C57BL/6 or C3^{-/-} mice in vitro to a single dose of 20 Gy or left them untreated and analyzed sera 4 and 24 hr after irradiation by immuno-blot. We did not detect any significant changes upon irradiation (data not shown). To investigate whether irradiated tumor cells can activate complement, we either or not exposed B16F10-OVA cells in vitro to a single dose of 20 Gy. Immediately after irradiation, 10-fold diluted serum from C57BL/6 mice or C3^{-/-} mice was added to irradiated or untreated B16F10-OVA cells. Complement activation was detected in the supernatants 24 hr after radiation. Because the culture serum was from C3^{-/-} mice, the complement should have been derived from the tumor cells (Figures 3A and 3B).

To identify the mode of tumor cell death upon a single dose of 20 Gy, we performed electron microscopy (EM) on B16F10-OVA tumors isolated at different time points after radiotherapy as indicated (Figure 3C). The tissue isolated before radiation showed normal nuclei, cell boundaries, intracellular organelles, and melanosomes (the dark vesicular structures) illustrating healthy

C3ar1^{-/-}, and C3ar1^{-/-}C5ar1^{-/-} mice showed no significant impact of radiotherapy on tumor progression, whereas BALB/c mice did (Figures 2B, 2C, and S2F). To avoid the issue of inherent different tumor growth rates in various genetically ablated strains, we blocked C3aR with an antagonist (C3aRA, SB290157) or C5aR with a monoclonal antibody (20/70) (Baelder et al., 2005) just before applying radiotherapy to tumor-bearing C57BL/6 mice. This treatment blocked the improved antitumor effect of radiotherapy (Figures 2D and S2G), which is in line with the results observed in C3^{-/-}, C3ar1^{-/-}, C5ar1^{-/-}, and C3ar1^{-/-}C5ar1^{-/-} mice. These data suggest that radiotherapy induces the intratumoral generation of anaphylatoxins, which are crucial to the therapeutic efficacy.

Radiotherapy-Induced Cell Death Locally Activates Complement

Despite the fact that complement deposition was observed in the vicinity of blood vessels, we failed to detect activated complement in serum irrespective of whether mice received radiotherapy or bore a tumor (Figure 4A). This excludes that

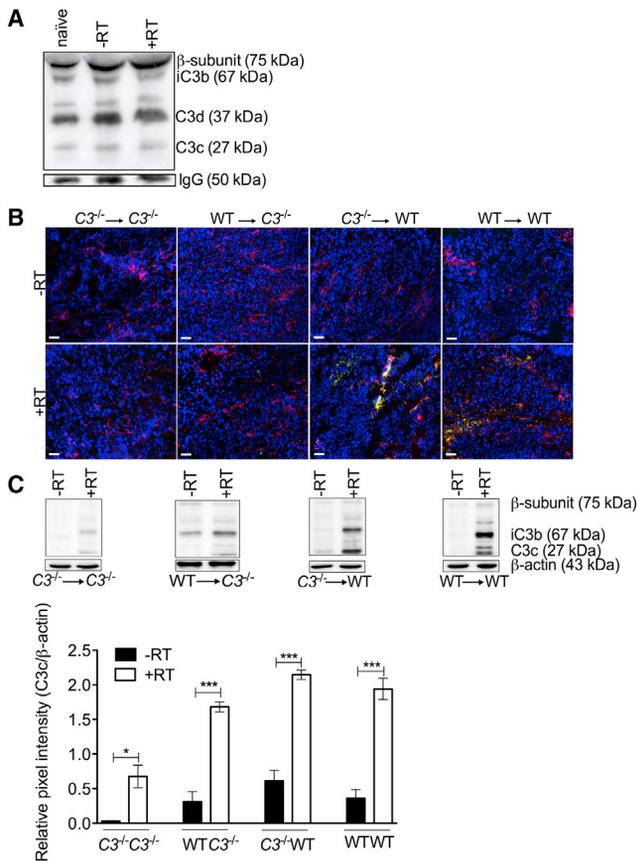


Figure 4. Radiotherapy-Induced, Tumor-Associated Complement Is Mainly Liver-Derived but Partially Produced Locally

(A) Measurement of complement activation by immuno-blot in pooled blood samples from naive, tumor-bearing, and irradiated tumor-bearing mice (24 hr after radiotherapy), (n = 3 mice per group). C3 cleavage products were detected using a polyclonal anti-C3 antibody. Radiotherapy, RT.

(B and C) Bone-marrow chimeras were injected with B16F10-OVA cells and received radiotherapy 13 days later. Tumors were collected 24 hr after radiotherapy (B) Immunofluorescence of irradiated and untreated tumors. Sections were stained with an antibody recognizing C3b, iC3b, and C3c (green), CD31 for blood vessels (red) and DAPI (blue). Scale bars represent 100 μm. Representative images from two independent experiments are shown (n = 4 mice per group). (C) C3 cleavage products in the tumors were analyzed by immuno-blot using a polyclonal anti-C3 antibody, (n = 3 mice per group). Relative expression measured by Bio-1D software. Representative data from two independent experiments are shown.

Data in (C) are shown as the mean ± SD. *p < 0.05, ***p < 0.0005 by two-way ANOVA with the Bonferroni correction.

tumor tissue. Already 1 hr after radiation, the first signs of radiation damage were detected in the form of extensive vacuolization of tumor cells with normal nuclei and cell boundaries. Already 4 hr after radiation, cell boundaries were dissolved and cellular content fragmented. Mitochondrial structures (M) were swollen and did not show normal cristae. This did not change any further between 4 and 18 hr after radiotherapy. We did not observe any nuclear fragmentation and apoptotic bodies; rather, the irradiated tissue showed marked fields of necrosis with many intracellular materials now entering the extracellular space.

Because immunoglobulin M (IgM) binds to necrotic cells, which results in complement activation (Ciurana et al., 2004;

Quartier et al., 2005), we performed the experiment described above using serum from *Rag1*^{-/-} mice. We observed reduced complement activation in supernatants containing *Rag1*^{-/-} serum (Figures 3D and 3E), suggesting that IgM binding to necrotic cells contributes to radiotherapy-induced complement activation. This suggests that necrotic tumor cells express or secrete factors that activate complement.

Microenvironmental Complement Is Produced by Immune Cells

Hepatocytes are the main source of complement proteins, but also extra-hepatic tissues and immune cells can produce them (Farrar and Sacks, 2014; Kolev et al., 2014; Pio et al., 2014; Strainic et al., 2008). To define the source of radiotherapy-induced, tumor-associated complement, we generated C3^{-/-} → WT, WT → C3^{-/-}, WT → WT, and C3^{-/-} → C3^{-/-} bone marrow chimeras. B16F10-OVA bearing chimeras received radiotherapy or not and tumors were processed 24 hr later. We detected high amounts of activated complement in C3^{-/-} → WT and WT → WT mice and less in WT → C3^{-/-} mice. The low amount of complement detected in irradiated tumors in C3^{-/-} → C3^{-/-} mice is presumably tumor-derived (Figures 4B and 4C). This is in agreement with the detection of basal expression of complement-related transcripts in cultured B16F10-OVA tumor cells and an increased release of complement proteins by B16F10-OVA cells upon in vitro irradiation (Figures 3A, 3B, and 4C). These experiments suggest that a large fraction of radiotherapy-induced, tumor-associated complement is produced systemically, with a contribution of local production by immune and tumor cells.

Radiotherapy-Induced DC Activation Depends on Anaphylatoxins

Because several immune cells can produce complement components (Li et al., 2007; Strainic et al., 2008), which are essential for full functional development of antigen-presenting cells (APCs) and T cell responses (Peng et al., 2009; Strainic et al., 2008), we investigated which immune cells produce complement or anaphylatoxin receptors in irradiated tumors. We sorted DCs, CD8⁺ T cells, CD4⁺ T cells, and other CD45.2⁺ cells from irradiated and untreated tumors (Figure S3A) and quantified the complement factor related transcripts *C3*, *C1s*, *Masp2*, *Cfb*, *C3ar1*, and *C5ar1* by qPCR. Because the impact of radiotherapy on tumor-associated DCs and T cells is apparent after 24 hr and 5–7 days, respectively (Gupta et al., 2012), we harvested tumors at these time points after radiotherapy exposure. DCs showed increased expression of *C3*, *Cfb*, *C3ar1*, and *C5ar1* 24 hr and 168 hr after radiotherapy (Figure 5A). We failed to detect expression of *C1s* and *Masp2* transcripts by DCs. The other CD45.2⁺ cells (mainly containing macrophages) showed upregulated *C5ar1* but no other complement-related transcripts at 168 hr after radiotherapy (Figure S3B).

Recent studies demonstrate that complement factors, in particular anaphylatoxins, directly bind to their receptors on DCs thereby supporting their maturation, which then induces T cell effector activation (Li et al., 2007; Peng et al., 2009; Strainic et al., 2008). Furthermore, we showed previously that radiotherapy-induced activation of tumor-associated dendritic cells locally supports the function of tumor-specific CD8⁺ T cells

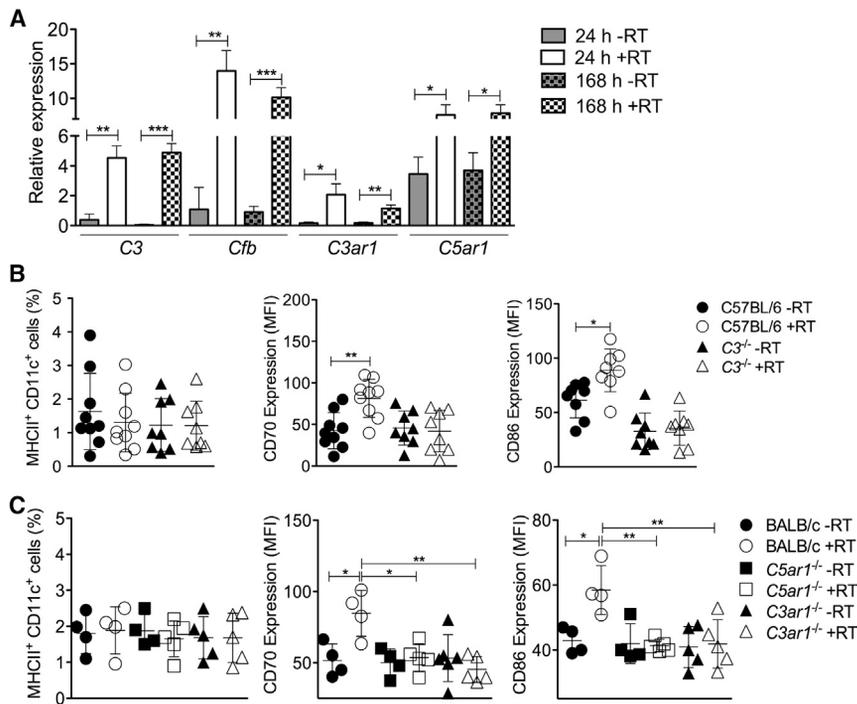


Figure 5. Radiotherapy Induces Upregulation of Transcripts for Complement and Anaphylatoxin Receptors by DCs and Is Essential to Their Maturation

(A) Mice were injected with B16F10-OVA cells and received radiotherapy 12 days later. DCs (CD45.2⁺ TCRβ⁻ CD11c⁺ MHC-II^{high}) gated on live singlets) were sorted from untreated and irradiated tumors 24 and 168 hr after radiotherapy. Complement-related transcripts were quantified by qPCR (n = 5 mice per group). Radiotherapy, RT.

(B) Control and C3^{-/-} mice were injected with B16F10-OVA cells and received radiotherapy 12 days later. CD70 and CD86 expression on tumor-associated DCs in irradiated (48 hr after radiotherapy) and untreated tumors plotted as geometric mean fluorescence intensity (gMFI).

(C) BALB/c, C3ar1^{-/-}, and C5ar1^{-/-} mice were injected with CT26 cells and received radiotherapy 12 days later. DCs were analyzed as described in (B).

(B and C) Each symbol represents an individual mouse. Pooled data from two independent experiments are shown. Data are shown as the mean ± SD. *p < 0.05, **p < 0.005, ***p < 0.0005 by two-way ANOVA with the Bonferroni correction. See also Figure S3.

and that this is crucial to therapeutic efficacy (Gupta et al., 2012). Therefore, we analyzed the activation status of DCs upon irradiation in C3^{-/-} and in C57BL/6 mice 2 days after radiotherapy. DCs were equally present in untreated and irradiated tumors of complement-proficient and -deficient mice, however, DC activation as measured by surface expression of CD70 and CD86 (Keller et al., 2008) was observed only in irradiated C57BL/6, but not in C3^{-/-} mice (Figure 5B). These results were confirmed using C3ar1^{-/-}, C5ar1^{-/-}, and BALB/c mice (Figure 5C). Thus, radiotherapy induces upregulation of anaphylatoxins, and their receptors in tumor-associated DCs controls radiotherapy-induced DC activation.

Radiotherapy-Induced CD8⁺ T Cell Activation Depends on Anaphylatoxins

T cells express complement components and anaphylatoxin receptors that are critical to T cell co-stimulation (Liszewski et al., 2013; Strainic et al., 2008). CD8⁺, but not CD4⁺ T, cells upregulated C3 and the anaphylatoxin receptors slowly at 168 hr, but not at 24 hr, after radiotherapy (Figures 6A and S3C), whereas C1s and Masp2 were undetectable. C5aR1 and C3aR1 signaling during cognate interaction between DCs and CD4⁺ T cells promotes IFN-γ production (Liu et al., 2008) and counteracts the development of FoxP3⁺ regulatory T cells (Strainic et al., 2013). We therefore analyzed the impact of radiotherapy on expression of *Ifng* and *Foxp3* and observed a strong upregulation of *Ifng* in CD8⁺ T cells 168 hr after the treatment (Figure 6A), but no changes of *Foxp3* within the CD4⁺ T cell compartment (data not shown).

To investigate the in vivo relevance of those observations, we analyzed the infiltrate in tumors from BALB/c and C3ar1^{-/-}C5ar1^{-/-} mice by flow cytometry 168 hr after radiotherapy. Radiotherapy resulted in higher numbers of CD45.2⁺

and CD8⁺ T cells in tumors, which was independent of anaphylatoxins (Figure 6B). Radiotherapy had no effect on CD4⁺ T cell numbers (Figures 6C and 6D). Therefore, it is unlikely that anaphylatoxins act as chemoattractants for T cells. We analyzed the global IFN-γ production by tumor-infiltrating CD8⁺ T cells in situ. Therefore, we injected mice with Brefeldin A 4 hr before euthanasia and measured intracellular IFN-γ without in vitro restimulation. IFN-γ production by CD8⁺ T cells was significantly increased in irradiated tumors in BALB/c, but not C3ar1^{-/-}C5ar1^{-/-} mice compared to untreated controls (Figures 6C, S4A, and S4B). This presumably involves anaphylatoxin-mediated DC maturation (Strainic et al., 2013), although a direct activity of anaphylatoxins on CD8⁺ T cells cannot be excluded. In agreement with earlier data (Strainic et al., 2013), we observed higher numbers of FoxP3⁺ T cells in C3ar1^{-/-}C5ar1^{-/-} mice independent of radiotherapy (Figures 6D, S4C, and S4D). Flow cytometry results thus confirmed the qPCR data on sorted cells (Figure 6A). We obtained similar results using B16F10-OVA-bearing C57BL/6 mice in which C3aR1 and C5aR1 were blocked (Figure S4E). A recent publication shows that blocking C3 improves the efficacy of fractionated radiotherapy given as multiple daily fractions of 1.5 Gy (Elvington et al., 2014), which is an apparent controversy with our findings. We found that 5 × 1.5 Gy equally diminished tumor growth as 1 × 20 Gy did. However, in contrast to a single high dose, 5 × 1.5 Gy resulted in prolonged activation of complement and did not support accumulation of CD8⁺ T cells nor their function in the tumor (Figures S4G–S4I).

To generate a complete immune cell profile related to irradiation responses, we analyzed CD19⁺ B cells and found low numbers (Figure S4F), regardless of radiotherapy or blockade of C5aR1 and C3aR1. Tumors contained substantial numbers of NK1.1⁺ cells that significantly increased upon

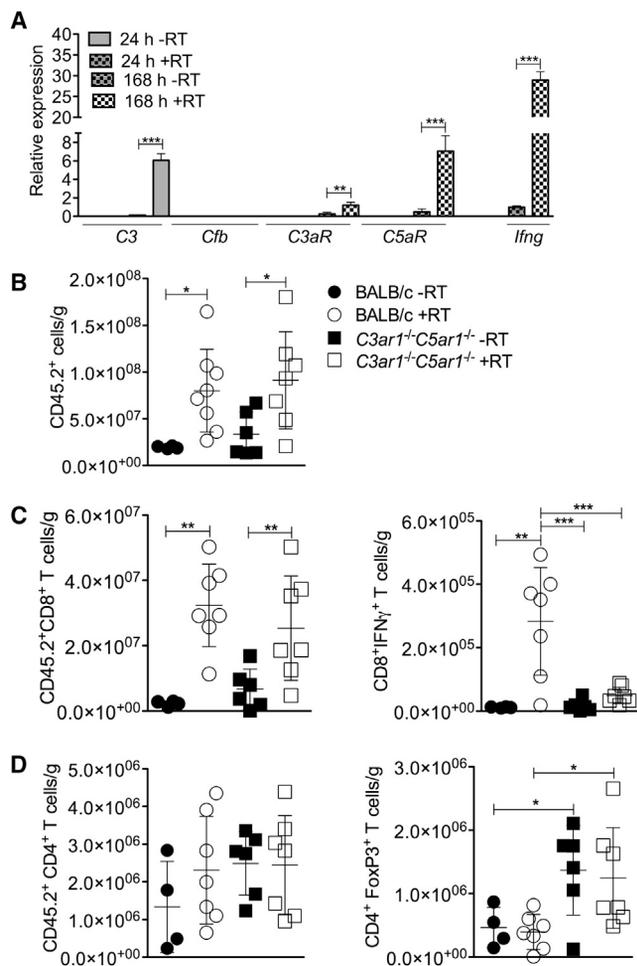


Figure 6. Radiotherapy Induces Upregulation of Transcripts for Complement and Anaphylatoxin Receptors by CD8⁺ T Cells and Supports Tumor-Specific Immunity

(A) C57BL/6 mice were injected with B16F10-OVA cells and received radiotherapy 12 days later. CD8⁺ T cells (CD45.2⁺ TCR β ⁺ CD8⁺ gated on live singlets) were sorted from untreated and irradiated tumors 24 and 168 hr after radiotherapy. Complement-related transcripts were quantified by qPCR (n = 5 mice per group). Radiotherapy, RT.

(B–D) BALB/c and *C3ar1*^{-/-}*C5ar1*^{-/-} mice were injected with CT26 cells and received radiotherapy 12 days later. Flow cytometric analysis was performed 7 days after radiotherapy. (B) The total number of infiltrating leukocytes (CD45.2⁺), (C) CD8⁺ cells (left panel), and CD8⁺ T cells that produce IFN- γ in vivo (right panel), (D) CD4⁺ (left panel) and FoxP3⁺ CD4⁺ T cells (right panel) were determined per gram tumor tissue. Mice were injected with Brefeldin A 4 hr before euthanasia.

Each symbol represents an individual mouse. Data are shown as the mean \pm SD. *p < 0.05, **p < 0.005, ***p < 0.0005 by two-way ANOVA with the Bonferroni correction. See also Figure S4.

radiotherapy and seeded independent of anaphylatoxin receptor blockade. We observed very low numbers of NKp46⁺ cells that remained similar in all four groups (Figure S4F). Thus, following a single dose of 20 Gy, the DC-CD8-arm of the immune system appears to be selectively activated in irradiated tumors, which is essential for tumor control and depends on complement.

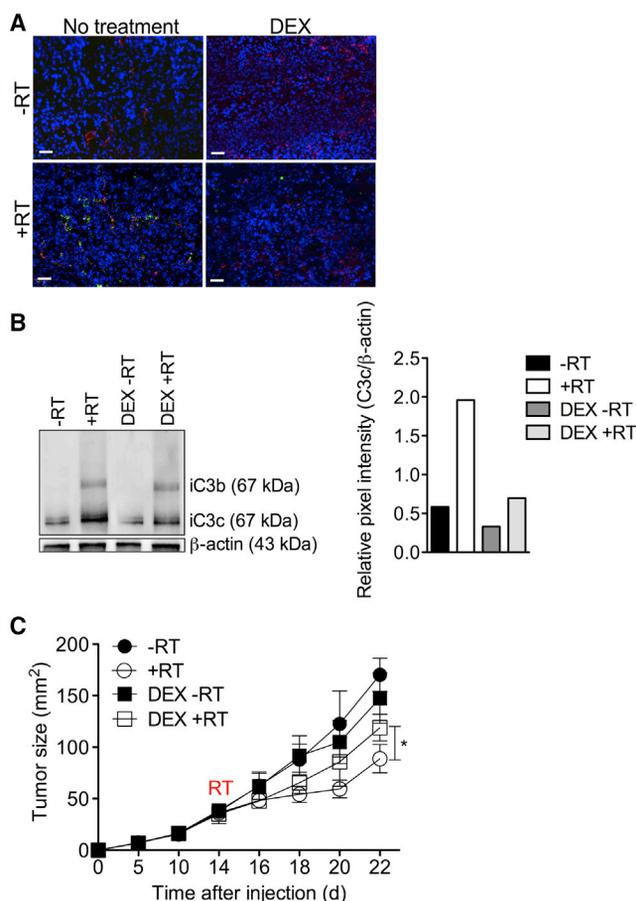


Figure 7. Dexamethasone Inhibits Complement Activation and Reduces the Clinical Efficacy of Radiotherapy

(A–C) Mice were injected with B16F10-OVA cells and received radiotherapy 14 days later. DEX (0.3 mg/kg) was injected daily from day 12 until day 22, (n = 6 mice per group). (A) Immunofluorescence of irradiated (24 hr) and untreated tumors. Sections were stained with an antibody recognizing C3b, iC3b, and C3c (green), CD31 for blood vessels (red), and DAPI (blue). Scale bars represent 100 μ m. Representative images are shown. (B) C3 cleavage products in the tumors 24 hr after radiotherapy were analyzed by immuno-blot using a polyclonal anti-C3 antibody. Relative expression measured by Bio-1D software. Representative data are shown. (C) Tumor growth curves.

The data are shown as the mean \pm SD. *p < 0.05 by two-way ANOVA with the Bonferroni correction. Radiotherapy, RT.

Dexamethasone (DEX) is a glucocorticoid with anti-inflammatory and immunosuppressive properties (Auphan et al., 1995). In addition, it inhibits the activation of complement (Engelman et al., 1995; Packard and Weiler, 1983). Given the important role of complement in promoting adaptive immunity and supporting the efficacy of radiotherapy, we treated mice with DEX starting 1 day before radiotherapy. DEX treatment significantly reduced the extent of local complement activation (Figure 7A) and importantly, also the efficacy of radiotherapy (Figures 7B and 7C).

Thus, radiotherapy-induced, local production of anaphylatoxins is essential to activation of DCs and protective effector function of CD8⁺ T cells in the tumor and as such to therapeutic efficacy.

DISCUSSION

In this study, we have demonstrated that radiotherapy induces an acute and transient local activation of complement, which is pivotal for tumor-specific immunity and therapeutic efficacy. Complement has traditionally been considered only to “complete” the action of the immune system in the antibody-mediated defense against pathogens. The current appreciation is that complement is involved in many different pathological processes such as transplant rejection, autoimmunity, neurodegeneration, and cancer.

The role of complement in cancer is still confusing as the production of complement-inhibiting proteins by tumor cells or stroma has been suggested to promote tumor growth (Kolev et al., 2011), whereas it is also proposed that complement in the context of chronic inflammation promotes tumor growth, migration and angiogenesis (Markiewski et al., 2008; Pio et al., 2014). This is in line with a previous publication (Elvington et al., 2014) showing increased efficacy of fractionated radiotherapy when C3 was blocked. Repeated irradiation might thus induce a chronic inflammatory response that interferes with protective adaptive immunity. In addition, the infiltrating T cells might be killed by the next dose before they could execute their anti-tumor effect. A different radiotherapy protocol that either introduces a radiotherapy holiday of 7–10 days between the fractions of radiotherapy or provides a single high-dose of radiotherapy might be required to optimally support tumor-specific immunity (Favaudon et al., 2014).

Pathogen- and damage-associated molecular patterns can activate C1q, MBL, and the alternative complement pathway. The latter can also be activated by spontaneous hydrolysis of C3 or by non-complement proteins (Markiewski and Lambris, 2007). Furthermore, modified membranes of late apoptotic and necrotic cells are potent activators of complement (Ricklin et al., 2010). In fact, every disturbance of homeostasis or assault might result in activation of complement (Kolev et al., 2014). Our data suggest that factors released from necrotic tumor cells upon radiotherapy are responsible for local complement activation. The leakiness of tumor-associated blood vessels (Carmeliet and Jain, 2011) might further promote accumulation of complement in the tumor.

The liver is the main source of complement, but many complement components can be produced by a variety of tissues and immune cells either constitutively or in response to stress (Kolev et al., 2014; Li et al., 2007). For example, locally produced C1q contributes to removal of apoptotic material and immune complexes (Roumenina et al., 2011) and supports T cell responses (Baudino et al., 2014). We have shown here that tumor-associated T cells, DCs, other CD45⁺ cells, as well as tumor cells can be a source of anaphylatoxins and their receptors in response to radiotherapy. It is plausible to consider that tumor-associated stroma might also contribute to the production of complement upon radiotherapy.

Sensing immune cell-derived complement during cognate interactions between T cells and DCs is essential for development of protective immunity (Lalli et al., 2007; Liszewski et al., 2013; Peng et al., 2009; Strainic et al., 2008). Moreover, when signaling through C3aR1 and C5aR1 is prevented during cognate interactions, CD4⁺ T cells develop into FoxP3⁺ regulatory T (Treg) cells

instead of effectors (Strainic et al., 2013), in line with our observation that tumors contained more Treg cells in the absence of anaphylatoxin receptor signaling. We found that tumor-associated DCs produced complement factors and upregulated the expression of C3aR1 and C5aR1 upon radiotherapy, which appeared to be essential for radiotherapy-induced DCs maturation. It has been shown that anaphylatoxins directly can induce DC maturation in vitro and that C3 upregulation precedes the expression of IL-1, IL-12, and IL-23 (Strainic et al., 2008), suggesting a direct effect of anaphylatoxins on DCs. DC maturation is crucial to development and/or maintenance of T cell effector function within the tumor and efficacy of radiotherapy (Gupta et al., 2012), and indeed, tumor-infiltrating CD8⁺ T cells failed to produce IFN- γ after radiotherapy in the absence of signaling through C3aR1 and C5aR1.

Given the importance of complement activation and immune response following local radiotherapy, the administration of glucocorticoids, anti-inflammatory, and immunosuppressive drugs for managing post-radiation symptoms (Hempfen et al., 2002; Hughes et al., 2005) might have a modulating impact on the efficacy of radiotherapy. Indeed, DEX given around the time of radiotherapy significantly diminishes its efficacy, suggesting that treatment with glucocorticoids or other anti-inflammatory or immunosuppressive drugs might decrease the clinical response of cancer patients to radiotherapy.

The stimulation of tumor-specific immunity by standard therapies including radio- and chemotherapy has been documented in several publications (Formenti and Demaria, 2012; Gupta et al., 2012; Matsumura et al., 2008; Reits et al., 2006; Sharma et al., 2013) and this phenomenon might actually be of great clinical importance: dormant metastases are intrinsically resistant to standard treatments that mainly target rapidly dividing cells but might still be susceptible to immune-mediated control (Koebel et al., 2007). The abscopal effect—a situation in which not only the irradiated tumor but also distant lesions show a clinical response—can be explained as such. When radiotherapy is combined with immune stimulation by anti-CTLA-4 antibodies, the abscopal effect becomes readily apparent (Postow et al., 2012; Verbrugge et al., 2014).

Our data expand the role of complement in the defense against tumors. Tumor-specific immunity is unleashed by locally produced anaphylatoxins in response to radiotherapy that activate DCs and then CD8⁺ T cells for optimal tumor control following radiotherapy.

EXPERIMENTAL PROCEDURES

Mice and Cell Lines

C57BL/6, C3^{-/-}, BALB/cJ, C5ar1^{-/-}, C3ar1^{-/-}, and Rag1^{-/-} mice were purchased from the Jackson Laboratory. C3^{-/-} mice were on a C57BL/6.129S4 background; C3ar1^{-/-} and C5ar1^{-/-} mice on a BALB/cJ background, Rag1^{-/-} mice were on a C57BL/6 background. We generated C5ar1^{-/-} C3ar1^{-/-} mice by crossing C5ar1^{-/-} and C3ar1^{-/-} mice. All mice were bred and maintained in specific pathogen-free facilities at the University of Zurich and University Hospital of Zurich. C57BL/6 \rightarrow C3^{-/-}, C3^{-/-} \rightarrow C57BL/6, C3^{-/-} \rightarrow C3^{-/-}, and C57BL/6 \rightarrow C57BL/6 bone-marrow chimeras were generated as previously described (Probst et al., 2003). All experiments were performed with age- and sex-matched mice in accordance with the guidelines of the Swiss federal and cantonal laws on animal protection.

B16F10 melanoma cells (ATCC) and B16F10-OVA (B16F10 stably transfected to express chicken ovalbumin as neo-antigen, provided by Melody

Swartz, EPFL) were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO Invitrogen), CT26 murine colon carcinoma (ATCC) cells in MEM medium (Invitrogen). Media were supplemented with 10% fetal calf serum, 5×10^{-5} M 2-mercaptoethanol, 10 mM sodium pyruvate 2 mM L-glutamine, and antibiotics.

In Vivo Experiments

2×10^5 B16F10-OVA, B16F10 or CT26 were injected s.c. in 100 μ l of a 1:1 mix of PBS and Matrigel Basement Membrane Matrix (BD Biosciences).

Local radiotherapy with a single dose of 20 or 5 Gy was performed at indicated time points using a Xstrahl 200 kV X-ray unit at 1 Gy/min as described (Gupta et al., 2012). Prior to radiotherapy, mice were anaesthetized by i.p. injection of 50 mg/kg ketamine and 10 mg/kg xylazine.

The C3aR1 antagonist (C3aRA) N2-[(2,2-Diphenylethoxy)acetyl]-L-arginine (SB290157, Calbiochem) was administered i.p. at 2 mg/kg body weight in 100 μ l PBS. Anti-C5aR1 mAb 20/70 (Hycult Biotech) or an isotype control (rat IgG2b anti-HLA-DR, clone SFRF8B6) was administered i.p. at 0.6 mg/kg in 100 μ l PBS (Baelder et al., 2005; Godau et al., 2004; Sun et al., 2011). C3aRA and anti-C5aR1 mAb were administered every second day starting at the day of radiotherapy until the end of the experiment.

To measure the cytokine production in vivo, we injected Brefeldin A (BFA, Sigma-Aldrich). A 20-mg/ml stock was prepared in DMSO. Further dilution to 0.5 mg/ml was made in PBS, and 500 μ l was injected i.p. 4 hr before mice were sacrificed (Liu and Whitton, 2005). DEX (Sigma-Aldrich) was administered daily at 0.3 mg/kg in PBS p.o., starting 1 day before radiotherapy. Tumors were measured with a caliper every 2–3 days in two dimensions (length and width). Excised tumors were weighed and processed for flow cytometry, histology, or isolation of RNA and proteins.

In Vitro Experiments

C57BL/6 and $C3^{-/-}$ mice were bled from the sublingual vein and sera were collected with Microtainer SST tubes (BD). Sera were diluted 1:10 with PBS, irradiated with 20 Gy (YXLON Y.SMART582, YXLON International GmbH, Hamburg, D) and subsequently incubated at 37°C. B16F10-OVA cells were cultured in 6-well plates (10^6 cells per well) and irradiated as described above. Immediately after irradiation, 60 μ l of serum from C57BL/6 or $C3^{-/-}$ mice diluted in 500 μ l of PBS was added to the cells. Control samples were not irradiated but treated similarly otherwise. Supernatants were taken 4 hr and 24 hr after irradiation and were stored at -80°C until further use.

Human Samples

Two sets of biopsies were provided by the Department of Dermatology, University Hospital Zurich. Patients signed informed consent. The ethical committee of the canton of Zurich approved this study (EK647). (1) Paired biopsies were collected from two patients with basal cell carcinoma and one with lentigo maligna melanoma immediately before and 24–36 hr after radiotherapy (1.5–2 Gy) and were snap frozen. (2) Four biopsies were collected from four patients (two with basal cell carcinoma, one with melanoma, and one with squamous cell carcinoma) 24–36 hr after radiotherapy (1.5–2 Gy) but without previous interventions and were snap frozen.

Statistical Analysis

Results are presented as mean \pm SD. Statistical significance was determined by ANOVA using GraphPad Prism 5 software (GraphPad). When multiple groups were compared, we used the Bonferroni post-test correction. When two groups were compared, we used the two-tailed Student's t test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.immuni.2015.03.009>.

AUTHOR CONTRIBUTIONS

L.S., A.K., A.G., and M.v.d.B. conceptualized the study and designed experiments; L.S. conducted the majority of the experiments; V.L. and A.B. per-

formed immuno-blot experiments; M.O. created heatmaps and performed bioinformatics analyses; A.O.F. and M.P. irradiated mice; V.C. sorted cells and performed some experiments; R.D. provided patient biopsies; H.J. and J.N. performed electron microscopy; and L.S. and M.v.d.B. wrote the manuscript.

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