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Glia-Selective Deletion of Complement *C1q* Prevents Radiation-Induced Cognitive Deficits and Neuroinflammation

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ABSTRACT

The adverse neurocognitive sequelae following clinical radiotherapy (RT) for central nervous system (CNS) malignancies are often long-lasting without any clinical recourse. Despite recent progress, the cellular mechanisms mediating RT-induced cognitive deficits (RICD) are poorly understood. The complement system is an immediate sensor of a disturbed inflammatory environment and a potent mediator of gliosis with a range of nonimmune functions in the CNS, including synaptic pruning, which is detrimental if dysregulated. We hypothesize that complement-mediated changes in glial cell function significantly contribute to RICD. The underlying alterations in CNS complement cascade proteins (*C1q*, *C3*), TLR4, and colabeling with glia (IBA1, GFAP) were examined using gene expression, immunofluorescence, and *in silico* modeling approaches in the adult mouse brain following 9 Gy cranial RT. Three-dimensional volumetric quantification showed elevated molecular signatures of gliosis at short- and long-term post-RT times. We found significant elevations in complement *C1q*, *C3*, and TLR4 post-RT accompanied by increased colabeling of astrocytes and microglia. To address the mechanism of

RT-induced complement cascade activation, neuroinflammation, and cognitive dysfunction, we used a genetic approach—conditional, microglia-selective *C1q* (Flox) knockdown mice—to determine whether a glia-specific, upstream complement cascade contributes to RICD. *C1q*-Flox mice exposed to cranial RT showed no cognitive deficits compared with irradiated WT mice. Further, irradiated *C1q*-Flox mice were protected from RT-induced microglial activation and synaptic loss, elevation of anaphylatoxin *C5a* receptor, astrocytic-*C3*, and microglial-TLR4 expression in the brain. Our findings demonstrate for the first time a microglia-specific mechanism of RICD involving an upstream complement cascade component, *C1q*.

Significance: Clinically-relevant radiotherapy induces aberrant complement activation, leading to brain injury. Microglia-selective genetic deletion of CNS complement *C1q* ameliorates radiation-induced cognitive impairments, synaptic loss, and neuroinflammation, highlighting the potential for *C1q* as a novel therapeutic target.

See related commentary by Korimerla and Wahl, p. 1635

Introduction

Clinical radiotherapy (RT) remains a first-line treatment for primary and metastatic brain cancers, along with surgery and chemotherapy. Unfortunately, longer-term survival comes at a cost, as there are now numerous clinical (1, 2) and preclinical studies (3) that have established the adverse side effects of such treatment on cognition without any clinical recourse, imposing significant socioeconomic burdens and impacting long-term quality of life (4). Therefore, cellular mechanisms and strategies for preventing RT-induced cognitive deficits (RICD) following cancer treatments such as cranial RT are clearly needed. Preclinical studies conducted by us and others have found that the irradiated central nervous system (CNS) exhibits robust and dynamic cycles of secondary reactive processes that follow complex

temporal dynamics (5, 6). Many of these changes can be linked to increased ROS (6), reduced hippocampal neurogenesis, damaged neuronal morphology, and elevated neuro-inflammation involving a range of cytokines along with persistent microglial activation (7–10). Although these factors are likely to disrupt CNS functionality, our current knowledge regarding the cellular mechanisms of RT-induced brain injury and neurocognitive sequelae are limited. Because cranial RT causes microglial activation, we hypothesized that detrimental change in the glial function leads to long-term cognitive dysfunction. Astrocytes form complex astroglial networks and modulate synaptic plasticity and network activity through the tripartite synapse (11). Disruption of astrocytic function following irradiation or brain injury may cause an imbalance in the global homeostasis of the brain, leading to functional deficits (12). Our data and reports in the literature have shown that radiation-induced elevation in inflammatory cytokines and reduced synaptic adenosine accompanied by astroglial gliosis are biochemical indicators of RICD (13–15). In addition, persistent activation of microglia is implicated in chronic neurodegeneration and spine loss in the irradiated brain (7, 16, 17). Thus, perturbations in glial function significantly contribute to RICD.

The complement system is comprised of over 40 proteins. Activation of the complement system generates powerful effector molecules of the immune system that protect tissues from infection, and also play important roles during development by synaptic pruning (18). Yet, an imbalance in complement cascades can also provoke a proinflammatory response in many degenerative conditions including rheumatoid arthritis, traumatic brain injury (TBI), and Alzheimer's disease (AD; refs. 18, 19). *C1q* synthesis is elevated as an early response to a variety of neurodegenerative conditions in both humans and rodents (19).

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C1q Knockdown Ameliorates RT-Induced Cognitive Dysfunction

Predominant microglial synthesis of C1q affords a reparative role of microglia to maintain normal brain function. Conversely, activation of C1 in complex with serine proteases (C1r, C1s) initiates an enzymatic cascade that generates C3a and C5a, both of which can mediate inflammation that is associated with synapse and neuronal loss, and cognitive dysfunction (20–22). As such, neuronal injury or radiation-induced activation of the complement cascade can potentially trigger collateral proinflammatory polarization, resulting in damaging microglial activation (15). Past studies from our laboratory have established the damaging role of radiation-induced chronic, elevated microglial activation in the irradiated adult brain (14, 16, 17). Astrocytes also express C1q under pathologic conditions including multiple sclerosis (23), temporal lobe epilepsy (24), and AD (25). On the basis of these observations, we hypothesize that cranial RT-induced aberrant activation of the CNS complement cascade contributes to brain injury. Using gene expression, dual-immunofluorescence, and *in silico* three-dimensional (3D) reconstruction and volumetric quantification approaches, this report addresses detrimental neuro-inflammatory consequences of complement activation in the irradiated brain. In addition, our data show that conditional, microglia-selective deletion of C1q in the mouse brain is neuroprotective against the detrimental neurocognitive impact of cranial irradiation, establishing a mechanistic link between RT-induced complement cascade activation and cognitive dysfunction.

Materials and Methods

Detailed methods are provided in the Supplementary Information section.

Animals, cranial irradiation, and tissue analysis

All animal use procedures and safety protocols were approved by UCI Institutional Animal Care and Use Committee and were consistent with NIH guidelines. Adult (5- to 6-month-old) male WT mice (C57Bl/6J) were randomly divided into two groups: 0 or 9 Gy head-only irradiation using a ^{137}Cs - γ irradiator at the dose rate of 1.2 Gy/min with shielding to the eyes, body, and the cerebellum. A recent study showed that the adult female brain is protected from RICD and neuronal damage (26), thus, as a proof of principle, we have used male mice to determine the detrimental consequences of cranial RT on complement cascade. Mice were divided into the following experimental groups (8–10 mice/group): 2 hours, 24 hours, 48 hours, 1-, 2-, 3-, and 4-weeks post-irradiation. An additional, 6-month post-RT group was also included for the gene expression analysis. Concurrent sham-irradiated controls were included at each time point. At the select post-RT times, brains were collected after intra-cardiac perfusion, fixed using 4% paraformaldehyde (PFA) and cryo-sectioned (30 μm , coronal). For gene expression analysis, the micro-dissected hippocampus was flash-frozen and stored at -80°C until analyzed. For RT-PCR, total mRNA was extracted from micro-dissected hippocampi (3–6 mice/group). Primers and experimental conditions for gliosis markers were optimized and followed as described previously (Supplementary Table T1; Supplementary Information; ref. 27). Gene expression data are presented as $2^{-\Delta\Delta\text{Ct}}$ values.

C1q-flox mice and behavior testing

Microglia-selective deletion of the C1qa gene was achieved by crossing $\text{C1qa}^{\text{FL/FL}}$ and microglia-specific deleter strain ($\text{Cx3cr1}^{\text{CreERT2/Wganf}}$) to generate $\text{C1qa}^{\text{FL/FL};\text{Cx3cr1}^{\text{CreERT2/Wganf}}}$ (C1q-Flox) mice as described previously (28). We have shown previously that C1q-Flox display CNS

and microglia-specific depletion of C1q, without the need of tamoxifen treatment, by the age of 8 weeks. The ablation of the C1qa gene did not impact peripheral or circulating C1q levels, thereby enabling the mechanistic determination of CNS C1q-mediated events in the irradiated brain. Adult (4-months old, male) littermate control (WT) and C1q-Flox mice were randomly divided into 0 or 9 Gy head-only irradiation groups (12–16 mice/group). Our past studies have shown that the group size of 8 to 10 mice/group for behavior and 4 to 6 mice/group for the molecular or tissue analyses achieved sufficient power ($\alpha = 0.05$) to reach the statistical significance (15, 16, 29). One-month post-RT, animals were administered a light–dark box (LDB) test to assess anxiety and a spatial memory testing using the novel place recognition (NPR) task as described previously (30, 31). The NPR task depends on animal's inherent preference to explore a novel location of an object and relies on intact hippocampal function (32, 33). Time spent interacting with the familiar versus novel object location was recorded and the data calculated as the discrimination index (DI): $([\text{Novel location exploration time}/\text{Total exploration time}] - [\text{Familiar location exploration time}/\text{Total exploration time}]) \times 100$. For the LDB, the time spent in light and dark chamber and number of transitions between compartments was assessed. A separate cohort of mice was also administered the fear extinction memory test as described (see Supplementary Information). All tests were scored by observers blind to the experimental groups to avoid bias. After conclusion of behavior testing, mice were euthanized via intra-cardiac perfusion and brains were fixed using 4% PFA for IHC.

Dual-immunofluorescence staining, confocal microscopy, 3D algorithm-based volumetric quantification, and cytokine analysis

PFA-fixed brains from each treatment group underwent dual-immunofluorescence staining (3–4 sections/brain, 6–8 brains/group) including GFAP, IBA1, C1q, C3, C5aR1, TLR4, CD68, synaptophysin, and SV2a. We used unbiased, 3D blinded deconvolution and volumetric quantification of glial surfaces, complement, and receptor puncta using AutoQuant X3 and Imaris modules. Details of primary and secondary antibodies, immunostaining, and *in silico* modeling are provided in the Supplementary Information. We did not observe significant differences between control groups (0 Gy) from each post-irradiation time point (2 hours to 4 weeks); thus, pooled control data from 48 hours and 4 weeks were used for all the comparisons. Data were expressed as mean percent immunoreactivity relative to the unirradiated control group. For the cytokine analysis, freshly dissected hemi-brains (3–6 brains/group) were homogenized in presence of protease inhibitor, centrifuged, and supernatants were assayed for cytokines using a Magnetic Bead-Based Customized Kit (Thermo Fisher Scientific).

Data analysis

Statistical analyses were performed using one-way ANOVA to confirm overall significance (GraphPad Prism, v8.0, RRID: SCR_002798). All data are expressed as the mean $\pm$ SEM. For comparisons between the controls (0 Gy) and irradiated groups, nonparametric, two-tailed unpaired *t* tests were performed with Holm–Sidak's correction. For the analysis of C1q deletion and cranial irradiation, two-way ANOVA along with Bonferroni's multiple comparisons test were performed. The Kruskal–Wallis test was used for the analysis of cytokine and synaptic proteins data. Wilcoxon matched-pairs signed rank test was used to compare exploration of familiar versus novel places by the same animals. All analyses considered a value of $P \leq 0.05$ to be statistically significant.

Results

Cranial irradiation elevates astrogliosis

To determine the impact of cranial RT on the hippocampal neuroinflammation status as a function of time, gene expression (RT-PCR, **Fig. 1A**) for gliosis and neuroinflammation-related genes and astrocytic morphology were characterized at early and delayed post-irradiation times (**Fig. 1B** and **C**). A subset of these genes and gene products are shown to be linked with inflammation-mediated microglial and astrocytic cross-talk, leading to synaptic damage (e.g., IL1a, Lcn2, Serpin1, Serpin3n, Amigo2, C1q, and Tnfa; ref. 27). RT significantly increased expression (2- to 6-fold) of gliosis-related genes Serpin1, P21, Amigo2, Tnfa, and C1q at early post-RT times (24 hours to 1 week, **Fig. 1A**). IL1a, Lcn2, and Serpin3n were increased (1.5- to 3-fold) at the delayed 6-month post-RT time, indicating a long-term effect of cranial RT that coincides with cognitive impairments (16, 17). Lcn2 expression was also reduced at 48 hours and 1 month post-RT. To determine the dynamics of astrocytic morphology as a function of time post-RT, the volume and area of individual astrocytes were quantified using 3D algorithm-based blinded deconvolution of high-resolution confocal z-stacks and unbiased volumetric quantification of reconstructed GFAP⁺ astrocytic surface (**Fig. 1B** and **C**). We found 1.3- to 1.7-fold increases in astrocytic immunoreactivity in the hippocampus as early as 48 hours that remained elevated at 4 weeks post-RT (**Fig. 1B**). The morphology of astrocytes showed longer and thicker processes in the irradiated brain compared with controls that is characteristic of astrocytic hypertrophy (**Fig. 1C**; ref. 34). Together, analysis of gene expression and astrocytic morphology indicated RT-induced persistent gliosis, which is coincident with cognitive deficits (16, 17), indicating elevated neuroinflammation.

Radiation exposure increases glial levels of complement C1q

In the murine brain, resident microglia are the major source of C1q (28). Moreover, C1q also colocalized with astrocytes in multiple sclerosis (23), temporal lobe epilepsy (24), and AD (25, 35). Interestingly, the status of C1q has not been characterized in context of normal tissue responses following cranial RT. Therefore, we characterized the time-dependent microglial (**Fig. 2**) and astrocytic (**Fig. 3A** and **B**) immunoreactivity of C1q at early and delayed post-irradiation times in the hippocampus. RT elicited a 2-fold increase in the total immunoreactivity of C1q at 2 and 24 hours post-exposure ($P < 0.01$ and 0.001 , respectively; **Fig. 2A**) that reached a peak level at 48 hours (2- to 3-fold, $P < 0.02$). However, by 2 to 3 weeks post-RT, the gross C1q immunoreactivity dropped to control levels and to less than control levels by 4 weeks post-irradiation (63%, $P < 0.01$). The volume of IBA1⁺ microglial immunoreactivity did not change except decreases at 48 hours and 2 weeks post-exposure (66% and 73% of controls, respectively; $P < 0.02$; **Fig. 2B**). Importantly, volumetric quantification of C1q⁺ puncta colocalized within 0.5 μ m of IBA1⁺ microglial surfaces indicated a 1.3-fold increase at 2 hours post-irradiation ($P < 0.01$, **Fig. 2C**). The colabeling with IBA1⁺ microglia reached a peak level at 48 hours post-RT (2.3-fold, $P < 0.001$) and then declined at 1 through 3 weeks (1.3- to 1.6-fold) and dropped below the control level by 4 weeks post-exposure (67%, $P < 0.02$; **Fig. 2C** and **D**). Concurrently, quantification of C1q⁺ puncta colocalized with GFAP⁺ astrocytic surfaces (**Fig. 3A**) followed a similar pattern as that observed for microglia. Increased colabeling of C1q with GFAP⁺ astrocytes was observed at 2 hours post-exposure that peaked between 24 to 48 hours ($P < 0.002$) and then declined in weeks 1, 2, and 3 post-RT ($P < 0.002$) before returning to control levels at 4 weeks post-exposure. As shown above (**Fig. 1C**), astrocytic hypertrophy was also evident in the C1q⁺

GFAP⁺ ramified processes of astrocytes in the irradiated brain (**Fig. 3B**).

Elevated astrocytic expression of complement C3 in the irradiated brain

In the brain, complement C3 is primarily expressed by astrocytes (36–38) and a recent report suggested that complement receptor 3 (CR3) knockout mice were protected against radiation-induced neuronal damage (26). Thus, we characterized the astrocytic expression of complement C3 via C3d chain immunoreactivity in the hippocampus following cranial RT as a function of time (**Fig. 3C–E**). We found a significant elevation in total C3 protein levels as early as 2 hours that remained elevated for over 4 weeks post-irradiation (2.5-fold, $P < 0.002$; **Fig. 3C**). Quantification of astrocyte-specific C3 similarly showed a 2.5- to 3.5-fold increase in expression from 2 hours to 4 weeks post-radiation exposure ($P < 0.002$; **Fig. 3D**). Typical C3⁺ immunofluorescent puncta were colocalized with the GFAP⁺ astrocytic processes in both control and irradiated cohorts (**Fig. 3E**).

Increased glial expression of TLR4 in the irradiated brain

Toll-like receptor 4 (TLR4) plays important roles in innate and acquired immune responses, particularly triggering microglial activation in the brain (39). The foregoing data showed peak increase in complement proteins (C1q, C3; **Figs. 2** and **3**) and astrogliosis (**Fig. 1B**) at 48 hours post-exposure. We showed a correlation between microglial activation and RICD 1 month later (16). On the basis of these observations, we assessed TLR4 at key post-irradiation time points 48 hours and 1 month (**Fig. 4**). We found a 2-fold elevation in total TLR4 immunoreactivity in the hippocampus at 48 hours and 1-month post-irradiation ($P < 0.04$; **Fig. 4A**). Further, quantification of glial-specific TLR4 indicated a 3.5- to 4-fold increase in microglial and astrocytic TLR4 expression (IBA1⁺ and GFAP⁺, respectively; $P < 0.001$; **Fig. 4B** and **C**) at 48 hours post-exposure. TLR4 expression remained high in astrocytes (3-fold, $P < 0.001$) and microglia at 1-month post-irradiation (3- and 1.5-fold, respectively; $P < 0.001$). Typical TLR4⁺ immunofluorescent puncta were colocalized with the IBA1⁺ microglial and GFAP⁺ astrocytic processes in both control and irradiated cohorts (**Fig. 4D** and **E**). Such persistent TLR4 elevation may contribute significantly to glial activation in the irradiated brain.

In summary, these data describing the time-dependent dynamics of astrogliosis (**Fig. 1**), complement component C1q and C3 (**Figs. 2** and **3**), and TLR4 (**Fig. 4**) expression in the irradiated brain clearly indicate elevated neuroinflammation. Radiation-induced neuroinflammation has been shown to be detrimental to neuron and synaptic structure and cognitive function (17). Our new data on astrogliosis and complement activation thus provide functional relevance to our past data showing that cranial RT (9 Gy)-induced significant impairments in cognitive function and microglial activation (CD68; ref. 16). Altogether, these data strongly suggest that RT-induced activation of the CNS classical complement cascade triggers neuroinflammation leading to functional impairments in the CNS.

Microglia-selective deletion of C1q prevents RT-induced cognitive impairments

To determine whether RT-induced CNS complement cascade activation leads to cognitive decrements, microglia-specific *C1q* knockdown mice (*C1q*-Flox) were exposed to cranial irradiation (**Fig. 5A**). *C1q*-Flox mice show a complete depletion of CNS C1q in the absence of tamoxifen by the age of 8 weeks without alteration in circulating C1q (28). Analysis of *C1q* expression (qPCR) in the

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Figure 1.

Cranial irradiation elevates expression of complement and inflammation-related genes and astroglia. **A**, Microdissected hippocampi from the irradiated (IRR) and control mice were analyzed for mRNA levels at early (24 hours, 48 hours, and 1 week) and delayed (1 and 6 months) post-irradiation times. Elevated expression for inflammation (IL1a, C1q, Tnfa, Tlr4), gliosis (Lcn2, Serping1, Serpina3n, Amigo2), and apoptosis (P21)-related genes was found at short-term post-irradiation. **B**, Volumetric analysis of GFAP⁺ hippocampal astrocytes (dentate hilus, dentate gyrus, and CA1) showed elevated immunoreactivity at early (2, 24, and 48 hours) and delayed (1, 2, 3, and 4 weeks) post-IRR as compared with controls, indicating ongoing astroglia. **C**, 3D algorithm-based z-stack deconvolution, surface reconstruction, and volumetric quantification of GFAP⁺ astrocytes showed thicker and longer stela, indicating IRR-induced astrocytic hypertrophy. Mean $\pm$ SEM (3–4 observations per group, **A**; 6–8 observations per group, **B**). *P* values were derived from one-way ANOVA, unpaired, two-tailed *t* test with Holm-Sidak's correction. *, *P* $\leq$ 0.05; **, *P* $<$ 0.03; ***, *P* $<$ 0.01 vs. control. Scale bar, 5 μ m.

hippocampus showed nearly complete ablation (<2–4%) in the 0 or 9 Gy irradiated C1q-Flox groups (Fig. 5B). One-month post-irradiation, C1q-flox and littermate control (WT) mice were habituated and tested on the hippocampal-dependent NPR task (Fig. 5C and D). Comparison of the exploration of familiar and novel location exploration times revealed significant differences between the WT (0 Gy, *P* = 0.0001), C1q-Flox (0 Gy, *P* = 0.02), and irradiated C1q-Flox (*P* = 0.001) mice, but not for the WT mice exposed to cranial irradiation (WT-IRR), indicating reduced exploration of the novel spatial location (Fig. 5C). We also found significant overall group differences between the cohorts for the discrimination index (Fig. 5D; $F_{(3, 48)} = 6.31$, *P* = 0.001). Irradiated WT mice (WT-IRR) showed a significantly reduced

preference for the novel location compared with the 0 Gy WT (*P* = 0.001), 0 Gy C1q-Flox (*P* = 0.02), and irradiated C1q-Flox (*P* = 0.001) mice. Importantly, irradiated C1q-Flox mice did not show reductions in the discrimination index and the novel place exploration was comparable with the unirradiated WT and C1q-Flox mice. In addition, a significant interaction for the genetic knockdown of C1q was found ($F_{(1, 48)} = 8.42$; *P* = 0.01). For the LDB test, we did not find significant overall differences between groups for the time spent exploring the open compartment (Supplementary Fig. S1). Moreover, for the fear extinction test, WT-IRR mice showed elevated freezing compared with WT-0 Gy mice (*P* = 0.03, Supplementary Fig. S2). Interestingly, both 0 and 9 Gy irradiated C1q-Flox mice showed elevated freezing on

Figure 2.

Elevated microglial expression of complement component C1q in the irradiated brain. **A**, 3D algorithm-based volumetric quantification of the total overall immunoreactivity of C1q⁺ puncta in the irradiated hippocampus (dentate hilus, dentate gyrus, and CA1) showed increased C1q short-term post-exposure (2–24 hours) that reached the peak at 48 hours and subsequently declined to control levels by 3 weeks post-irradiation. **B**, Radiation exposure reduced IBA1⁺ microglial immunoreactivity at 48 hours and 2-week post-exposure. **C**, Volumetric analysis of IBA1⁺ microglia showed a significantly elevated expression of complement component C1q from 2 to 48 hours post-irradiation that gradually declined from 1 to 4 weeks post-irradiation. **D**, The confocal z stacks (top) for microglia (IBA1, red) and C1q (green), and 3D surface and spot reconstruction (bottom) for the microglial (IBA1, red) colabeling with C1q (sky blue) are shown for the control and irradiated groups at the 48 hours time point. Data are presented as mean $\pm$ SEM ($N = 4$ –8 observations per group). P values were derived from one-way ANOVA and unpaired, two-tailed t test with Holm-Sidak's correction. *, $P < 0.02$; **, $P < 0.01$; ***, $P < 0.001$ compared with control. Scale bar, 5 μ m (**D**).

the extinction test ($P = 0.03$ and 0.001 respectively; Supplementary Fig. S2). In summary, genetic depletion of microglial C1q is neuroprotective against cranial radiation-induced, hippocampal-dependent, cognitive impairments.

Microglial C1q knockdown reduced neuroinflammation

To determine if microglia-specific knockdown of *C1q* prevents radiation-induced neuroinflammation, we evaluated cytokine levels (Fig. 5E), CD68 (microglial activation marker; Fig. 6A), C5aR1 (a downstream complement or anaphylatoxin receptor; Fig. 6B), astrocytic C3 (GFAP-C3; Fig. 6C), and microglial TLR4 (IBA1-TLR4; Fig. 6D). Exposure to cranial RT significantly increased levels of proinflammatory cytokines in the WT-IRR brains (e.g., TNF α , IL1 β , IL6, IL1 α , CCL2, and IL18; Fig. 5E) as compared with irradiated C1q-Flox mice. Further, we found significant overall group effects for the quantification of CD68 ($F_{(3, 20)} = 33.57$, $P < 0.0001$). CD68 expression in the WT-IRR group was significantly greater than that of the 0 Gy WT, 0 Gy C1q-Flox, and

irradiated C1q-Flox mice ($P < 0.0001$; Fig. 6A). A significant overall group effect was found for C5aR1 immunoreactivity ($F_{(3, 28)} = 13.52$; $P < 0.0001$). C5aR1 expression was significantly elevated in the WT-IRR hippocampus compared with WT-0 Gy ($P = 0.002$), C1q-Flox 0 Gy ($P = 0.0001$), and C1q-Flox IRR ($P = 0.002$) groups (Fig. 6B). Radiation exposure did not increase CD68 or C5aR1 in C1q-Flox mice. Cranial irradiation significantly elevated astrocytic colabeling of C3 in the WT-IRR brains ($P = 0.001$), whereas C3 expression was comparable in the WT-0 Gy, C1q-Flox 0 Gy, and C1q-Flox IRR mice. Similarly, quantification of microglial TLR4 immunoreactivity indicated radiation-induced elevation in the WT-IRR group ($P = 0.04$ and 0.002 compared with WT-0 Gy and C1q-Flox 0 Gy, respectively). Conversely, irradiated C1q-Flox mice showed a significant decrease in microglial TLR4 expression compared with the WT-IRR group ($P = 0.04$). Overall, these data show that microglia-specific deletion of *C1q* prevented cranial RT-induced neuroinflammation and gliosis that was coincided with intact cognitive function.

C1q Knockdown Ameliorates RT-Induced Cognitive Dysfunction

Figure 3.

Increased glial C1q and C3 colabeling in the irradiated brain. **A**, Quantification of C1q⁺ puncta on the surface of GFAP⁺ astrocytes showed radiation-induced elevation in the hippocampus (dentate hilus, dentate gyrus, and CA1) at 2 and 24 hours post-exposure that peaked at 48 hours and declined with time to the control levels by 4 weeks post-irradiation. **B**, The confocal z stacks (top) for astrocytes (GFAP, red) and C1q (green), and 3D surface and spot reconstruction (bottom) for the astrocytic C1q (GFAP, red; C1q white) are shown for the control and irradiated groups at the 48 hours time point. **C**, Volumetric quantification of the overall total C3 immunoreactivity (C3d) in the irradiated hippocampus showed consistently increased C3 expression levels at all post-exposure times. **D**, Volumetric analysis of GFAP⁺ astrocytes showed significantly elevated levels of complement component C3 at all post-irradiation times. **E**, The confocal z stacks (top) and 3D surface and spot reconstruction for astrocytic C3 (GFAP, magenta; C3, green) are shown for the control and irradiated groups at the 48 hours time point. Data are presented as mean $\pm$ SEM ($N = 5-8$ observations per group). P values were derived from one-way ANOVA and unpaired, two-tailed t test with Holm-Sidak's correction. *, $P < 0.002$; **, $P < 0.001$ compared with control. Scale bar, 5 μ m (**B** and **E**).

C1q knockdown protects against radiation-induced synaptic loss

C1q has been shown to play an important role in synapse elimination in pathologic conditions (18). To determine the downstream impact of deleting microglial C1q expression in the irradiated brain, we quantified immunoreactivity of synaptic markers synaptophysin and SV2a (Fig. 7A and B). Exposure to cranial RT significantly reduced synaptophysin and SV2a immunoreactivity in the CA1 stratum radiatum (Fig. 7, a1-a4 and b1-b4) in WT-IRR mice compared with the unirradiated WT controls ($P = 0.01$ and 0.03 , respectively). In contrast, irradiated C1q-Flox mice did not show decreased synaptophysin or SV2a immunoreactivity,

indicating a neuroprotective role of CNS C1q ablation against radiation-induced synaptic loss.

Discussion

Over the past two decades, numerous studies have shown cranial RT-induced early and delayed neuropathologies, which culminate in long-term cognitive impairments (3, 7) linked with elevated neuroinflammation (7, 16, 17, 40-42). This study sought to address the underlying radiation-induced proinflammatory signaling mechanisms, leading to the brain injury. In the CNS, complement factors C1q and C3 serve not only as mediators of innate immunity, but also

Figure 4.

Glial expression of TLR4 is increased in the irradiated brain. **A**, Volumetric quantification of the overall TLR4 immunoreactivity in the irradiated hippocampus (dentate hilus, dentate gyrus, and CA1) showed increased TLR4 at early (48 hours) and delayed (4 weeks) post-exposure times. **B** and **C**, Volumetric analysis of IBA1⁺ microglia and GFAP⁺ astrocytes showed a significantly elevated glia-specific TLR4 expression at 48 hours and 4 weeks post-irradiation. **D** and **E**, The confocal z stacks (top) and 3D surface and spot reconstruction for microglial (IBA1, green; **D**) and astrocytic (GFAP, green; **E**) colabeling with TLR4 (white) are shown for the control and irradiated groups at the 48 hours time point. Data are presented as mean $\pm$ SEM ($N = 5-8$ observations per group). P values were derived from one-way ANOVA and unpaired, two-tailed t test with Holm-Sidak's correction. *, $P < 0.04$; **, $P < 0.001$; ***, $P < 0.0001$ compared with control. Scale bar, 8 μ m.

coordinate microglia-mediated synapse pruning during circuit development (18, 43). In the diseased or injured state, activation of the complement system is linked with the loss of spines and increased neuroinflammation (6–9). To the date, two studies have shown the adverse effects of hippocampal C3 or CR3 signaling in radiation-induced brain injury models (26, 44). We characterized radioresponse of complement C1q and C3 that may, in presence of injured cells, induce tagging of damaged or “weak” synapses for pruning or elimination by microglia and trigger long-term neuroinflammation (via C3a or C5a). Importantly, microglia-selective deletion of the upstream complement C1q allowed us to study the CNS-specific contribution of classical complement cascade activation in the RICD, neuroinflammation, and synaptic loss.

Radiation exposure induces astrogliosis

Preclinical experimental models and clinical cases have provided sporadic evidence that astrocytes play a role in neuropathologic

conditions (27, 45). However, unlike microglial activation, astrogliosis is still an understudied area of research in CNS radiobiology. Our past data and the literature have shown that radiation-induced elevation in inflammatory cytokines, and reduced synaptic adenosine accompanied by astrogliosis, are functional indicators of radiation-induced brain injury and cognitive deficits (5, 14). Moreover, Barres and colleagues showed that microglia-mediated astrocytic activation via C1q, IL1 α , and TNF α upregulated the production of C3 and neurotoxins that were destructive to synapses (27). The molecular signatures of astrogliosis were substantiated by acute and delayed alterations morphologic characterization of individual astrocytes (Fig. 1B), as illustrated by elongation and thickening of the astrocyte intermediate filaments (GFAP). Acute reactive astrogliosis observed within hours after irradiation is thought to be neuroprotective by repairing BBB integrity, detoxifying ROS, reducing peripheral immune cell infiltration and slowing down neurodegeneration (34). However, persistent astrogliosis at delayed times has been shown to be neurotoxic where

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Figure 5.

Microglia-selective deletion of complement C1q protect against radiation-induced cognitive impairments and elevated proinflammatory cytokines. **A**, Four-month-old C1q-Flox (*C1qa^{FL/FL}.Cx3cr1^{CreERT2}*) and littermate control (WT) mice were exposed to 0 or 9 Gy cranial irradiation and one month post-irradiation mice were administered hippocampal-dependent NPR task. **B**, Quantification of C1q expression (qPCR) *in vivo* showed a significant reductions (96–98%, $P = 0.0001$) in the C1q-Flox 0 and 9 Gy irradiated brains. **C**, Irradiated (IRR) WT mice spent significantly less time exploring novel spatial location. Time spent exploring the novel placements of objects during the test phase of the NPR task showed that 0 Gy WT, 0 Gy C1q-Flox and irradiated C1q-Flox mice spent significantly more time exploring novel versus familiar location, whereas irradiated WT mice spent comparable time exploring both locations. **D**, The tendency to explore novel spatial location was derived from the Discrimination Index, calculated as [(Novel location exploration time/Total exploration time) – (Familiar location exploration time/Total exploration time)]

elevated levels of proinflammatory cytokines, complement components, and activated microglia limit regenerative neuronal, synaptic, and glial responses.

We characterized *in vivo* gliosis, inflammation, and apoptosis/cell-cycle arrest (P21)-related gene expression in the hippocampus from 24 hours to 6 months post-irradiation (**Fig. 1**). We found increased expression of Serping1, Amigo2, P21, TNFα, and C1qa genes at early (24 hours to 1 week) times, suggesting an early trigger for radiation-induced inflammation. Serping1 (encoding C1-INH) regulates complement cascade activation by inhibiting activated C1r and C1s (46). A recent report showed that knockdown of circulating C1-INH caused upregulation of CNS C1-INH that led to neurovascular impairment and loss of BBB integrity, elevated astrogliosis, and hippocampal-dependent cognitive deficits (47). The latter two degenerative characteristics have also been observed in the irradiated brain (14, 16). Amigo2 regulates the PDK1–Akt signaling pathway that regulates cell survival, adhesion, neurite development, axon tract formation, and angiogenesis (48). In the irradiated hippocampus, elevated expression of Serping1 and Amigo2 (24 hours post-RT) was synchronized with increased P21 and C1q genes, suggesting an adaptive CNS response that may facilitate the removal of apoptotic cells, cellular debris, and facilitate repair of the irradiated tissue bed at 24 hours post-exposure time, but at 48 hours elicits persistent and damaging inflammation (TNFα). Elevated classical complement pathway proteins (C1, C4, and C3) were implicated in neurodegeneration, synapse elimination, and cognitive impairments (43). Our present and past studies on late CNS radio-responses (16, 17) found similar increases in proinflammatory gene expression and gliosis along with behavioral deficits at 1 and 6 months post-irradiation respectively, linking CNS activation of the complement cascade with cognitive impairment. In fact, we found long-term hippocampal elevation in cytokine IL1α and lipocalin 2 (Lcn2). Secretion of Lcn2 by reactive astrocytes was shown to damage neurons and elicit neurodegeneration (49). McBride and colleagues characterized a sharp elevation in proinflammatory cytokines including TNFα and IL1α 4 hours post-exposure to high acute dose cranial-RT (25 Gy) with recurrent cyclic upregulation between 48 hours and 1 month post-RT (5, 50). We also found elevated proinflammatory cytokines (TNFα, IL1β, IL6, IL1α, CCL2, and IL18; **Fig. 5E**) 1 month post-RT, coinciding with RICD. Those data provided premise to characterize time-dependent changes in gliosis and complement cascade proteins at early post-irradiation times. While the resultant short- and long-term gene expression profiles (**Fig. 1A**) remain to be validated at the protein level, they do suggest a radiation-induced proinflammatory astrocytic response that may lead to neurotoxic events *in vivo*.

Taken together, our new data and the literature suggest two key points in the context of the CNS radio-response: (i) early upregulation of inflammation, apoptosis, complement-related gene

× 100. Cranial irradiation significantly impaired cognitive function in the WT mice as shown by the reduced preference towards the novel place compared with the 0 Gy WT, 0 Gy C1q-Flox, and irradiated C1q-Flox mice ($P = 0.01$; $P = 0.02$; $P = 0.001$, respectively). Importantly, irradiated C1q-Flox mice did not show a decline on the spatial exploration. **E**, Multiplex quantification of cytokines showed radiation-induced elevations in the proinflammatory factors TNFα, IL1β, IL6, IL1α, CCL2, and IL18 in the WT-IRR brains, whereas irradiated C1q-Flox mice did not show increases in the cytokine levels. Data are presented as mean ± SEM ($N = 4$ observations per group, **B**; $N = 16$ observations per group, **C** and **D**; $N = 3$ to 5 observations per group, **E**). P values were derived from Kruskal–Wallis test (**B** and **E**), Wilcoxon-matched pairs signed rank test (**C**) and, two-way ANOVA and Bonferroni's *post hoc* test (**D**). NS, not significant; *, $P = 0.03$; **, $P = 0.02$; ***, $P = 0.01$; ****, $P = 0.001$; ###, $P = 0.0001$.

expression, and gliosis are suggestive of an adaptive, reparative radio-response, but that (ii) persistent inflammation and reactive astrogliosis are detrimental responses to RT that may lead to functional deficits in cognition.

Radiation elevates glial colabeling of complement cascade proteins

We found a sharp elevation in C1q immunoreactivity in the early phases (hours) of the radiation response, which preceded by 1 month (Fig. 2), consistent with mRNA expression (Fig. 1). Our past genetic study using the microglia-selective C1q-Flox mouse, with intact peripheral C1q levels, showed that resident microglia are the prominent source of C1q in the CNS (28). Indeed, dual staining for IBA⁺ and microglial C1q showed similar patterns of C1q upregulation that peaked at 48 hours and declined by 1 month post-irradiation. These data indicate that radiation-induced complement activation may synergize with elevated cytokine signaling at a short-term (2–48 hours) post-irradiation interval. Radiation-induced brain injury may also induce activation of other complement proteins, including C3a, iC3b, and C5a, leading to the proinflammatory polarization of microglia and astrocytic hypertrophy. We found elevated C3 in the irradiated brain (Fig. 3) that coincides with elevated expression of the danger recognition receptor TLR4 and proinflammatory cytokine signaling (Fig. 4 and microglial activation in the short-term and, subsequently long-term cognitive deficits (1 month; Fig. 5C and D). These data suggest RT-induced complement cascade activation lead to long-term inflammatory injury.

Interestingly, we found elevated C1q also associated with astrocyte intermediate filaments (GFAP, Fig. 3). Recent studies using LPS-induced CNS insult have suggested that microglia-mediated astrogliosis via C1q, IL1a, or TNF α increased the production of C3 and neurotoxins that were destructive to synapses (27). Similarly, we and others have shown persistent microglial activation that may share some commonalities with the LPS-induced inflammatory cascade (7, 17, 41). C3 gene expression was found to be elevated following 20 Gy RT in the juvenile brain (51), but genetic C3 knockout prevented radiation-induced learning disabilities and gliosis (44). Further, Hinkle and colleagues showed neuroprotective effects of the CR3 knockdown against radiation-induced damage to neuron structure, spine loss, and microglial activation (26), further suggesting a neurotoxic role of iC3b in RICD. A caveat of this study is that the global knockdown of CD11b gene to produce mutant CR3 that does not allow mechanistic characterization of peripheral- versus CNS-specific effects of complement cascade-mediated signaling *in vivo*. Nonetheless, this study revealed that radiation-induced detrimental complement signaling leads to synaptic damage. In total, radiation-induced glial elevation in complement cascade proteins are linked with cognitive impairments and neuroinflammation.

Microglial C1q knockdown protects against cranial RT-induced cognitive deficits

RT-induced complement cascade activation, commencing with C1q (Fig. 2), triggers detrimental microglial activation in the irradiated hippocampus that may lead to cognitive decline as shown in other neurodegeneration models. Thus, to validate the proinflammatory role of complement system in the irradiated brain, we utilized conditional C1q-Flox mice. We have shown selective knockdown of microglial C1q by 8 weeks of age, in absence of tamoxifen treatment (28) and, established microglia as a major source of C1q in the brain. Our study also showed selective depletion of C1q in the brain without altering

C1q in the blood (28), thus allowing the delineation of CNS-specific C1q-mediated events in the irradiated brain. Indeed, cranially irradiated C1q-Flox mice did not exhibit cognitive impairments on the hippocampal-dependent NPR task, as indicated by comparable behavior with the 0 Gy control and C1q-Flox mice (Fig. 5C and D). In contrast, littermate controls exposed to cranial RT showed significant decrements on a hippocampal-dependent novel spatial recognition task. Using a fear extinction test, we found increased freezing behavior by the WT-IRR mice, indicating RT-induced disruption in the dissociative learning process (Supplementary Fig. S2), whereas WT-0 Gy mice were able to show reduced freezing. Interestingly, both 0 and 9 Gy irradiated C1q-Flox mice showed elevated freezing on the extinction test. To address this finding, we quantified cytokines in the WT and C1q-Flox brains exposed to cranial RT. Activated microglia and elevated proinflammatory cytokines play a disruptive role in fear memory consolidation (52–56). Our multiplex data (Fig. 5E; Supplementary Fig. S2) showed significant elevations in TNF α , IL1 β , IL6, IL1 α , CCL2, and IL18 in the WT-IRR brains that suggest proinflammatory cytokine-mediated disruption in dissociative learning. However, we did not observe significant differences in the cytokine levels in the 0 or 9 Gy irradiated C1q-Flox brains. Additional cytokine analyses in the brains of WT and C1q-Flox mice with or without experiencing foot shock and after fear extinction training also did not show any significant differences (Supplementary Fig. S3). These behavior and cytokine data may indicate that cytokines are not playing any role in the elevated freezing observed in the C1q-Flox mice and that possible impact of the C1q gene inactivation on the dissociative learning and memory consolidation processes in the brain. In fact, a recent study, using CD55-mediated inhibition of classical CNS complement pathway, suggested the dependency of dissociative learning or memory consolidation processes on the microglial C1q (57).

Importantly, analysis of irradiated C1q-Flox mouse brains (with undetectable C1q, Fig. 5B) did not show microglial activation (CD68, Fig. 6A), C5aR1 (Fig. 6B), astrocytic-C3 or microglial-TLR4 (Fig. 6C and D) elevation compared with irradiated littermate control mice providing evidence that deletion of microglial C1q protected the brain from the detrimental downstream effects of cranial RT. Activation of the complement cascade, starting from C1q, produces effector anaphylatoxins, C3a and C5a, which are chemotactic for microglia (37, 58). Of these, C5a is more potent in triggering neuroinflammation and C5aR1 signaling also synergizes with the danger recognition TLRs (19, 59). C1 complex, along with C3b and iC3b, also play important roles in microglia-mediated synaptic pruning (43). Radiation-induced aberrant activation of complement cascade may also facilitate overzealous synaptic pruning, leading to functional impairments. Importantly, cranial RT-induced loss of synaptic proteins synaptophysin and SV2a (Fig. 7) was not found in the irradiated C1q-Flox mice, indicating neuroprotective effect of ablating C1q. These data also show that microglia-selective targeting of C1q prevented radiation-induced downstream damaging events.

Conclusion

This study provides novel insights into induction of complement cascade components by radiation exposure that impact brain function and lead to cognitive impairments. Our studies linking activation of the complement cascade shortly post-radiation exposure to the long-term neuroinflammation and cognitive dysfunction have a unique potential for impacting the quality of life for cancer survivors. Particularly, our genetic approach, microglia-selective deletion of C1q, addresses the contribution of an upstream CNS complement cascade

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Figure 6.

Microglial C1q knockdown prevents radiation-induced neuroinflammation. Immunofluorescence staining, confocal microscopy, and 3D algorithm-based, volumetric quantification for the CD68 (**A**) and C5aR1 (**B**) immunoreactivity (CD68 and C5aR1, red; blue, DAPI nuclear counterstain; **a1-a4** and **b1-b4**, respectively) showed that cranial irradiation (IRR) significantly elevated CD68 ($P = 0.001$; **A**) and C5aR1 ($P = 0.002$; **B**) in the WT hippocampus (DH, dentate hilus; DG, dentate gyrus) compared with unirradiated controls. Exposure of C1q-Flox mice to the cranial IRR did not elevate CD68 or C5aR1 immunoreactivity, indicating prevention of inflammation (**A** and **B**). Similarly, volumetric analysis for C3-GFAP (**C**, **c1-c4**) and IBA1-TLR4 colocalization (**D**, **d1-d4**) showed radiation-induced elevation in the colabeling of GFAP⁺ astrocytic surfaces with C3 (GFAP surface rendering, magenta; C3 spot rendering, yellow, **c1-c4**) and IBA1⁺ microglial surfaces with TLR4 (IBA1, green; red, TLR4; blue, DAPI, **d1-d4**) in the hippocampal DH. Irradiated C1q-Flox brains did not show elevated C3-GFAP or IBA-TLR4, indicating reduced neuroinflammation. Data are presented as mean $\pm$ SEM ($N = 6-8$ observations per group). P values were derived from ANOVA and Bonferroni's *post hoc* test. Scale bars, 40 μ m (**a1-a4** and **b1-b4**) and 5 μ m (**c1-c4** and **d1-d4**).

mechanism playing a detrimental role in RICD. Importantly, our strategy to target the CNS complement cascade also needs to be confirmed in presence of tumor, as activation of the complement cascade has been proposed to promote glioma stem-like cell proliferation, decreased apoptosis, immunosuppression and angiogenesis

within tumor vasculature, thereby promoting invasion (60-64). Moreover, the impact of fractionated RT should be studied to evaluate the nature of complement and microglia activation in the normal tissue bed. Nonetheless, our study establishes the link between radiation-induced gliosis, neuroinflammation, and cognitive dysfunction (16)

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Figure 7.

C1q depletion in microglia prevents radiation-induced synaptic loss. **A** and **B**, Immunofluorescence staining, confocal microscopy, and 3D algorithm-based quantification for the synaptic proteins synaptophysin (red, DAPI nuclear counterstain; **A**, a1-a4) and SV2a (red, DAPI⁺ nucleus; **B**, b1-b4) showed that cranial irradiation (IRR) significantly decreased synaptic proteins in the WT CA1 subregion (pyr, pyramidal cell layer; sr, stratum radiatum) compared with unirradiated controls, 0 Gy C1q-Flox, and irradiated C1q-Flox groups (**A** and **B**). Exposure of C1q-Flox mice to the cranial IRR did not reduce synaptic protein immunoreactivity, indicating neuroprotection against irradiation (**A** and **B**). Data are presented as mean $\pm$ SEM ($N = 5-8$ observations per group). P values were derived from ANOVA and Bonferroni's *post hoc* test. Scale bars, 40 μ m.

with complement activity. These data highlight the intricacies of the aberrant glial responses following radiation exposure in the brain and provide a unique opportunity to develop pathway branch point-specific therapeutics that may not need to directly affect neuronal function in the irradiated brain.

Authors' Disclosures

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Authors' Contributions

M. Markarian: Data curation, formal analysis, validation, investigation, methodology. **R.P. Krattli Jr:** Data curation, formal analysis, investigation, methodology. **J.D. Baddour:** Data curation, formal analysis, investigation, methodology. **L. Alikhani:** Data curation, formal analysis, investigation, methodology. **G. Giedzinski:** Resources, methodology. **M.T. Usmani:** Investigation, methodology. **A. Agrawal:** Formal analysis, investigation, methodology. **J.E. Baulch:** Conceptualization, resources, supervision, project administration, writing-review and editing. **A.J. Tenner:** Conceptualization, resources, writing-original draft, writing-review

and editing. **M.M. Acharya:** Conceptualization, resources, data curation, supervision, funding acquisition, writing-original draft, project administration, writing-review and editing.

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Glia-Selective Deletion of Complement *C1q* Prevents Radiation-Induced Cognitive Deficits and Neuroinflammation

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SUPPLEMENTAL INFORMATION

Markarian *et al.*, “*Glia-selective deletion of complement C1q prevents radiation-induced cognitive deficits and neuroinflammation*”

Supplemental Figure S1.

Supplemental Figure S1. Cranial irradiation did not affect anxiety-like behavior. No significant differences were observed between littermate controls (WT) and C1q-flox mice exposed to 0 or 9 Gy cranial irradiation (IRR) on anxiety-like behavior measured by light-dark box test. Time spent (percentage) in the open compartment (**A**) and number of transitions between the dark and light compartment (**B**) did not show significant differences one month post-irradiation. Data are presented as mean $\pm$ SEM ($N = 12$ for WT 0 Gy, C1q-flox IRR, C1q-flox IRR and, $N = 8$ for C1q-flox 0 Gy).

Supplemental Figure S2.

Supplemental Figure S2. Behavior of 0 or 9 Gy irradiated WT and C1q-Flox mice on the fear extinction memory task. Freezing behavior of WT-IRR on the extinction test showed a significant elevation compared to the WT - 0Gy group ($P = 0.03$) indicating the adverse impact of radiation exposure on the dissociative learning process. Both C1q-Flox groups of mice (0 and 9 Gy irradiated) showed elevated freezing compared to the WT - 0 Gy controls ($P = 0.03$ and 0.001 respectively). Data are presented as mean $\pm$ SEM ($N = 16$ mice per group). P values were derived from ANOVA and Bonferroni's post hoc test.

Supplemental Figure S3.

Supplemental Figure S3. Cytokine analyses of 0 or 9 Gy irradiated WT and C1q-Flox mice. Multiplex cytokine analysis was carried out in mice brains exposed to no foot shock, 30 minutes after experiencing 3 pairs of foot-shocks (0.6 mA for 1 sec) and auditory stimulus (16 kHz tone, 80 dB, lasting 120 sec) or 30 minutes after completion of extinction training (experiencing just the tone for 3 days). Significant elevation in the pro-inflammatory cytokines (TNF α , IL-1 β , IL-6, IL-1 α , CCL2, and IL-18) was observed in the WT-IRR mice (no foot shock) compared to WT-0Gy (no foot shock) group (as shown in Fig. 5E). No significant differences were found for the cytokine profile in the C1q-Flox mice exposed to no foot shock, experiencing foot shock or the tone (extinction training). Data are presented as mean $\pm$ SEM ($N = 3$ to 6 mice per group). *, $P < 0.05$ compared with WT-0Gy (no foot shock) group (as in Fig. 5E).

SUPPLEMENTAL METHODS

Animals and irradiation procedure:

Animals were maintained in standard housing conditions (20 °C±1 °C; 70% ± 10% humidity; 12h:12h light and dark cycle). At the select post-RT time, WT mice (C57Bl/6J, Jackson Laboratories, Cat#000664, RRID:IMSR JAX:000664) were anesthetized using isoflurane and euthanized via intra-cardiac perfusion (30 mL of saline with heparin, 10 U/mL, and 75 mL of 4% paraformaldehyde, PFA, Sigma). Dissected brains were kept in 4% PFA overnight and transferred to PBS with 0.02% sodium azide (pH 7.5, Sigma). Sucrose gradient infiltration steps (10 to 30% w/v, Sigma) were used for each brain to protect and cryosection (30 µm, coronal) using a cryostat (Leica Microsystems). For the gene expression analysis (RT-PCR), the hippocampus was micro-dissected from saline-heparin perfused brains, flash-frozen in liquid nitrogen and stored at -80 C° until analyzed.

Supplemental Table T1. Primers used for the gene expression analysis as in Fig. 1A.

Gene	Forward primer	Reverse primer
<i>Il1a</i>	CAGTGAGACCTTCACTGAAG	TTGTCCACATCCTGATATATAG
<i>Lcn2</i>	CATTTGTTCCAAGCTCCAGG	CTCCTTGGTTCTTCCATACAG
<i>Serping1</i>	CCAAAGCACCAACTTAAAGATG	AAAGAATTCCAGTTTCTCCATG
<i>Serpina3n</i>	TTAAAGCCAAATGGAAGGTGC	CATTTCTGTGTACTTCAGC
<i>P21</i>	ACCAGCCTGACAGATTTCTATC	CAGCGTATATACAGGAGACGTT
<i>Amigo2</i>	ATTACGGAATAACTCTAACGG	CAGTGTGTGGAACCTTAACG
<i>C1qa</i>	TCTCTAACACCAACAACAAGG	TGAGGAATCCGCTGAAGATG
<i>Tnfa</i>	ATGAGCACAGAAAGCATGATC	CCATTTGGGAAGTTCTCATC
<i>Cd14</i>	AATCTACCGACCATGGAGCGT	CCAAACAATTGAAAGCGCTGGA

Gene expression analysis

Dissected hippocampi from each brain (3 to 6 mice per group, 24 h, 48 h, 1-week, 1- and 6-months post-irradiation) were placed in RNeasy lysis solution (Qiagen, Life Technologies, CA, USA) and stored at -20°C. Tissues were lysed in RNA extraction buffer and Trizol (Ambion) for homogenization followed by treatment with BCP reagent (1-bromo-3-chloropropane, Molecular Research Center, OH, USA) to separate the RNA layer from DNA and protein. The extract was centrifuged (12000 × g, at 4°C for 15 minutes), and RNA was precipitated using isopropanol, washed twice (75% ethanol), and dried (vacuum centrifugation). The RNA pellet was re-suspended in 20µL of nuclease-free water (Ambion). RT-PCR was carried out using Applied

Biosystems Step One RT-PCR machine with a 48-well plate format. Primers and experimental conditions for the astrogliosis markers were optimized and followed as described (1). The primer sequence for each gene is provided in the **Supplement Table T1**. Gene expression data is presented as $2^{-(\Delta\Delta Ct)}$ values.

Cytokine analysis

For the cytokine analysis, freshly dissected hemi-brains (3-6 brains/group) were homogenized in presence of protease inhibitor cocktail (Thermo Fisher Scientific), centrifuged (500g, at 4°C for 10 min), washed using sterile PBS (15000g, 4°C for 5 min) and supernatants were assayed for cytokines (TNF α , IL-1 β , IL-6, IL-1 α , CCL2, CCL5, IL-10 and IL-18) using a magnetic bead-based customized kit (Thermo Fisher Scientific).

Behavior testing

One month post-irradiation, littermate controls and C1q-flox mice receiving 0 or 9 Gy cranial RT were administered an anxiety-related light-dark box test and a spatial memory testing using novel place recognition (NPR) task as described previously (2,3). The LDB arena consisted of a light compartment (30 x 20 x 27 cm, 915 lux) connected to a dark compartment (15 x 10 x 27 cm, 4 lux) via a small opening (7.5 x 7.5 cm). Thus, the LDB test contrasts the natural propensity of mice to explore new environments with their degree of anxiety to be in a well-lit space. Mice were placed in the arena for 10 minutes, and the time spent in each chamber and number of transitions between compartments were recorded by a blind observer. NPR task depend on animal's inherent ability to explore novel object location and reply on the intact hippocampal function (4,5). Briefly, mice were habituated to 3 days (15 min per day) to a dimly lit (48 lux) custom-made empty arena (30 x 30 x 30 cm) with a layer of fresh bedding and recorded using a ceiling-mounted digital USB COMS camera (Imaging Source, North Carolina, US). In between familiarization and test phases, all bedding was replaced and the arena was thoroughly cleaned with 10% ethanol. The following testing day, two plastic objects (duplo blocks, similar size and shape) were magnetically affixed 16 cm apart in the arena and the mouse was allowed 5 min to explore the objects. The mouse was then returned to the home cage for 5 min while the location of one object was move to a novel spatial location after cleaning the objecting using 10% ethanol. The mouse was then returned to the arena for 5 min of further exploration. All trials were scored by observers blind to the experimental groups to avoid bias. Time spent interacting (nose within 2 cm radius) with familiar versus novel object location was recorded and the data was calculate as the discrimination index (DI): $([\text{Novel location exploration time} / \text{Total exploration time}] - [\text{Familiar location exploration time} / \text{Total exploration time}]) \times 100$.

To determine if cranial irradiation microglial C1q gene knockdown affects amygdala-hippocampal circuit-dependent fear conditioning learning and fear memory consolidation, we performed fear extinction behavior reliant on hippocampal function (6,7) . Testing occurred in a behavioral conditioning chamber (17.5 × 17.5 × 18 cm, Coulbourn Instruments) with a steel shock floors (3.2 mm diameter slats, 8 mm spacing). Throughout the conditioning, memory consolidation (extinction training) and extinction testing phases, the bottom acrylic collection plate was scented with a spray of 10% acetic acid in water. For the initial fear conditioning phase (day 1), mice were allowed to habituate to the chamber for two minutes. Three pairings of an auditory conditioned stimulus (16 kHz tone, 80 dB, lasting 120 sec; CS) co-terminating with a foot shock unconditioned stimulus (0.6 mA, 1 sec; US) were presented at two minute intervals. On the following two to three days of extinction training, mice were initially habituated to the same context for two minutes before being presented with 20 non-US reinforced CS tones (16 kHz, 80 dB, lasting 120 sec, at 5 sec intervals). On a final day of fear testing mice were presented with only three non-US reinforced CS tones (16 kHz, 80 dB, lasting 120 sec) at two minute intervals in the same context. Freezing behavior was recorded with a camera mounted above the chamber and scored by an automated measurement program (FreezeFrame, Coulbourn Instruments). FreezeFrame algorithms calculate a motion index for each frame of the video, with higher values representing greater motion. An investigator blinded to the experimental groups set the motion index threshold representing immobility for each animal individually, based on identifying a trough separating low values during immobility and higher values associated with motion. Freezing behavior was defined as continuous bouts of one second or more of immobility. The percentage of time each mouse spent freezing was then calculated for the final day of extinction test.

Immunohistochemistry

PFA-fixed brains were collected from each treatment group for dual-immunofluorescence staining (3 to 4 sections per brain, 6 to 8 brains per group). Primary antibodies included: rabbit anti-IBA1 (1:200, ionized calcium binding adapter molecule 1, Wako Cat# 019-19741, RRID:AB_839504), goat anti-IBA1 (1:200, Novus Cat# NB 100-1028, RRID:AB_521594), rabbit anti-C1q (1:100, clone 4.8, Barres B. A., RabMAb, Abcam Cat# ab182451, RRID:AB_2732849), mouse anti-C3d (1:750, C3d chain, Quidel Cat# A207, RRID:AB_452479), rat anti-mouse CD68 (1:200, Bio-Rad Cat# MCA1957GA, RRID:AB_324217), mouse anti-TLR4 (1:100, toll-like receptor 4, Novus Cat# NB100-56566, RRID:AB_2205129), rat anti-C5aR1 (1:500, Abcam Cat# ab117579, RRID:AB_10902319), mouse anti-GFAP (1:500, glial fibrillary acidic protein, Biolegend Cat# 840001, RRID:AB_2565444), mouse anti-SV2 (1:750 DSHB Cat# SV2, RRID:AB_2315387),

mouse anti-synaptophysin (1:1000 Sigma-Aldrich Cat# S5768, RRID:AB_477523). For the fluorescence detection, following secondary antibodies were used: donkey anti-rabbit and anti-mouse Alexa Fluor 647 (1:500, Thermo Fisher Scientific Cat# A-31573, RRID:AB_2536183 and Thermo Fisher Scientific Cat# A32787, RRID:AB_2762830), donkey anti-goat Alexa Fluor 488 (1:400, Invitrogen, (1:400, Thermo Fisher Scientific Cat# A-11055, RRID:AB_2534102), donkey anti-rabbit and anti-mouse Alexa Fluor 568 (1:400, Abcam, Cat# ab175470, RRID:AB_2783823 and Thermo Fisher Scientific Cat# A10037, RRID:AB_2534013),), donkey anti-goat Alexa Fluor 568 (1:400, Thermo Fisher Scientific Cat# A-11057, RRID:AB_2534104), horse anti-goat biotinylated (1:200, Vector Laboratories Cat# BA-9500, RRID:AB_2336123), Texas Red Streptavidin (1:200, Vector Laboratories Cat# SA-5006, RRID:AB_2336754).

Tissue sections for each staining procedure were washed three times in the phosphate buffered saline (PBS, 100 mM, pH 7.4, Gibco) and antigen retrieval was facilitated by citrate buffer incubation (10 mM, pH 6.0 with 0.05% Tween-20, Sigma) at 70°C for 45 minutes. Tissues were then recovered by borate buffer (pH 8.5, 100mM, 10 minutes) and PBS incubation (3 washes, 5 min each). Prior to the primary antibody incubation, tissues were blocked with serum (certified, pathogen free; Jackson Immuno Research) from the respective secondary antibody host (10% normal donkey, goat or horse serum, NDS, NGS or NHS respectively in PBS with 0.01% Triton X-100 (NDS, Jackson ImmunoResearch Labs Cat# 017-000-121, RRID:AB_2337258; NGS, Jackson ImmunoResearch Labs Cat# 005-000-121, RRID:AB_2336990; NHS, Jackson ImmunoResearch Labs Cat# 008-000-121, RRID:AB_2337040) . After the blocking step, tissues were incubated in the primary antibodies for glial and complement components or receptors. Primary antibody solutions were made in PBS containing 0.01% Triton X-100 (Sigma) and 3% NDS, NGS or NHS. Tissues were incubated overnight at 4°C in a shaker incubator (Enviro-Genie Scientific). After 24 hours, the tissues were washed with PBS and incubated in the secondary antibody for one hour to visualize the target antigens (dual immunofluorescence). Lastly, all the sections were counterstained with DAPI nuclear dye (1 µmol/L, Thermo Fisher Scientific Cat# D1306, RRID:AB_2629482, in PBS for 15 min). Immunostained sections were washed with PBS and mounted on super-frost slides (Fisher Scientific) using SlowFade Anti-Fade Gold mounting medium (Thermo Fisher Scientific Cat# A10037, RRID:AB_2534013).

***In silico* modeling: 3D algorithm-based volumetric quantification**

Immunostained sections were imaged with a laser-scanning confocal microscope (Nikon Eclipse Ti C2) equipped with a 40x and 60x oil-immersion objective lens (1.4 NA) and NIS element AR module (v4.3, Nikon). The high-resolution (1024 to 2048p) z stacks (0.5 µm thick) were scanned

through the 25-30 μm thick section. An adaptive, 3D blinded deconvolution method (AutoQuant X3, v3.2, Media Cybernetics, MD, USA, RRID:SCR_002465) was used to deconvolute images to improve the signal resolution with respective fluorescent wavelengths (510nm, green; 594 nm, red; 447 nm, UV; and 647 nm for IR range). The deconvoluted images were converted to IMS format for 3D algorithm-based Imaris analysis (v9.5, Bitplane Inc., Zürich, Switzerland, RRID:SCR_007370). In Imaris, IBA-1 and GFAP were 3D modeled using the surface-rendering tool to create volume of individual glial cells, and a 3D spot analysis was conducted to detect the complement/synaptic proteins and specific receptors (C1q, C3, C5aR1, TLR4, synaptophysin and SV2a). Using an unbiased, dedicated co-localization channel, the number of complement puncta or receptor on the surface of IBA1 and GFAP was individually calculated by selecting spots at a distance of -0.5 μm to 0.5 μm from the surface created. The total (gross) expression and the expression level of the receptor (TLR4) or complement protein (puncta) on each surface, the area and volume of IBA1 and GFAP, and the total number of puncta were used for quantitative comparison between the control and irradiated groups. Similarly, total expression level of C5aR1 or synaptic proteins were rendered using surface tool to determine the volume of immunoreactive puncta. All *in silico* analyses were conducted using automated batch processing modules and criteria applied uniformly for all experimental groups to avoid bias.

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A Complementary Strategy to Mitigate Radiation-Induced Cognitive Decline

Navyateja Korimerla and Daniel R. Wahl

Cranial radiation activates an upstream complement cascade component, C1q, leading to brain injury. Microglia-specific deletion of C1q prevents astrocyte and microglial activation, synaptic loss, neuroinflammation, and cognitive impairment. Therapeutically

inhibiting complement activation may help mitigate radiation-induced cognitive decline.

See related article by Markarian *et al.*, p. 1732

In this issue of *Cancer Research*, Markarian and colleagues define therapeutically relevant mechanisms linking the complement cascade to radiation-induced cognitive decline. Radiotherapy is a critical treatment for many primary and metastatic brain tumors (1). The efficacy of radiotherapy is counterbalanced by its capacity to cause neurocognitive decline, which occurs to some extent in many patients surviving more than several months after treatment. Radiation-induced cognitive decline often presents as memory difficulties, decreased attentiveness, and difficulty with higher-order cognitive tasks. While these changes are often difficult to quantify without advanced neurocognitive testing, they are frequently detrimental to patient quality of life (2). Radiation-induced cognitive decline is often biphasic, with reversible changes occurring several months after radiation and more permanent decline developing later. In rare cases, radiation-induced cognitive decline causes a progressive and fatal dementia-like syndrome, especially when high doses of whole-brain radiotherapy are used in combination with some cytotoxic chemotherapies. Several strategies are currently used to mitigate radiation-induced cognitive dysfunction, including using focal radiotherapy rather than whole-brain radiation when possible, avoiding high dose radiation to the hippocampus, and coadministering anti-dementia drugs targeting the N-methyl-D-aspartate receptor along with radiotherapy. While these efforts are efficacious, they have not eliminated radiation-induced cognitive decline, and a full understanding of cellular and molecular mediators of this phenomenon is needed to design more effective mitigation strategies.

The cellular and molecular processes underlying the clinical entity of radiation-induced cognitive change are complex. Within hours to days after radiation, cellular components of the normal brain become activated. Astrocytes, which are a major supporting cell type in the brain, undergo a number of reactive changes when they are activated by radiation or other insults. Such changes include altered morphology, increased expression of the glial fibrillary acidic protein (GFAP), and increased expression of proinflammatory genes. Microglia, which are the major resident immune cells of the central nervous system, also

undergo reactive changes following radiation. These changes include an altered morphology and increased expression of proinflammatory cell surface and secreted proteins. These early inflammatory changes are followed months later by microstructural changes such as demyelination, vascular abnormalities, and white matter necrosis, which temporally coincide with clinical neurocognitive decline.

Astrocyte and microglial activation are causally related to the development of radiation-induced cognitive decline. Pharmacologic elimination of microglia in mice prevents the development of radiation-induced cognitive decline (3). Some aspects of astrocyte function are also important for the development of radiation-induced cognitive decline. Radiation causes astrocytes to increase the clearance of the protective neurotransmitter adenosine, and interrupting this axis can mitigate radiation-induced neurodegeneration and cognitive decline (4). *In vitro* experiments using isolated and mixed-culture systems have suggested that soluble factors derived from microglia are required for radiation to activate astrocytes. However, such ideas have not been explored *in vivo* and a full characterization of the molecules linking microglial and astrocytic activation after radiation is lacking (5).

In this study, Markarian and colleagues investigate whether the complement system—which is a key mediator of innate immunity and microglia-mediated synapse regulation—is a link between radiation, glial activation, and the development of radiation-induced cognitive decline. Dysregulation of the complement system evokes proinflammatory signatures and can result in the pathogenesis of autoimmune and neurodegenerative diseases. The authors have previously observed elevated levels of the complement component proteins C1q (a proximal component of the complement cascade) and C3 (a downstream component of the complement cascade, which is activated by C1q) in irradiated brains compared with nonirradiated control brains (6). C1q is synthesized by microglia in response to radiation, while astrocytes express C1q under pathologic conditions such as multiple sclerosis and temporal lobe epilepsy (6, 7).

Here, the authors provide additional evidence that the complement system links microglia and astrocyte activation after radiation and is a critical mediator of radiation-induced cognitive decline. The authors show that the proximal complement factor C1q increases in abundance within 2 hours after radiation and colocalizes with both activated microglia and astrocytes. The more downstream complement component C3 also increases in abundance within 2 hours of radiation, but primarily colocalizes with reactive astrocytes. These findings suggest a mechanism by which radiation activates microglia, causing them to produce C1q, which then activates astrocytes. Notably, such a mechanism clearly exists for other neurotoxic stimuli (7).

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The authors utilize a genetically engineered mouse model in which C1q is deleted specifically in microglia to test this hypothesis. Mice lacking microglial C1q produce lower levels of numerous inflammatory cytokines in response to radiation than controls. Furthermore, the deletion of C1q in microglia prevents the deposition of C3 on astrocytes, their upregulation of the complement receptor C5aR1, and astrocyte activation (as assessed by CD68 expression). Deletion of microglial C1q also prevents the radiation-induced upregulation of the danger receptor TLR4 on microglia, suggesting that C1q may act on microglia in addition to astrocytes.

These cellular mechanisms are relevant to neurocognition. Deletion of microglial C1q prevents radiation-induced cognitive decline as assessed by the novel place recognition test, which is a hippocampal-dependent test of memory. Why was memory spared? The authors wisely recognize the physiologic role of the complement cascade in synaptic pruning and hypothesized that overactive complement following radiation could cause aberrant synaptic pruning, leading to cognitive decline. Consistent with this hypothesis, multiple synaptic proteins are decreased in irradiated control mice but remain at normal levels in irradiated mice lacking microglial C1q.

In sum, the authors have described a novel mechanism by which radiation acutely stimulates microglia to release C1q and the resulting complement cascade mediates neuroinflammation and astrocyte activation, synapse loss, and cognitive decline. This mechanistic understanding has important therapeutic implications, as it suggests that pharmacologic inhibition of the complement cascade could mitigate radiation-induced cognitive decline. C1q-targeted complement inhibitors are currently in preclinical development for a variety of neurodegenerative diseases, and inhibitors of other components of the

complement system are FDA-approved for complement-mediated diseases such as paroxysmal nocturnal hemoglobinuria (8). Whether C1q-directed therapeutics might be useful clinically to prevent radiation-induced cognitive decline will depend on whether these agents successfully cross the blood-brain barrier to inactivate the complement cascade intracranially. Another determinant of potential efficacy in patients is to what extent these therapies interact with the radiation-induced killing of cancer cells. Such interactions may vary by cancer type. Inhibiting complement activation augments the efficacy of radiotherapy in some cancer models (9), but reduces radiotherapy efficacy in others (10). Thus, how microglial-derived C1q influences the effects of radiation therapy on a variety of intracranial cancers should be carefully studied prior to clinical investigation. While clinical implementation of these strategies is not yet at hand, these findings raise the possibility that targeting the complement cascade may improve quality of life for patients receiving cranial radiotherapy, especially when combined with some of the other mitigation strategies currently in use. If these advances provide sufficient cognitive protection, they may also prompt the reinvestigation of therapies that were effective from an anticancer standpoint, but abandoned due to neurotoxicity, such as whole-brain radiation for CNS lymphoma.

Authors' Disclosures

D.R. Wahl reports grants and personal fees from Agios, Inc. outside the submitted work; in addition, D.R. Wahl has a patent for Methods of predicting brain tumor treatment response pending. No disclosures were reported by the other author.

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Preclinical Evaluation and Phase Ib Study of Prexasertib, a CHK1 Inhibitor, and Samotolisib (LY3023414), a Dual PI3K/mTOR Inhibitor

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Blocking TIM-3 in Treatment-refractory Advanced Solid Tumors: A Phase Ia/b Study of LY3321367 with or without an Anti-PD-L1 Antibody

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Genetic Ancestry Contributes to Somatic Mutations in Lung Cancers from Admixed Latin American Populations

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Modeled Reductions in Late-stage Cancer with a Multi-Cancer Early Detection Test

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Antitumor Effects of CAR T Cells Redirected to the EDB Splice Variant of Fibronectin

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An Improved Murine Premalignant Squamous Cell Model: Tobacco Smoke Exposure Augments NTCU-Induced Murine Airway Dysplasia

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EMT Transcription Factor ZEB1 Represses the Mutagenic POLθ-Mediated End-Joining Pathway in Breast Cancers

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Phase I Study of Everolimus, Letrozole, and Trastuzumab in Patients with Hormone Receptor–positive Metastatic Breast Cancer or Other Solid Tumors

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Multiorgan Signaling Mobilizes Tumor-Associated Erythroid Cells Expressing Immune Checkpoint Molecules

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Combined BRAF, MEK, and CDK4/6 Inhibition Depletes Intratumoral Immune-Potentiating Myeloid Populations in Melanoma

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Estrogen Receptor Alpha Mutations in Breast Cancer Cells Cause Gene Expression Changes through Constant Activity and Secondary Effects

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Assessing Impact of HPV Vaccination on Cervical Cancer Incidence among Women Aged 15–29 Years in the United States, 1999–2017: An Ecologic Study

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The Prognostic Role of Macrophage Polarization in the Colorectal Cancer Microenvironment

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Breast Cancer Chemoprevention: Use and Views of Australian Women and Their Clinicians

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Expanded Low Allele Frequency *RAS* and *BRAF* V600E Testing in Metastatic Colorectal Cancer as Predictive Biomarkers for Cetuximab in the Randomized CO.17 Trial

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A Phase Ib Study of Alpelisib or Buparlisib Combined with Tamoxifen Plus Goserelin in Premenopausal Women with HR-Positive HER2-Negative Advanced Breast Cancer

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Progesterone Receptor Is a Haploinsufficient Tumor-Suppressor Gene in Cervical Cancer

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