Biology Contribution

NF-κB Blockade by NEMO Binding Domain Peptide Ameliorates Inflammation and Neurobehavioral Sequelae After Cranial Radiation Therapy in Juvenile Mice

Christine A. Beamish, PhD,* Janice A. Zawaski, PhD,† Taeko Inoue, PhD,† Poonam Sarkar, PhD,‡ David R. Grosshans, MD, PhD,§ Omaima M. Sabek, PhD,*¶ and M. Waleed Gaber, PhD§

*Department of Surgery, Houston Methodist Hospital, Houston, Texas; †Department of Pediatrics, Hematology-Oncology Section, Dan L. Duncan Cancer Center, Baylor College of Medicine, Houston, Texas; Departments of ‡Radiation and §Experimental Radiation Oncology, University of Texas MD Anderson Cancer Center, Houston, Texas; ¶Department of Cell and Microbiology, Weill Cornell Medical College, New York, New York; and §Dept. of Molecular Physiology & Biophysics, Baylor College of Medicine, Houston, Texas

Received Jun 26, 2020. Accepted for publication Nov 25, 2020.

Purpose: Cranial radiation therapy (CRT) is a common treatment for pediatric brain tumor patients. However, side effects include significant neurobehavioral dysfunction in survivors. This dysfunction may in part be caused by inflammation, including increased production of tumor necrosis factor alpha (TNFα) and its receptor TNFR1, which can activate the nuclear factor kappa light-chain enhancer of activated B cells (NF-κB). The TNFα blockade abrogates this inflammatory response, although it presents immunologic risks. Thus, modulation of pathway subsets may be preferable. Here, we test whether inhibition of NF-κB activation using an NF-κB essential modulator binding domain (NBD) peptide mitigates CRT-induced neuroinflammation and improves behavioral outcomes.

Methods and Materials: Male C57BL/6J 28-day old mice were randomized to saline (sham), 5 Gy whole-brain CRT, or CRT + NBD-peptide. Brain tissue was collected after 4 hours or 3 months for Western blot or immunohistochemistry. The cortex, corpus callosum (CC), and dentate gyrus were variably imaged for NF-κB-p65, IκBα, proliferation, apoptosis, necroptosis, TNFα, TNFR1, IBA-1, doublecortin, CD11c, and GFAP. Neurobehavioral changes were assessed by open field and elevated plus maze tests 3 months post-CRT.

Results: NF-κB expression increased in whole and nuclear fractions 4 hours after CRT and was abrogated by NBD treatment. Cell death increased and proliferation decreased after CRT, including within neuronal progenitors, with some loss mitigated...
by NBD. Increased levels of TNFα, IBA-1, and GFAP were found in the CC and cortex months after CRT and were limited by NBD. The anti-NF-κB peptide also improved neurobehavioral assessments, yielding improvements in anxiety and exploration.

**Conclusions:** Results suggest a role for NF-κB modulation by NBD peptide in the reduction of neuroinflammation and mitigation of behavioral complications after pediatric radiation therapy. © 2020 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

**Introduction**

Cranial radiation therapy (CRT) is a standard treatment for brain tumors in children; however, significant side effects, including cognitive and neurobehavioral impairments, are common in survivors. Inflammation is an inherent contributor to radiation-induced brain injury, and is present in the early and late phases of damage. Blocking tumor necrosis factor alpha (TNFα) using a specific antibody has been shown to abrogate increases in astrogliosis, vessel permeability at the blood-brain barrier, leukocyte adhesion, and arteriole vasoconstriction in irradiated rodent brains. TNFα is the primary proinflammatory cytokine, part of the innate immune response to injury. The two TNF receptors, TNFR1 and TNFR2, function in opposing modes: the ubiquitously expressed TNFR1 (p55, CD120a) acts primarily in the canonical pathway via its death domain, promoting apoptosis, necroptosis, and inflammation, whereas TNFR2 (p75, CD120b) acts in a protective, antiapoptotic capacity. The TNFα pathway has been a critical immune-modulating target, but because it also impacts TNFR2, its modulation is not without serious risks.

The nuclear factor kappa light-chain enhancer of activated B cells (NF-κB) is a large family of transcription factors which responds to a multitude of adverse stimuli. Although basal levels are prosurvival, cytokine-triggered activation of NF-κB is involved in inflammatory and immune responses. Furthermore, whereas the canonical pathway demonstrates an inflammatory stimulus causing NF-κB (p65/RelA) activation through binding of TNFα to TNFR1 and inhibition of apoptosis, other DNA-dependent mechanisms have been shown to activate NF-κB, which suppresses antiapoptotic genes. CRT activates and induces the expression of NF-κB, directly damages both neurons and microglia, and is further confounded by vascular injury. As such, anti-NF-κB treatment strategies have been proposed, including NF-κB essential modulator (NEMO)/IKKγ–binding domain (NBD) peptide, which does not affect basal levels of NF-κB but blocks proinflammatory activation of the IKK complex. Linkages between inflammation, brain cell death, and cognitive-behavioral alterations have been demonstrated. In one report, activated microglia were inhibited by the polyphenol Corilagin, which inhibits NF-κB. Others showed that microglia were inhibited by sertraline hydrochloride, an antidepressant with anti-inflammatory properties that bound to TNFR1 and consequently inhibited microglia activation via the NF-κB pathway. Importantly, NBD has been shown to alleviate hypoxic-ischemic neonatal rat brain injury and to reduce microglial activation with associated neurobehavioral protection in diverse models of fronto-temporal dementia and impaired spatial learning tasks.

Here, a single cranial radiation dose in young mice was used to evaluate the involvement of inflammation and neurobehavioral outcomes after CRT and to determine whether TNFα pathway modulation can effect a benefit using NBD. Due to the developmental age of the mice, fractionated radiation therapy and a higher total CRT dose were not possible due to adverse animal health outcomes. Moreover, the experimental intention was to evaluate the contribution of NF-κB signaling in the absence of overwhelming radiative cell damage. Five Gy radiation therapy is akin to single fractionation dosing paradigms (hypofractionation), which is thought to improve the therapeutic index and to have clinical benefit in treating metastases. In addition to alterations in NF-κB, changes in cell turnover markers for early (cleaved caspase-3) and late (terminal deoxynucleotidyl transferase dUTP nick end labeling, TUNEL) apoptosis, inflammatory programmed cell death (necroptosis) by high mobility group 1 (HMGB1), and cell proliferation (Ki67) were quantified 4 hours following radiation therapy. Months later, sustained inflammation was evaluated for the proportional presence of cellular TNFα, GFAP, CD11c, and IBA-1. These effects were examined in 3 discrete anatomic brain regions responsible for dynamic functions, including the hippocampal dentate gyrus (DG), a site of rare postnatal neurogenesis that has roles in spatial, anxiety, and olfactory processing; the cerebral cortex, responsible for motor function, cognitive planning/organization, and processing of sensory information; and the corpus callosum (CC), which ensures the communication of motor, sensory, and cognitive information between brain hemispheres. CRT has widely been shown to thin the CC myelinated nerve fiber layer, and has demonstrated increased inflammation by TNFα and reactive astroglia by GFAP expression in the CC, cerebral cortex, and hippocampus. The extent and location-specific effects from a single CRT dose, and its long-term impact on behavior, demonstrate both immediate and sustained impacts on multiple brain regions, and which may be modified by NBD administration.
Methods and Materials

Animal experiments were approved by the Baylor College of Medicine IACUC. Male 28-day C57BL/6J mice (Jackson Laboratory, Bar Harbor, Maine) were housed in same-sex groups on a 14/10-hour light/dark cycle. Body weight was assessed weekly.

Cranial radiation therapy, drug treatment, tumor model, and Western blotting

Mice were randomly assigned to either a sham, CRT, or CRT + TAT-NBD group (selective NF-kB inhibitor NEMO binding domain peptide coupled to the transduction sequence of the HIV-TAT protein, sequence: YGRKRRQRR-TALDWSWLQTE\textsuperscript{18}). Under isofluorane anesthesia, CRT-treated mice received 5 Gy whole-brain radiation 1.5 cm from behind the eyes and to the back of the ears using a RadSource 2000 x-ray irradiator (306 cGy/min 160kVp, 25mA, Rad Source Technologies, Inc, Suwanee, Georgia). The body was shielded with ¼-inch lead. TAT-NBD was delivered 1 ug/g (body weight) intra-nasally 1 hour before CRT\textsuperscript{18} and 3 times thereafter, each a week apart, to maintain NF-kB inhibition during the timeframe of radiation therapy “sterile inflammation”\textsuperscript{18} and to minimize chronic inflammation’s contribution to late CRT effects.\textsuperscript{4} Sham mice received the anesthetic and saline but no radiation. The Western blotting and tumor model procedures are provided in the Supplemental Materials.

Immunofluorescent histology

Mice (\(n = \text{4–6/ group}\)) at 28 days (4 hours, acute effects) and 4 months (3 months post-CRT, late effects) were deeply anesthetized and intravenously perfused with 10% formalin. Whole brains were sliced into 2-mm coronal sections, paraffin-embedded, and sectioned at 5 μm. Antibody details are available in Table E1. Antigen retrieval was performed for 30 min. Tissue was blocked for 8 minutes using Background Sniper (Biocare Medical, Inc, Concord, MA). Fluorescent secondary antibodies conjugated to 488, 555, and 647 nm fluorophores (1/500, Alexafluor, Invitrogen, Carlsbad, CA) were incubated for 2 hours. Nuclei were visualized by 4,6-diamidino-2-phenylindole (DAPI) (1/500, Invitrogen).

Slides were imaged using a Nikon A1 confocal microscope (Tokyo, Japan) at 20X and were analyzed using ImageJ software (v.1.50i, NIH, Bethesda, MA). Every NF-kB-, IκB-α-, TNF-α-, TNFR1-, and GFAP-expressing cell was imaged within 2 to 3 representative images in both the corpus callosum (CC) and the cerebral cortex above the CC, extending to the cortical surface and correlating to the primary somatosensory area and posterior parietal association areas. Every TUNEL-, HMGB1-, cleaved caspase-3— (CIC-3), human homolog of Drosophila Melanogaster Embryonic Lethal Abnormal Vision proteins antigen C/D (HuC/D)-, IBA-1-, CD11c-, and Ki67-expressing cells within the dentate gyrus (DG), CC, and cortex. The CC was imaged at the body, excluding the cingulum bundle. Myelin thickness 3 months post-CRT was measured within the CC by myelin basic protein immunostaining with distance normalized to pixel size. Regions of interest (ROIs) corresponded to images 70 to 75 of the p56 coronal Allen mouse brain atlas (http://mouse.brainmap.org/). ROIs were landmarked by DAPI-stained morphology. All manually counted cell numbers were representative of a percent total by DAPI within the stated region, with the operator blinded to treatment group and with average numbers of total cells counted per region of 905 ± 38 in the cortex, 251 ± 10 in the CC, and 869 ± 83 in the DG.

Behavioral assessments

Behavioral assessments were measured in mice 3 months post-CRT (\(n = \text{15–16/group}\)) by the open field test (OFT) and the elevated plus maze (EPM) to assess anxiety, stress, and exploration in an unconditioned setting,\textsuperscript{26} as described by Lacaria et al.\textsuperscript{27} All tests were conducted at approximately 700 lux illumination and 60 dB background white noise, with no training sessions. At the start of testing, mice were placed into and allowed to move freely throughout the open chamber. OFT was recorded over 30 minutes using the VersaMax Legacy (Omnitech Electronics, Inc, Columbus, OH) system for metrics related to distance, rotations, rearing number and time, and movement, recorded in 2-minute bins. EPM was assessed the day after OFT. For EPM, position within the maze and time spent in the open or closed arms were tracked using ANY-maze\textsuperscript{TM} Behavior Tracking Software (Stoelting Co, Wood Dale, IL) for 10 minutes and assessed for distance, time, and latency.

Statistical analysis

Data were analyzed using GraphPad Prism 6 by 1-way analysis of variance (ANOVA) for cell proportion, Western blots, and EPM, and 2-way ANOVA for weight over time and OFT, with Tukey’s multiple comparisons comparing all means. Data are expressed as % means ± standard errors of the mean (SEMs). For Western blots and the EPM % time spent in the open/closed arms, sham values were normalized to 1, with other groups’ mean data reported as the fold change relative to sham. Outliers from all experiments were excluded using the robust regression and outlier removal function (Prism), based on the false discovery rate (FDR), with Q (the maximum desired FDR) set to 1%. Significance was set to a minimum of \(P < .05\) and analyzed using parametric (normal) distribution. Levels of significance were set at \(P < .05\), \(P < .01\), \(P < .001\), and \(P < .0001\), and are presented on graphs to denote group comparison post-test analysis.
Fig. 1. NF-κB modulation decreases NF-κB protein after cranial radiation therapy. (A) NF-κB protein (green) was examined 4 hours after CRT by immunofluorescence, showing increased total NF-κB-p65 in the (B) cortex and (C) CC relative to sham. Similar findings were shown by (D, E) whole cell or (F, G) nuclear fraction Western blots in cortex + CC tissue. (B–G) The addition of TAT-NBD before CRT demonstrated lower NF-κB-p65 protein versus CRT alone. Data represent mean ± SEM using a 1-way ANOVA. *Versus sham; †Versus CRT; *∗∗∗P < .001; n = 5/group. Abbreviations: ANOVA = analysis of variance; CC = corpus callosum; CRT = cranial radiation therapy; TAT-NBD = NF-κB essential modulator (NEMO) binding domain (NBD) coupled to the transduction sequence of the HIV-TAT protein; NF-κB = nuclear factor kappa light-chain enhancer of activated B cells; TBP = TATA-binding protein. (A color version of this figure is available at https://doi.org/10.1016/j.ijrobp.2020.11.067.)
Results

Inflammation and NF-κB-p65 protein expression increase early after CRT

The experimental goal was to effect a phenotypic response without significant morbidity,28 and to model radiation outcomes in mice analogous to preadolescent humans, before complete brain development. At 28 days, mice weighed $15.78 \pm 0.29$ g; thereafter, NBD-treated mice displayed minimally blunted weight gain versus other groups >4 weeks posttreatment (Fig. E1; $P < .05$).

To confirm an inflammatory response 4 hours after CRT, brain sections were examined by immunofluorescence for total and nuclear-specific NF-κB-p65 (Fig. 1A, green; IkBε, red). A proportional doubling of NF-κB-p65+ stained cells was shown in total cortical (Fig. 1B; $P < .05$) and CC cells (Fig. 1C; $P < .05$) in CRT-treated versus sham mice and abrogated by TAT-NBD (Fig. 1B and C). The TNFR1 proportion (Fig. E2A, green) also increased in the cortex and CC 4 hours post-CRT, and remained elevated with TAT-NBD (Fig. E2B and C; $P < .05$). Nuclear NF-κB-p65 protein proportion was elevated in these 2 regions after CRT exposure (Fig. 1B and C; $P < .05$), whereas NBD treatment levels in CRT mice were similar to those of sham mice. Confirming histology results, a Western blot analysis of the mouse cortex (including CC) 4 hours post-CRT yielded a significant increase in total (Fig. 1D and F; $P < .001$) and nuclear protein...
Fig. 3. CRT increases regional-specific brain cell death. (A,B) CRT increased necroptosis by cytoplasmic-HMGB1 (red and closed arrow heads) in the DG of CRT-treated mice at 4 hours postirradiation, and (B) increased HMGB1-negative cell proportion (arrows) primarily within the DG inner fold. (A) There was significantly more apoptosis by TUNEL (A, green, open arrow head) in the (F) CC and (C) CIC-3 (red) immunostaining of CRT-treated mice in the (G) DG and (H) CC, and (D) decreased proliferation by Ki67 proportion (red), particularly in the (I) DG and (J) CC. (E–H) TAT-NBD lowered cell death but showed differential protection on proliferation in the (I) DG versus the (J) CC. Data represent mean ± SEM by 1-way
fractions of NF-κB-p65 (Fig. 1E and G; \( P < .05 \)) relative to sham mice; in both total and nuclear protein, NBD-treatment before CRT blunted the increase in NF-κB-p65 (Fig. 1D–G).

To identify cell type changes 4 hours post-CRT, HuC/D + neurons (Fig. 2C–G, red) displayed primarily cytoplasmic colocalization with NF-κB-p65 (Fig. 2C–E, green) in sham mouse brains; after CRT, cytoplasmic NF-κB-p65 + HuC/D + cells were less common (Fig. 2F), and some rare nuclear NF-κB-p65 + expressing cells displayed astrocyte characteristics by GFAP immunostaining (Fig. 2G, red) and were negative for the reactive microglia marker IBA-1 (not shown). In CRT + TAT-NBD brains, cytoplasmic NF-κB-p65 (Fig. 2H, green) was observed in some astrocytes (Fig. 2H, red, arrow), whereas nuclear NF-κB-p65 + GFAP + astrocytes and cytoplasmic NF-κB-p65 + HuC/D + neurons were still present (not shown). Thus, utilization of TAT-NBD appears to dampen NF-κB-p65 pathway activation early after CRT, specifically in astrocytes. In our hands and within the current model and time point examined, however, we did not observe IkBα protein degradation after CRT (Fig. E3A-E).

**NF-κB blockade reduces acute cell death resultant from cranial radiation therapy**

Cell turnover was examined early (4 hours) after CRT for apoptosis by TUNEL (Fig. 3A, green, arrow head) and CIC-3 (Fig. 3C, red); necroptosis by HMGB1 (Fig. 3A, B, red); and proliferation by Ki67 immunostaining (Fig. 3D, red). Necrotic cells are HMGB1-negative versus apoptotic cells demonstrate cytoplasmic HMGB1 (Fig. 3B, red). Necrotic cells were less common (Fig. 3B, arrows; Figure E4A; \( P < .01 \)) in CRT versus sham mice (Fig. 3B, red). Necrotic cells displayed primarily cytoplasmic HMGB1 (Fig. 3B, red, arrow heads), and necrotic cells are HMGB1-negative versus the nuclear prototype.29 The cytoplasmic-HMGB1 proportion was tripled (Fig. 3E; \( P < .01 \)) in CRT versus sham mice cells of the DG granule cell layer (neuro-epithelium, interior fold) at 4 hours, and there was a significant increase in the HMGB1-negative proportion (Fig. 3B, arrows; Figure E4A; \( P < .01 \)). CRT + TAT-NBD–treated mice had fewer cytoplasmic-HMGB1 cells (Fig. 3E) and HMGB1-negative cells (Fig. E4A) in the DG than CRT. There was a significant increase in the CC TUNEL-positive cell proportion in CRT-treated mice versus those in other groups (Fig. 3F; \( P < .05 \)), but a similar trend in HMGB1-negative cells (Fig. E4B) but no other significant changes in HMGB1-negative or cytoplasmic proportions in the CC or cortex (Fig. E4C-E), nor changes in TUNEL labeling of DG and cortical cells (Fig. E4F-G). After CRT, the CIC-3 proportion was higher in the DG, CC (Fig. 3G-H), and cortex (Fig. E4H), and lower in TAT-NBD–treated DG and CC cells (Fig. 3G and H). Correspondingly, there were reductions in Ki67 + cells in the DG and CC after CRT (Fig. 3I and J); although TAT-NBD did not prevent loss of cell proliferation from CRT in the CC (Fig. 3J; \( P < .001 \) vs sham mice), there was some protection of proliferative capacity in the DG (Fig. 3I; \( P < .05 \) vs sham mice); there was no significant effect on proliferation in the cortex with any treatment at 4 hours (Fig. E4I). The HMGB1-negative and TUNEL-positive cells were also primarily located in the DG neuro-epithelium (Fig. 3A), an area representing migrating neuronal precursor cells in an “inside-out” action,30 and which demonstrated immunostaining for doublecortin (DCX; Fig. E5, red). Most Ki67- and CIC-3-expressing cells in the DG were also located in the neuro-epithelium (Fig. 3C and D), and ~70% of the Ki67 + cells expressed DCX (Fig. E5, red) across all treatment groups, indicating alterations in neuronal progenitor proliferation31 (Fig. E5A and B, green, arrows). Moreover, although the early apoptosis marker CIC-3 (Fig. E6, red) was found primarily in IBA-1 + cells (Fig. E6A, green), in some GFAP + cells (Fig. E6B, green), and in only rare HuC/D + (mature neuronal) cells (Fig. E6C, green) after CRT, ~12% of CIC-3 + cells expressed DCX (Fig. E6D, green) across all treatment groups in the DG. CIC-3 + cells in the cortex and CC were also primarily positive for IBA-1 (not shown). Combined with significant loss of cellular proliferation and concomitant increased death in multiple brain regions, including the DG neuro-epithelium, these data implicate the direct reduction of neuronal progenitor cells resulting from CRT, which may contribute to later neurocognitive deficits. Importantly, many of these negative outcomes were abrogated by TAT-NBD.

To assess the impact of TAT-NBD on radiation therapy (RT) efficacy within the tumor context, mice were cranially implanted with glioma GL261-Luciferase tumors, treated with saline or TAT-NBD, then exposed to 6 Gy radiation therapy. Survival was significantly lower in glioma-only versus glioma + RT (± drug treatment) mice (Fig. E7), indicating that TAT-NBD did not mitigate the ability of CRT to reduce the tumor burden.

**Long-term inflammation and astrogliosis are prevented by TAT-NBD administration before CRT**

A long-term evaluation of radiation therapy showed sustained alterations in inflammatory markers 3 months post-CRT versus sham mice, shown by increased astrocytosis (Fig. E8A, green, GFAP), more so within the CC than the cortex (Fig. E8C and D). Curiously, there was a region-specific effect of NBD peptide with CRT, demonstrating lower GFAP immunostaining in the CC (Fig. E8C; \( P < .01 \)), but not in the cortex (Fig. E8D; \( P < .0001 \)). Similarly, the TNFα + cell proportion (Fig. E8A and F, red) was higher in CRT versus sham mice in the CC and cortex (Fig. E8E and F). These TNFα + expressing cells demonstrated a neuronal...
phenotype by HuC/D colocalization (Fig. E8Bi and Bii, green). TAT-NBD–treated mice displayed lower inflammation in the CC (Fig. E8E), but a sustained TNFα presence in the cortexes of both CRT mice and CRT + TAT-NBD mice (Fig. E8F). The reactive microglia proportion similarly increased after CRT in all regions examined (IBA-1, red; Fig. 4A–C, D [DG], E [CC], and F [cortex]). This higher IBA proportion was remedied by TAT-NBD, primarily in the CC (Fig. 4E; P < .01) and less so in the cortex (Fig. 4F). Crucially, a subset analysis of dual-immunostained IBA+ Cd11c+ cells demonstrated significant increases in all examined regions of the CRT cohort (Fig 4A–C [green] and G–I), suggesting sustained neuroinflammation. Pretreatment with TAT-NBD before CRT effected the prevention of neuroinflammation in these microglia, particularly within the CC and cortex (Fig. 4H and I). However, despite persistent changes in multiple brain regions after CRT, the CC thickness was not different between groups at 4 months (Fig. E9).

**Longstanding behavioral sequelae from CRT are mitigated by NF-κB blockade**

The effects of cranial radiation on behaviors for anxiety and exploration were assessed 3 months after CRT. The OFT showed that the distance traveled was significantly shorter in CRT versus sham mice (Fig. 5A; P < .0001) and was

---

**Fig. 4.** Long-term reactive microglia and neuroinflammation after CRT. (A–C) IBA-1 (red) proportions were significantly increased 3 months after CRT versus sham in the (A, D) DG, (B, E) CC, and (C, F) cortex. (D–F) The prior addition of TAT-NBD reduced IBA-1 proportions in all regions relative to CRT. (A–C) A subset analysis showed that CD11c expression (green; arrows) within IBA-1+ cells was also higher (G–I) with CRT treatment in all regions, but primarily in (I) the cortex (P < .001), and was lowered by prior TAT-NBD exposure. Size bars denote 25 μm. Data represent mean ± SEM by 1-way ANOVA. From a Tukey’s multiple comparisons test analysis: *Versus sham; †versus CRT; **,**††P < .05, **,**†††P < .01, **,**††††P < .001; n = 3 to 5/group. *Abbreviations:* ANOVA, analysis of variance; CC, corpus callosum; CRT, cranial radiation therapy; DG, dentate gyrus; TAT-NBD, NF-κB essential modulator (NEMO) binding domain (NBD) coupled to the transduction sequence of the HIV-TAT protein; NF-κB = nuclear factor kappa light-chain enhancer of activated B cells. (A color version of this figure is available at https://doi.org/10.1016/j.ijrobp.2020.11.067.)
Fig. 5. TAT-NBD with CRT ameliorates anxiety and movement by open field test assessment. CRT mice demonstrated reduced (A) distance, (B) margin distance, (C) center distance, (D) horizontal activity, (G) clockwise revolutions, (E)
mitigated by anti–NF-kB peptide (Fig. 5A; P < .01). Comparable effects were shown by margin distance (Fig. 5B; P < .0001) and center distance traveled (Fig. 5C; P < .05). There was decreased horizontal activity in CRT versus sham mice (Fig. 5D; P < .01). Sham mice spent more time moving (Fig. 5E; P < .01) and less time at rest (Fig. 5F; P < .01) than CRT mice. There were fewer clockwise revolutions by CRT mice versus those in other treatments (Fig. 5G; P < .05). Significant changes between cohorts were primarily noted in the early test period. Sham mice spent significantly less time in the center of the maze (Fig. 5I; P < .05) and more time at the margins (Fig. 5J; P < .05) versus mice in other groups by 2-way ANOVA, and sham mice spent less time rearing (vertical time) overall than those in other groups (Fig. 5H; P < .05; 2-way ANOVA), although Tukey’s multiple comparisons posttest was unable to discriminate a significant bin. The total rearing time in sham-treated mice also trended lower versus CRT mice (Fig. E10; Student t test). There were significant changes over time but not between groups for number of movements, counterclockwise revolutions, vertical activity, rearing activity, and vertical movement number by 2-way ANOVA (not shown).

EPM results demonstrated that CRT mice ventured a shorter distance than sham mice (Fig. 6A; P < .01), which was rectified by TAT-NBD (Fig. 6A; P < .001), shown by a 1-way ANOVA and Tukey’s test. Similar findings were exhibited in the center and closed distance (Fig. 6B and C) assessment. CRT mice had fewer center entries than sham mice (Fig. 6D; P < .05), which was abrogated in the CRT + TAT-NBD cohort (Fig. 6D; P < .05). The open latency and closed latency times were reduced in CRT- versus sham-treated mice, but were unchanged with previous TAT-NBD exposure plus CRT (Fig. 6E and F; P < .05). CRT mice showed a trend toward reduced line crossings versus sham mice and CRT + TAT-NBD—treated mice (P = .093 and data not shown, respectively); there were no significant findings between groups for mean speed, open or closed entries, open or closed time, or open distance, nor the percent of time spent in the open/closed arms of the maze (data not shown). Thus, CRT mice ventured shorter distances and were less risk averse than sham mice, whereas TAT-NBD appeared to prevent some of the irradiation-resultant behavioral deficits.

Discussion

A single 5-Gy dose of cranial radiation to young rodents has been shown to elicit sustained elevations in inflammation, cell death, and DNA damage, in addition to poorer behavioral outcomes. Although injury to healthy tissue as a consequence of radiation therapy intervention cannot be avoided entirely during lifesaving pediatric cranial cancer treatment, progress is needed to minimize these side effects and to maintain/promote long-term cognitive function after treatment conclusion. Here, we show that the collateral damage resulting from CRT-induced inflammation acts, in part, through NF-kB. Critically, treatment with the anti–NF-kB peptide TAT-NBD before CRT improved subclinical and clinical outcomes, as seen through the direct, early mitigation of NF-kB-p65 brain protein; decreased cell death and some protection of the neuronal progenitor pool in the DG; reduced astrogliosis and microgliosis; fewer TNFα-expressing neurons; and consequent maintenance in movement, changes in risk aversion, and reduced anxiety behaviors months after radiation therapy ended. Altogether, these data support our hypothesis.

CRT induces astrocytosis and microglia activation,1,2,10,12,15,22 illustrated here by the nuclear localization of NF-kB in astrocytes, increases in GFAP+ and IBA-1+ cells, the presence of neuroinflammation by dual CD11c+ IBA-1+ cells, and enhanced cell death in astrocytes and microglia. Others have similarly shown that inhibition of astroglial NF-kB signaling improved gliosis, white matter structure, and memory in a model of vascular dementia,32 supporting our findings. As the resident immune cells of the CNS, microglia activate in response to neuronal damage. Cunningham et al.33 showed that microglia regulate the number of neural precursors in the developing cerebral cortex and that prenatal pharmacologic microglial activation decreased the neural progenitor pool. Hence, the increased presence of apoptotic IBA-1+ cells early after CRT, combined with the increased proportion of IBA-1+ cells in the DG months after CRT observed here, suggests a fundamental alteration of available neuroprogenitor cells and may contribute to long-term cognitive-behavioral declines, particularly as the neurons themselves demonstrate persistent inflammation and are already, presumptively, reduced in number. Indeed, others showed that the elimination of microglia improved cognition after cranial irradiation34 and effected neuro-protection after TAT-NBD treatment following neonatal cerebral rat hypoxia-ischemia (HI), reducing both apoptosis and nuclear and mitochondrial accumulation of p53, as well as directly increasing neuronal survival by decreasing cell death.50 NBD improved long-term sensi-motor and cognitive outcomes in the same model of cerebral HI damage.19 Our data showing reductions of cell death in multiple regions (CIC-3 in the cortex, DG, and CC; TUNEL in the CC; movement time, (H) rearing time, and (J) margin time, and increased (F) rest time and (I) center time 3 months post-CRT relative to sham mice. (A–G) TAT-NBD treatment mitigated some of these effects. (A–J) Data in all panels represent mean ± SEM by 2-way ANOVA. P < .05 between groups. (A–G) Cohort significance from a Tukey’s posttest: *Versus sham; †Versus CRT; *† P < .05; **† P < .01; ***† P < .0001; n = 15 to 16/group. Abbreviations: ANOVA = analysis of variance; CRT = cranial radiation therapy; TAT-NBD = NF-kB essential modulator (NEMO) binding domain (NBD) coupled to the transduction sequence of the HIV-TAT protein; NF-kB = nuclear factor kappa light-chain enhancer of activated B cells.
and both cytoplasmic HMGB1 and HMGB1-negative rates in the DG); differential proportional preservation of proliferation in the DG, CC, and cortex; decreased neuro-inflammation shown by lower CD11c+ IBA-1+ cell proportion in these regions; and accompanying maintenance of exploratory behaviors after CRT by inhibiting NF-κB via TAT-NBD align with others’ data and contribute to knowledge on the importance of reducing inflammation for minimizing negative outcomes resulting from cancer therapy.

The hippocampal dentate gyrus contributes to the spatial exploration of new environments and may inform stress and depression responses. Intriguingly, the DG represents one of few brain regions, in addition to the subventricular zone, shown to exhibit postnatal neurogenesis, which may explain its sensitivity to tissue

---

**Fig. 6.** EPM results demonstrate damage of CRT in developing mouse brains. (A) Distance, (B) center distance, (C) closed distance, (D) center entries, and (E) open latency were reduced, and (F) closed latency increased in CRT versus sham mice at 3 months post-CRT. (A–D) Mice given TAT-NBD showed significant improvements in most tests. Data represent mean ± SEM by 1-way ANOVA. From a Tukey’s multiple comparisons test analysis: * Versus sham; † Versus CRT; † † P < .05, † † † P < .01; n = 15 to 16/group. Abbreviations: ANOVA = analysis of variance; CRT = cranial radiation therapy; EPM = elevated plus maze; TAT-NBD = NF-κB essential modulator (NEMO) binding domain (NBD) coupled to the transduction sequence of the HIV-TAT protein; NF-κB = nuclear factor kappa light-chain enhancer of activated B cells.
insult.\textsuperscript{15,24} HMGB1 is a nuclear factor and secreted protein, released by activated immune cells or dying cells, thereby amplifying inflammatory responses.\textsuperscript{25} Gao et al.\textsuperscript{37} showed that the release of HMGB1 from inflamed cells can act on microglia to activate NF-κB, driving neuro-inflammation and consequent neuronal damage. Similarly, traumatic brain injury was shown to induce the activation of HMGB1/NF-κB, and HMGB1 inhibition reduced NF-κB levels.\textsuperscript{39} Our findings of increased cell death by TUNEL, cytoplasmic HMGB1, and increased presence of CIC-3, and decreased cell proliferation by relative Ki67 proportion, specifically in the DG neuronal progenitor (DCX\textsuperscript{+}) population, illustrate the profoundly negative impact of CRT on the developing brain, and imply a direct mechanism for cognitive-behavioral diminishment after radiation therapy. Importantly, TAT-NBD significantly protected against cell death, and showed location-specific protection in the loss of cellular proliferation, with some restoration in the DG but not in the CC. NBD specifically blocks proinflammatory activation of the IKK complex without inhibiting basal NF-κB activity,\textsuperscript{13} which supports the dual role of NF-κB. This dual role is strengthened by our data showing that animal survival after tumor implantation was not affected by TAT-NBD when administered with RT, which is in agreement with other reports of NF-κB inhibition in cancer therapy.\textsuperscript{39}

Radiation-associated hippocampal damage at an early developmental age has been shown to elicit worsened cognitive outcomes\textsuperscript{38} and is associated with inflammation, reduced neurogenesis, and microglial activation.\textsuperscript{15} Lesions in the ventral DG have previously been shown to correlate with alterations in anxiety-based behaviors by the EPM test.\textsuperscript{22} Genetic knockout of exon IV of the p75 neurotrophin receptor (p75NTR) yielded anxiolytic changes in both EPM and OFT tests, as well as width reduction in the dorsal, but not ventral, DG.\textsuperscript{40} In a model of alcohol withdrawal—induced anxiety, supplementation of the serotonin 5-hydroxytryptamine was shown to modulate anxiety behaviors by EPM and OFT and to restore neurogenesis by BrdU incorporation and the density of DCX\textsuperscript{+} immature neurons in the dorsal DG.\textsuperscript{31} Traumatic brain injury after juvenile radiation exposure demonstrated cognitive impairment and spatial memory retention deficits, with particular damage found in the hippocampus, specifically showing a reduction in the number of new bromodeoxyuridine (BrdU)+ neurons (NeuN+ cells) and increased activated microglia proportion.\textsuperscript{17} Within the corpus callosum, demyelination and structural abnormalities due to cuprizone administration were associated with abnormal anxiety-like behavior without impaired spatial learning or memory, concurrent with myelin thinning.\textsuperscript{41} In a Spock3 knockout model, comparable structural changes were noted in the CC, and the mice demonstrated altered anxiety-like and social behaviors; Spock3 putatively contributes to neuronal development.\textsuperscript{42} CRT itself has widely been shown to thin the CC myelinated nerve fiber layer after 10 or 30 Gy fractionated dosing,\textsuperscript{3,14} yet even mild myelin disruption has been shown to alter neural progenitor proliferation in the subventricular zone, consequently altering behavior.\textsuperscript{43} CRT has also been demonstrated to increase inflammation by TNFα,\textsuperscript{14} and reactive astroglia by GFAP expression in the CC,\textsuperscript{14} cerebral cortex,\textsuperscript{3} and hippocampus.\textsuperscript{2,22} Considering the functional roles of both the cortex and the corpus callosum in motor and sensory integration, processing, and cognition, it is unsurprising that destructive cell turnover changes and both acute and sustained inflammation in these regions are paralleled by negative impacts on behavior. Our results are striking given the otherwise lack of overt toxicologic or morphologic effects manifested from this CRT dose, shown here by only a small blunting in total body weight gain, and the nonsignificant change of the CC thickness 3 months after 5 Gy of CRT; the impacts from higher CRT exposure or a larger NBD dose compel further interrogation. Regardless, the implications of these proof-of-principle data from relatively noninvasive, intranasal TAT-NBD treatment should generate new avenues for pediatric neural protection and inflammation blockade.

As with any research study, limitations to our interpretations exist. Direct extrapolations from young animals to human experiments are limited by radiation-dose versus animal-size considerations and developmental differences, explaining our radiation dosing paradigm. We also have not examined sex differences in our experimental cohorts and will include female mice in the future. Moreover, we did not observe the canonical degradation of IkBα using Western blot and immunofluorescence; however, others have reported IkBα-independent NF-κB activation,\textsuperscript{44} specifically in radiation injury,\textsuperscript{45} and the kinetics and amplitude of IkBα phosphorylation may be conditionally dependent on cytokine activation or DNA damage.\textsuperscript{7} Although we focused on radiation-induced inflammatory responses through the expression of TNFα, CRT-induced DNA damage alternatively might be responsible for NF-κB activation;\textsuperscript{7} ultraviolet light or doxorubicin exposure similarly activate NF-κB, which suppresses antiapoptotic genes and represses TNF transactivation of NF-κB.\textsuperscript{8} Furthermore, the empirical but untitrated TAT-NBD dosage, as well as drug durability, are still unknown. These reported limitations present opportunities for future investigation. Conclusions Results suggest a role for NF-κB modulation by NBD peptide in the reduction of neuro-inflammation and mitigation of behavioral complications after radiation therapy.

References


5. Probert L. TNF and its receptors in the CNS: The essential, the desirable and the deleterious effects. *Neuroscience* 2015;302:2-22.


