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The Characterization of Chimeric Chaperone Flagrp170 as a Novel Radioprotectant

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This thesis is submitted in partial fulfillment of the requirements for the Master of Science degree at Virginia Commonwealth University

By

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Abstract

THE CHARACTERIZATION OF CHIMERIC CHAPERONE FLAGRP170 AS A NOVEL RADIOPROTECTANT

By Tyler Nguyen, M.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2017

Major Director: Dr. Xiang-Yang (Shawn) Wang, Ph.D., Professor, Department of Human and Molecular Genetics

Radiation therapy (RT) is restricted by toxic effects on adjacent normal tissue, which limits RT efficacy in cancer treatment. Damage to normal tissue, such as radiosensitive intestine and bone marrow compartments, results in acute radiation damage. To reduce normal tissue injury in the setting of RT, we examine the potential radioprotectant, Flagrp170, a chimeric protein. Flagrp170 is comprised of glucose-regulated protein-170 (Grp170) and a NF-κB activating sequence derived from flagellin. We show that Flagrp170 can protect normal tissues post irradiation, indicated by TUNEL and clonogenic assays. However, treatment with Flagrp170 does not influence tumor response to RT. Studies indicate that Flagrp170 activates the transcription factor NF-κB, a strong pro-survival signal. In addition, Flagrp170 can induce production of radioprotective cytokines as well. Data suggests that Flagrp170 has potential as a novel radioprotectant in the setting of RT. The combination of Flagrp170 therapy and RT may lead to improved treatment outcomes.

Introduction

Exposure to high doses of ionizing radiation (IR) results in genetic damage, mutation, and cell death through apoptosis or mitotic death.[1] Radiation induced cell death occurs within the first cell division or a few divisions thereafter.[2] Thus, highly proliferative cells are often the most sensitive towards genotoxic damage. Modern medicine takes advantage of this highly targeted radiation induced damage in radiation therapy (RT) as cancerous cells are inherently mitotically active[DS1] compared to most normal tissue. In addition, tumor cells have little means of recovery from genetic damage, whereas adjacent normal tissue may recover if the therapeutic radiation dosage is tolerable or if the cellular supply of functional cells are regenerated by progenitor cells. As of today, nearly 50% of patients diagnosed with cancer will eventually receive RT throughout the course of their treatment. RT is used to shrink tumors, cure early stages of cancer, prevent cancer from recurring, or improve symptoms of advance stages of cancer such as pain or obstruction.[3] RT, however effective and widespread, is still limited by the genotoxic effects on normal adjacent tissue.

Acute early reactions to radiotherapy usually occur within a few weeks of treatment. This type of radiation damage is most prominent in normal tissue with high cellular turnover, such as the gastrointestinal tract and the bone marrow compartment which are continuously regenerating.[1] Radiation[DS2]-induced symptoms occur when functional cells are lost and are not recovered quickly enough to maintain homeostasis. The slow recovery of the gastrointestinal compartment after radiation is likely due to damage incurred by the clonogenic crypt cells. Repression of the hematopoietic compartment is due to multipotent progenitor cell death.[2]

Though these early acute reactions to RT rarely result in death, they restrict the effective therapeutic radiation dosage and may lead to chronic side effects such as fibrosis and persistent mucositis. Studies have revealed that aggravated acute reactions can lead to consequential late effects (CLE) as well. CLE often occurs after organ systems in which physiological barriers cease to protect against mechanical or chemical stress (gut, urinary bladder, oral mucosa, and the skin).[4-7] Persistent acute reactions cause a breakdown of the mucosal barriers, which often results in additional damage to connective and endothelial tissue. The additional damage incurred by connective and endothelial tissue sensitizes patients to CLE.

In treating cancer, the tolerable radiation dosage for separate human organs are based on published guidelines which clinicians follow. However, these guidelines have been largely derived from retroactive data and empirical observations.[8] In severe cases[DS3], it is necessary to operate outside of the established guidelines and treat patients with unconventional and aggressive irradiation protocols. Unfortunately, in such cases, aggressive radiation treatments are often associated with the aggravated acute reactions, which entails more severe and longer lasting symptoms as well as higher risk of CLE. The exact tolerable RT dosage is also difficult to pinpoint since radiation sensitivity can differ from person to person. Although there has been an effort to create genetically predictive tests[DS4], there has been no convincing evidence for the applicability of these tests.[9] This highlights the importance of radioprotectants. Radioprotectants can improve the efficacy of RT either by reducing the severity of radiation sickness, improving normal tissue recovery, or widening the therapeutic window of RT with higher tolerable dosages. Currently, there is only one radioprotectant approved by the FDA, Amifostine, a prodrug which becomes a[DS5] reactive oxygen species (ROS) scavenger once it is hydrolyzed by alkaline

phosphatases. However, Amifostine produces inconsistent radioprotective results when applied to a range of radiotherapies.[10] Consequently, the treatment is only FDA approved for radiation therapy treating head and neck cancers as Amifostine consistently reduces xerostromia.[11] Amifostine also has limiting side effects such nausea, vomiting, and most severely, hypotension.[12]

In this project, we characterize Flagrp170, a chimeric chaperone protein, as a potential radioprotectant. Previous studies have characterized Flagrp170 as an immune modulator, highly effective in mobilizing and restoring antitumor immunity. Flagrp170 is derived from a large chaperone protein, glucose-regulated-protein-170 (Grp170), conjugated with a flagellin derived pathogen-associated molecular pattern (PAMP).[13] Grp170 has been previously shown to be an effective immune-stimulatory adjuvant for therapeutic immunizations against cancers such melanoma and prostate cancer in mice.[14] The flagellin derived PAMP conjugated to Grp170 is danger signal that acts as a ligand that simulates the immune system through[DS6] the Toll-like receptor 5 (TLR5). The TLR5 signaling pathway culminates in the activation of transcription factor, NF-κB.[15] Flagrp170 may act as an effective radioprotectant as the activation of NF-κB is known method of radioprotection.[1,16,17] In addition, the molecular chaperone functions of Flagrp170 may further enhance the ligand/receptor interactions between the NF-κB activating PAMP and TLR5.[13]

The innate immune system relies on a set of evolutionary conserved pattern recognition receptors (PRR) to recognize a variety of microbial components and elicit an appropriate immune response. Toll-like receptors are among an assortment of PRRs located on various immune cells such as macrophages, dendritic cells, B cells and neutrophils as well as non-immune cells like fibroblasts, epithelial cells, and keratinocytes.[15] TLRs can recruit a set of adapters that triggers a

downstream signaling cascade after recognizing a PAMP that leads to the activation of the NF-κB pathway. TLR5, a member of the TLR family, senses the microbial component, flagellin. Once activated, TLR5 recruits myeloid differentiation primary-response protein 88 (MyD88), which in turns recruits interleukin receptor associated kinase 4 (IRAK4) associated with IRAK1. IRAK4 will [DS7]phosphorylate IRAK1. IRAK1,[DS8] once phosphorylated, associates with tumor necrosis factor receptor-associated factor 6 (TRAF6). The complex formed by p-IRAK1 and TRAF6 will dissociate from the receptor[DS9] and form yet another complex with transforming growth factor beta-kinase 1 (TAK1), TGF-beta activated kinase 1 binding protein 1 (TAB1), and TAB2. TAK1 and TAB2 are phosphorylated as the complex forms. The phosphorylation of TAK1 and TAB2 leads to the ubiquitylation of TRAF6 which activates TAK1. TAK1 phosphorylates the inhibitor of nuclear factor κB (IκB)-kinase complex (IKK complex), which is composed of IKKα, IKKβ, and IKKγ. The IKK complex will phosphorylate IκB, which is constitutively bound[DS10] to transcription factor NF-κB. phosphorylated, IκB will undergo ubiquitylation and subsequently degrade, freeing NF-κB to translocate to the nucleus.[18-20]

Once activated, NF-κB induces the transcription of cytokines and chemokines necessary for the immunologic and hematopoietic response which may alleviate radiation damage by enhancing recovery from acute radiation syndrome. NF-κB can induce the transcription of radioprotective cytokines and growth factors such interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), granulocyte macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF). Previous studies have shown IL-6 plays a significant role in hematopoietic recovery after irradiation and is an essential contributor to natural radioresistence.[21] Like IL-6, TNF-α has been also been shown to be a natural contributor to

radioresistance[DS11]. Anti-TNF-α antibodies reduces survival of irradiated mice and studies have shown that administration of TNF-α shortly after irradiation significantly protect irradiated mice. In addition, TNF-α can also induce the transcription of manganese super oxide dismutase (MnSOD), an effective ROS scavenger.[22] GM-CSF and G-CSF induces radioprotection by stimulating progenitor stem cells. These growth factors induce proliferation and differentiation in the hematopoietic compartment, reducing and preventing radiation induced myelosuppression and neutropenia.[23] G-CSF treatment has been shown to accelerate hematopoietic regeneration and enhance survival after lethal irradiation in mouse models.[24]

DNA damage caused by radiation induces apoptosis,[DS12] most prominently in progenitor of the gastrointestinal and hematopoietic compartments. A substantial lack of intestinal crypt cells and hematopoietic progenitor cells is the prevalent cause of acute radiation syndrome. Untreated, acute radiation reactions can cause oral and gut epithelial mucositis as well as hematopoietic repression. Prolonged mucositis can disrupt the integrity of mucosal barriers. Hematopoietic repression leaves the immune system vulnerable to opportunistic infections.[2] We predict that Flagrp170 can effectively protect against radiation damage and acute radiation effects by reducing apoptosis through the activation of NF-κB which incites the production of anti-apoptotic proteins as well as radioprotective cytokines. Studies have shown that increasing NF-κB activity inhibits apoptosis in a variety of normal cells lines.[16,25] Cancer cell lines often have NF-κB constitutively active as a mechanism of escape. The mechanisms behind the anti-apoptotic effects of NF-κB still has not[DS13] been completely elucidated, however, it is understood that NF- induces numerous agents that plays a role in desensitizing cells to apoptotic signals. Several genes regulated by NF-κB has been identified and shown to have a significant role in blocking

apoptosis. These genes include the anti-apoptotic members of Bcl-2 family (A1/Bfl1, Bcl-Xl, and NRl3) and inhibitors of apoptosis (cIAP-1, cIAP-2, TRAF1, and TRAF2).[25,26] The anti-apoptotic Bcl-2 family members promote cellular survival and inhibit[DS14] pro-apoptosis signals.[27] Inhibitors of apoptosis cIAP-1/2 proteins and TRAF1/2 bind caspases and suppress their activation, however the exact mechanism that inhibits their activity has not been well defined.[28,29] Previous studies have demonstrated the effect of radioprotective agents that activate NF-κB.[30,31] Flagellin injected prior to irradiation could reduce morbidity in lethally irradiated mice as well as rescue bone marrow cells. Vijay-Kumar et al. revealed that flagellin induced radioprotection required TLR5 and MyD88, thus it[DS15] is likely to require NF-κB activation.[31] Similarly, research conducted on CBLB502, an agonist of TLR5, concluded its effective radioprotective capabilities in mouse and primate models.[30]

Chaperone proteins, otherwise known as stress proteins, support the folding of client proteins or bind folding intermediates to prevent aggregations. Molecular chaperones can maintain and stabilize the structure, conformation, and function of conjugated proteins. In cancer immunotherapy, chaperone proteins can induce a superior immune response when conjugated with an antigen compared to an antigen[DS16] alone because of their natural function and ability to hold and condense multiple antigens.[32] As immune modulators, they are effective in binding an assortment of antigens, allowing for the efficient uptake of antigen via receptor specific interactions, and can interact with or stimulate innate immune components.[33,34] Of various families of chaperone proteins, Grp170 has been shown to have a superior capacity to hold client proteins and has been shown to induce a more significant antitumor response.[35,36] In addition, Grp170 has been previously shown to facilitate the sensing of PAMP by intercellular

TLR-9.[37] Flagrp170 retains the protein holding capabilities of Grp170 and its antigen cross-capabilities.[13]

We reason that Flagrp170 can effectively protect normal cells after radiation by reducing apoptosis and inducing radioprotective cytokines via the activation of NF-κB. Furthermore, Flagrp170 can effectively facilitate signal induction of TLR5, stabilize and perhaps enhance the function of the flagellin derived sequence, and induce a stronger cytokine induction via receptor specific interactions.

Materials and Methods

Mice strains and Cell lines

C57BL/6 and Balb/c mice were purchased from National Cancer Institute Mouse Repository (Frederick, MD). NF-κB-Luc-Tg mice was purchased from Taconic (Albany, NY). All experimental procedures were conducted according to the protocols approved by the VCU Institutional Animal Care and Use Committee (IACUC). CT-26 cells and SF-21 cells were purchased from American Type Culture Collection (Manassas, VA) and Clontech (Mountain View, CA) respectively. CT-26 cells and SF-21 cells were maintained by RPMI1640 media (GE Healthcare, Glen Allen, VA) with 10% FBS and 1% penicillin streptomycin and TNM-FH media (GE Healthcare, Glen Allen, VA) with 10% fetal bovine serum (FBS) and 1% penicillin streptomycin respectively.

Protein Expression and Purification

SF-21 cells were cultured with complete TNM-FH media in 15 mm plates. After confluency reached 90%, SF-21 cells were infected with Flagrp170 encoded baculovirus[DS18] (Invitrogen, Carlsbad, CA) per plate and cultured for an additional 48-72 hours until cells were granular and non-adherent. Between 48-72 hours SF-21 cells were extracted and lysed with prepared lysis buffer with proteinase inhibitors PMSF, Pepstatin A, benzamidine, and Levpeptin for 4 hrs at The lysate was then centrifuged at 10,000 RPM at 4°[DS20]C for 1 hour. After centrifugation, the supernatant was incubated with Ni-NTA agarose beads (Qiagen, Hilden, Germany) overnight. The Ni-NTA agarose beads was filtered through the Poly-Prep Chromatography Columns (BioRad, Hercules, CA). 10 mL of binding was added to the column followed by 5 mL of wash buffer. Lastly, 10 mL of elute buffer was pour though the column and collected. The elute buffer diluted with

1x phosphate buffered saline (PBS) and was concentrated using a Vivaspin 50,000 MVCO PES column (GE Healthcare, Glen Allen, VA).

SDS-page and Western blot

Purified protein was diluted with 5x SDS, denatured and loaded on a 15% SDS-PAGE followed by Coomassie Brilliant Blue staining (BioRad, Hercules, CA). Image was processed and analyzed via ImageJ, image processing software. For Western Blot analysis, protein sample was transferred from a 15% SDS-PAGE to a nitrocellulose membrane (BioRad, Hercules, CA). After transfer, the membrane was blocked with 5% bovine serum albumin (BSA) in milk followed by incubation with primary antibody overnight. Membrane was washed with 1x Tris-buffered saline-Tween-20 (TBST) and then treated with secondary HRP-conjugated rabbit IgG antibody (Santa Cruz, Dallas, TX). Membrane was then treated with Peroxide solution and Luminal Enhancer solution (Thermo Fisher Scientific, Waltham, MA) to visualize bands. Images were processed on X-ray films (Phenix, Richmond, VA) using SRX-101A medical image processor (Konika Minolta, Tokyo, Japan).

Preparation of Bone marrow derived macrophages

Balb/c mice were sacrificed via CO_2 followed by cervical dislocation. Bone marrow was flushed from mice femurs using a 6 mL syringe with 22 gauge needle containing serum free RPMI1640 media. Bone marrow was suspended in single cell suspension. Cells were then centrifuged at 1500 RPM for 5 minutes at 4˚[DS21]C. Serum free RPMI1640 media was removed and pellet was suspended with 70% RPMI1640 media and 30% L929 condition media containing G-CSF. 2×10^6 cells were plated in a 6 well plates and incubated at 37˚[DS22]C and 5% CO_2 for 7 days.

Preparation of Bone marrow derived dendritic cells

Bone marrow derived dendritic cells were prepared in a similar manner to bone marrow derived macrophages, however the culture media used to induce dendritic cell differentiation is 98% RPMI1640 media and 2% B78H1 condition media containing GM-CSF.

qPCR

Total RNA was extracted via TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA) following standard procedures from Thermo Fisher (Waltham, MA). Reverse transcription preceded with materials (RNase Inhibitor, dNTP mix, Reverse Transcriptase, Oligo$(dT)_{18}$ Primer) from Thermo Fisher (Waltham, MA). Reverse transcriptase PCR reaction occurred on MJ mini personal thermal cycler (BioRad, Hercules, CA). qPCR was conducted using materials and primers from Applied Biosystems (Foster City, CA) and processed on 7900 HT Fast Real Time PCR system (Applied Biosystems, Foster City, CA). Gene expression was relative to β-actin expression and normalized by negative control groups by standard (−ΔΔCT) calculation.

ELISA

Capture antibody (Biolegend, San Diego, CA) diluted in 1x Coating buffer (Biolegend, San Diego, CA) in ddH_2O was pipetted at 100 µL per well in the ELISA MAX uncoated plate (Biolegend, San Diego, CA) and incubated at 4˚[DS23]C overnight. Coating buffer was removed and plate was times with 0.1% TBST in ddH_2O by ELx50 automation (BioTek, Winooski, VT). All further wash procedures will proceed in a similar manner. Blocking buffer was prepared with 1x blocking buffer solution (Biolegend, San Diego, CA) in PBS. Plate was then blocked with 200 µL blocking buffer per well and incubated at room temperature (RT) for 1 hour. After 1 hour, blocking buffer was removed and plate was washed. After wash, 100 µL of sample were incubated in wells at RT for

2 hours. Samples were removed and plate was washed. After wash, the plate was incubated with Avidin-HPR solution (Biolegend, San Diego, CA) for 30 minutes. Avidin-HRP solution was removed and ELISA plate was thoroughly washed. TMB substrate solution (Biolegend, San Diego, CA) was incubated in ELISA plate in the dark at RT for 5-30 minutes depending probe.

Reporter Mice Imaging

NF-κB-Luc-Tg mice were treated protein or vehicle i.p. 1 hour prior to imaging. 10 minutes prior to imaging mice sedated via isoflurane (Henry Schein, Melville, NY) dispersed with an IVIS isoflurane vaporizer. Once sedated mice were inject i.p. with 200 µL of D-luciferin at concentration of 30 mg/mL (GoldBio, Olivette, MO). After injection mice were allowed to regain consciousness for 5 minute of activity in order to circulate luciferin. After 5 minutes mice were again sedated and placed in to the IVIS 200 In Vivo Imaging System (PerkinElmer, Waltham, MA). Image and fluorescence was processed by the IVIS living image software. ROI photon/sec measurement was determined by IVIS living image software as well. Immediately after imaging, mice were sacrificed via CO_2 followed by cervical dislocation and organs were harvested. Within a 30 minute timeframe beginning with cervical dislocation, liver, spleen, small intestines, and colon samples were harvested and placed on clean black paper. Organs were then placed into the IVIS 200 In Vivo Imaging system. Image and fluorescence from organs were processed by the IVIS living image software. ROI photon/sec measurement was determined by IVIS living image software as well.

Radiation Induced Tissue Injury Model

Mice were treated with protein or vehicle i.p. 1 hour prior to irradiation. Shortly prior to irradiation, mice were sedated with an i.p. injection of 200 µL of ketamine. Once fully sedated, mice were placed sparsely into a container which was placed into Cesium irradiator for a lethal dose of whole body radiation. Mice fully recovered from ketamine sedation before undergoing sacrifice or examination. Bone marrow and intestines was collected. Bone marrow cell viability was determined via clonogenic assay and normal tissue damage was measure via TUNEL stain.

Radiation Treatment of Tumor-bearing Mice

Tumors were induced via injection of 5 x 10^6 of CT-26 cells s.c. into the abdominal area. Tumor growth was recorded every 4 days. When tumors reached 5 mm in size mice were randomized. Once tumor were of size, mice were treated with either protein or PBS injection. Shortly prior to irradiation, mice were sedated with an i.p. injection of 200 µL of ketamine. Mice were placed into a container and restrained. Non-tumor-bearing portions were protected from radiation exposure by lead plate. Mice underwent a sub-lethal partial body irradiation dose. Mice fully recovered from ketamine sedation before undergoing sacrifice or examination. Normal tissue was collected. Radiation damage was measured via TUNEL staining.

TUNEL/DAPI Immunohistochemistry

Microscopic sections were incubated in 4% Paraformaldehyde in 1x PBS for 20 minutes. Slides were then rinsed twice in PBS and washed in PBS for 30 minutes at room temperature. After slides were washed, they were then incubated in 0.1% Triton and 0.1% Sodium Citrate for 2 minutes at 4°[DS24]C. Slides are then rinsed twice in PBS and dried. Once completely dried wax was drawn around the tissue samples on the slide. The TUNEL solution (Roche, Basel,

Switzerland) was prepared according to manufacturer's protocol. 40 µL of TUNEL staining solution was pipetted on to tissue section and incubated in the dark for at 37°C for 1 hour. 1 hour slides were washed for 5 minutes in PBS 3 times. Afterwards slides were dried and cover slipped using mounting media with DAPI (Vector Laboratories, Burlingame, CA). Fluorescence intensity was determined by ImageJ.

Clonogenic Assay

Balb/c mice were sacrificed via CO_2 followed by cervical dislocation. Bone marrow was flushed from mice femurs using a 6 mL syringe with a 22 gauge needle containing serum free RPMI1640 media. Bone marrow was suspended in single cell suspension. Cells were then centrifuged at 1500 RPM for 5 minutes at 4°C. Serum free RPMI1640 was removed and pellet was re-with 98% RPMI1640 media and 2% B78H1 condition media containing GM-CSF. Bone marrow cells were plated at 5 x 10^6 cells per plate in 6 well plate and 1 x 10^6 per plate in 12 well plate. Cells were incubated at 37°C and 5% CO_2 for 10 days. After 10 days, culture media was and cells were incubated with crystal violet dye for 30 minutes. After 30 minutes, crystal violet dye was removed and washed with ddH_2O and left to dry. Cell count was automated with ImageJ.

Effect of Flagrp170 on Tumor Response to Radiation Therapy

Tumors were induced via injection of 5 x 10^6 of CT-26 cells s.c. into the right dorsal flank of mice. Tumor growth was recorded every 4 days. When tumors reached 5 mm in size mice were randomized and injected s.c. with protein or vehicle. 1 hour post injection, mice underwent local radiation therapy directed at the flank where the tumor resided. Treatment with either protein with RT or PBS with RT was repeated the following day and once more the day after. A group of

tumor-bearing mice received neither injection nor RT. Experiment was terminated when tumor completely recessed or the diameter reached 2 cm.

Statistical Analysis

Statistical significance was determined by the Student t-test[DS28] or ANOVA. P values less than were considered to be statistically significant.

Figures

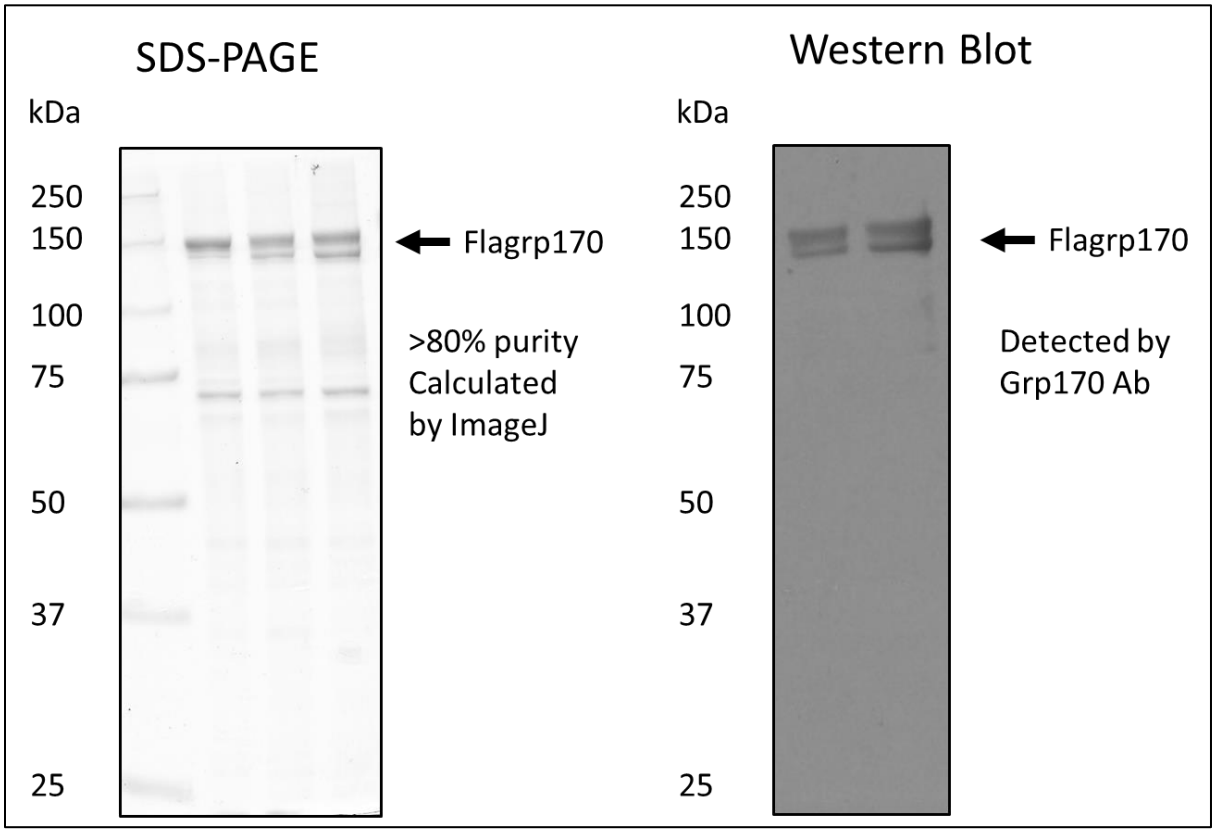
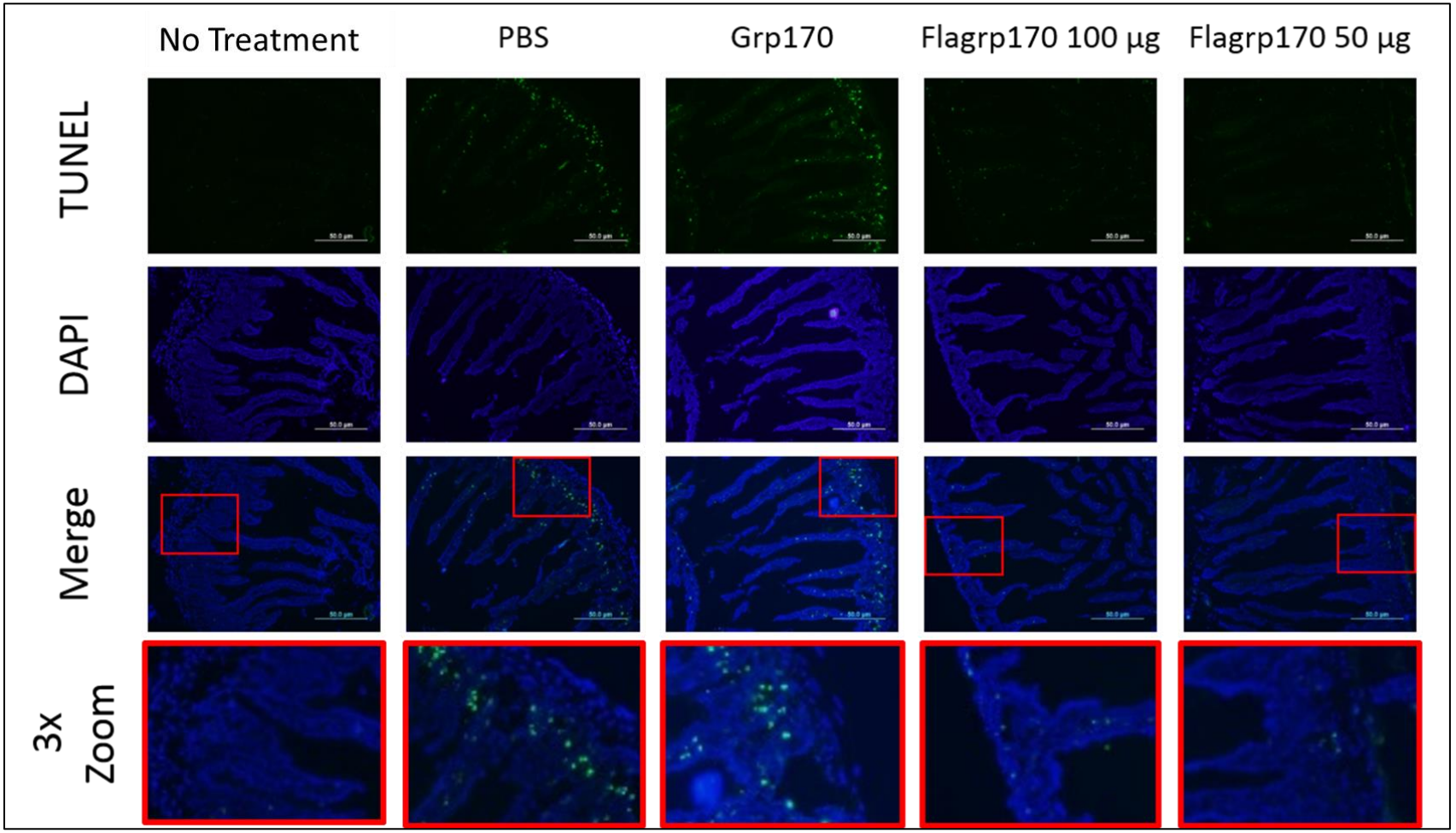


Figure 1. Characterization of Flagrp170 preparation. Flagrp170 protein stock was purified from SF-21 cells infected with baculovirus encoding Flagrp170. Flagrp170 protein from infected SF-21 cell lysate was extracted via nickel affinity chromatography. Flagrp170 was ran through SDS polyacrylamide gel (SDS-PAGE) to determine purity which was estimated by band density via ImageJ, image processing software. Purified protein also was analyzed via western blot with Grp170 antibody detection to confirm that purified protein was indeed Flagrp170.

A1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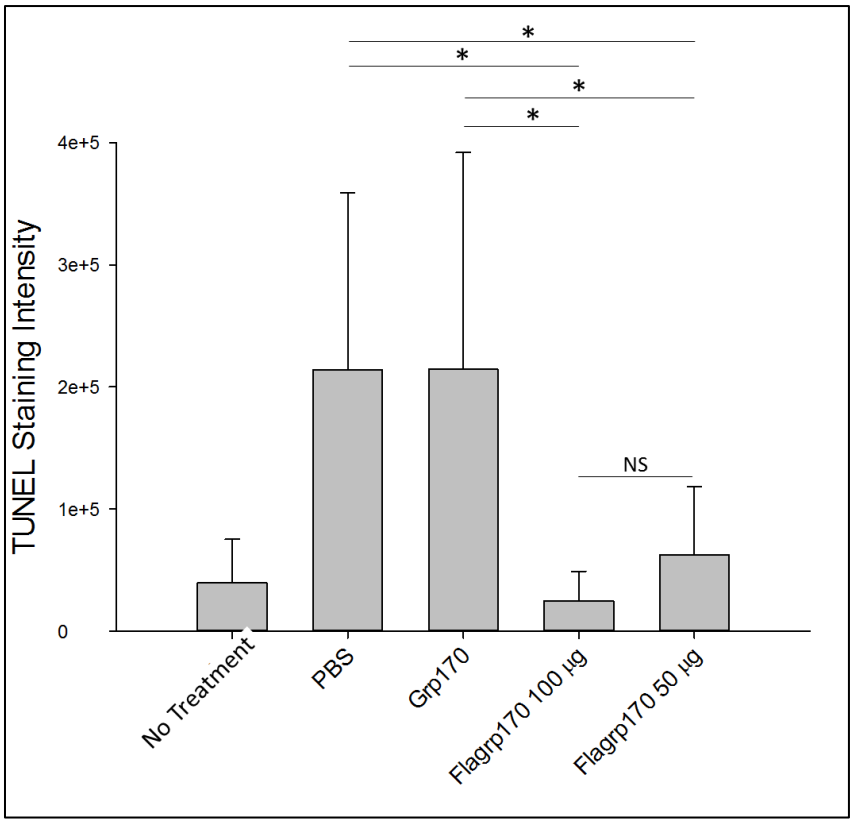
Flagrp170 50 µg
TUNEL
DAPI
Merge
3x Zoom

A2


TUNEL Staining Intensity
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3e+5
2e+5
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0
*
*
*
*
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Grp170
Flagrp170 100 µg
Flagrp170 50 µg

B

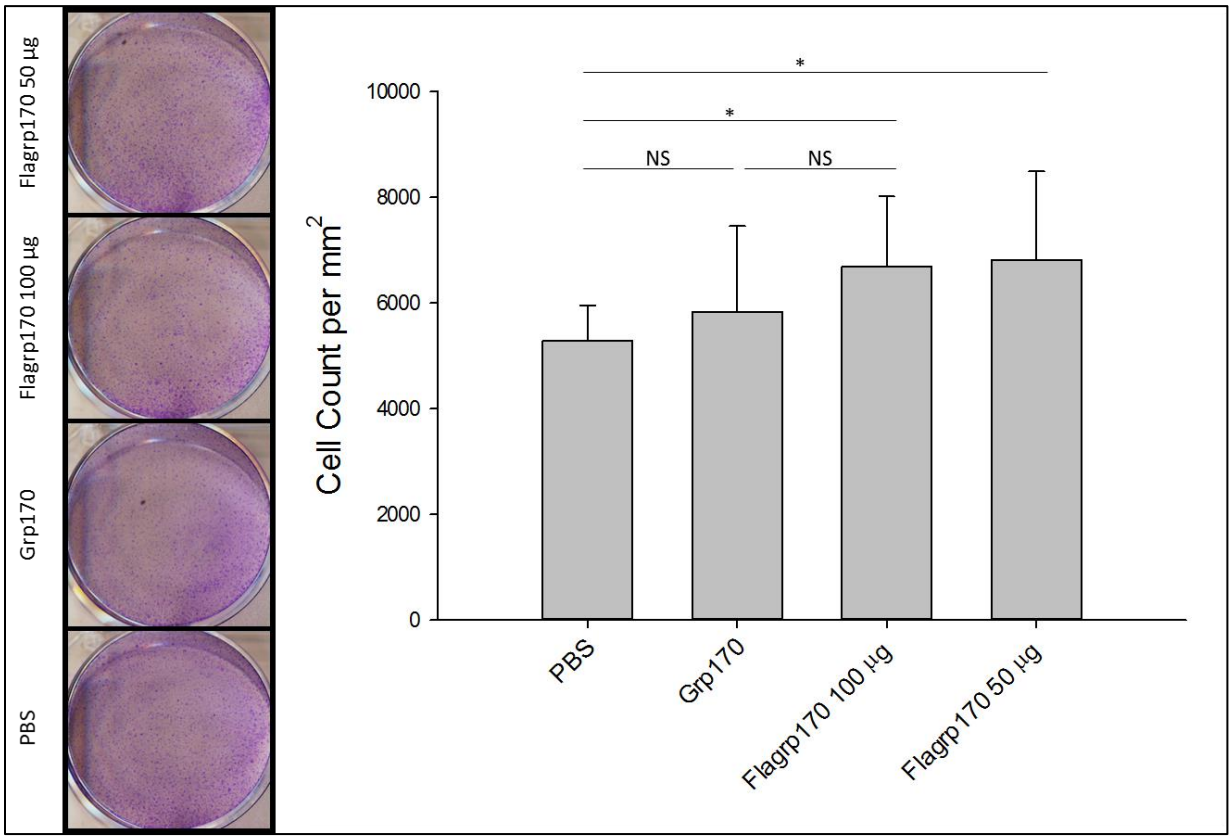
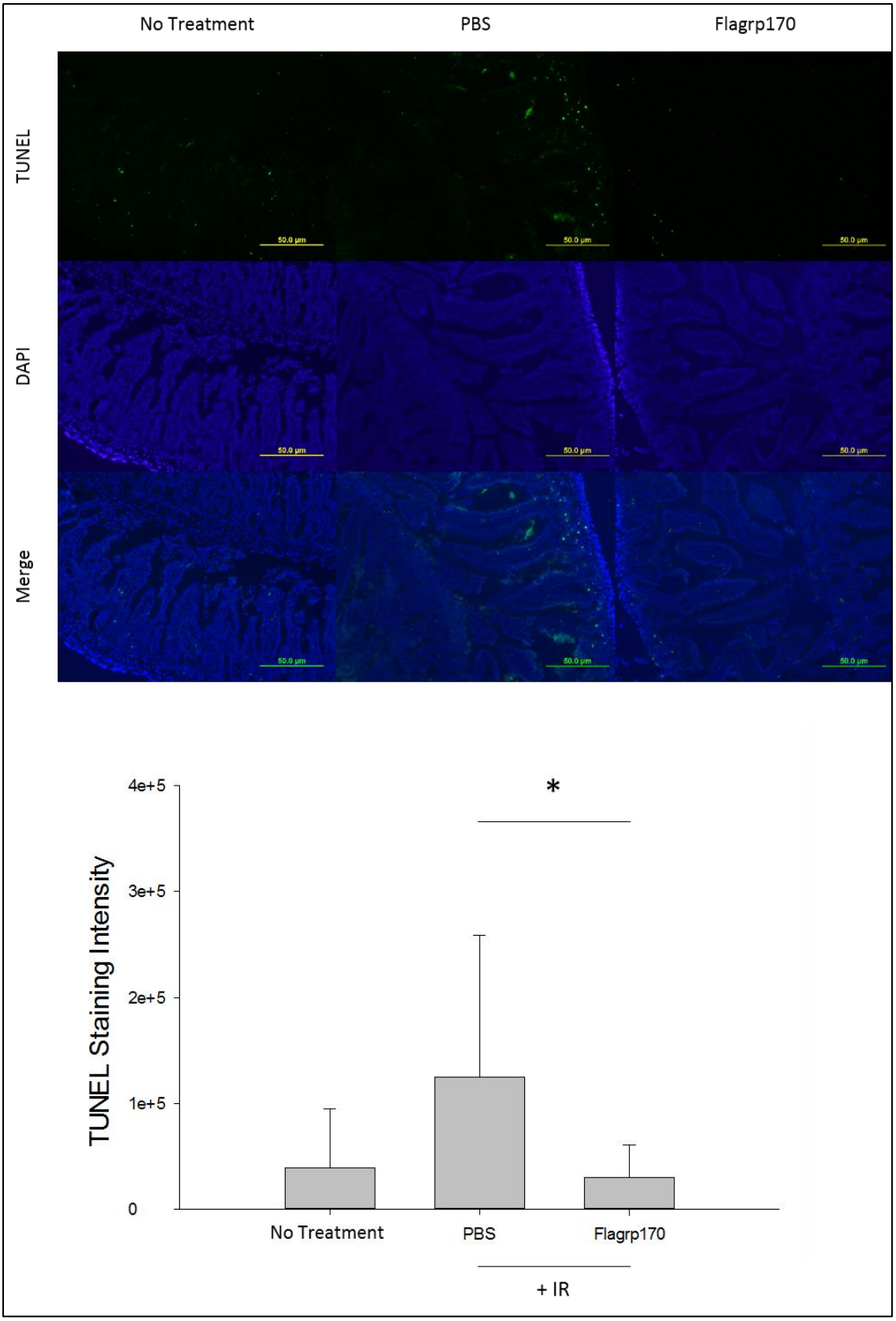

Flagrp170 50 µg
Flagrp170 100 µg
Grp170
PBS
Cell Count per mm2
10000
8000
6000
4000
2000
0
*
*
NS
NS
PBS
Grp170
Flagrp170 100 µg
Flagrp170 50 µg

Figure 2. Flagrp170 reduces normal tissue injury and improves bone marrow cell viability after irradiation. (A) Balb/c mice (n=2) were intraperitoneally (i.p.) injected with PBS, Grp170, Flagrp170 (100 µg), or Flagrp170 (50 µg). All mice underwent a lethal dose of ionizing whole body irradiation (9 Gy) via cesium irradiator 1 hour after treatment. Random intestine sections were collected, encased in OCT and stored in -80°[DS29]C. Tissue samples were sectioned on microscopic slides and apoptosis was analyzed by TUNEL assay. Results indicate there a significantly higher frequency of apoptotic cells in intestine samples from irradiated PBS treated mice and Grp170 treated mice when compared to both groups of Flagrp170 treated mice. (B) Bone marrow samples were harvested 5 hours after irradiation from the femora of all mice groups for clonogenic assay. Bone marrow cells (BMCs) were plated at 5 x 10^6 cells per well. All BMC samples were cultured with in complete RPMI1640 media with GM-CSF in six well plates. BMCs were cultured for 10 days. On the 10^{th} day, each plate was stained with crystal violet dye and cell count was estimated via ImageJ. Results indicate there is a significantly higher cell per mm^2 in the plates cultured with BMCs from Flagrp170 treated mice when compared to BMCs from PBS treated mice. There was no significant difference between the Grp170 treated group and the Flagrp170 treated group. There was no significant difference between the Grp170 treated group and the PBS treated group. *, $P < 0.05$; NS, not significant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Flagrp170
TUNEL
DAPI
Merge
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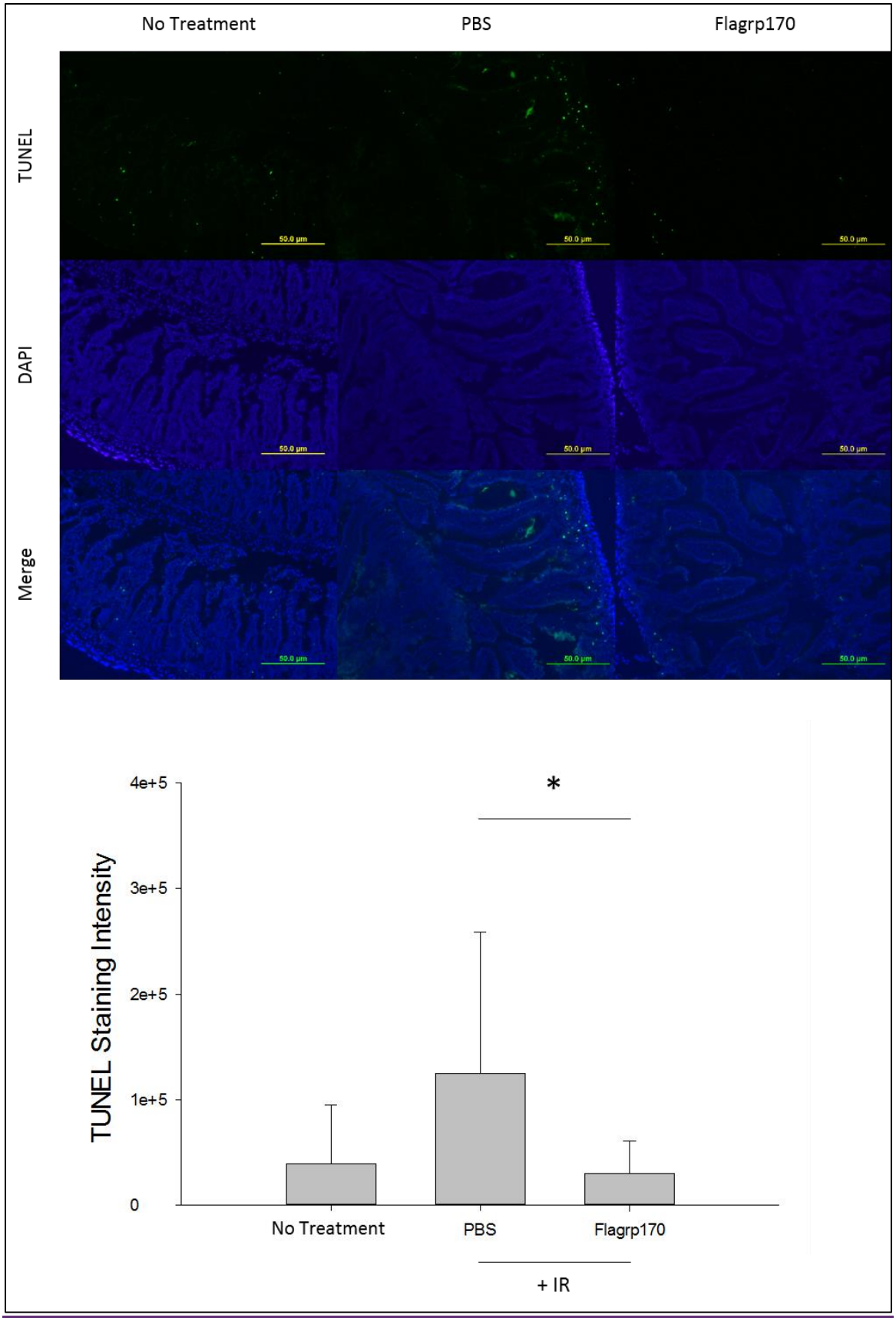
+ IR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50.0 µm
TUNEL Staining Intensity
4e+5
3e+5
2e+5
1e+5
0
*
No Treatment
PBS
Flagrp170
+ IR

Figure 3. Flagrp170 has a protective effect on normal tissue in the context of radiation therapy. Balb/c mice (n =3) were subcutaneously (s.c.) injected in the abdominal area with 5 x 10^6 cells CT-26 to induce tumor. After 1 week, mice were i.p. injected with 50 µg of Flagrp170 or PBS. 1 hour after treatment, Flagrp170 and PBS treated mice group underwent 10 Gy of partial body irradiation (PBI) in the abdominal area where the tumor resided. Mice left un-irradiated were used as controls. Random intestines were collected, encased in OCT, and stored in -80°[DS30]C. samples were sectioned and stained with DAPI/TUNEL. Results shows a higher frequency of apoptotic cells in irradiated intestines from PBS treated mice when compared to Flagrp170 treated mice. *, $P < 0.05$

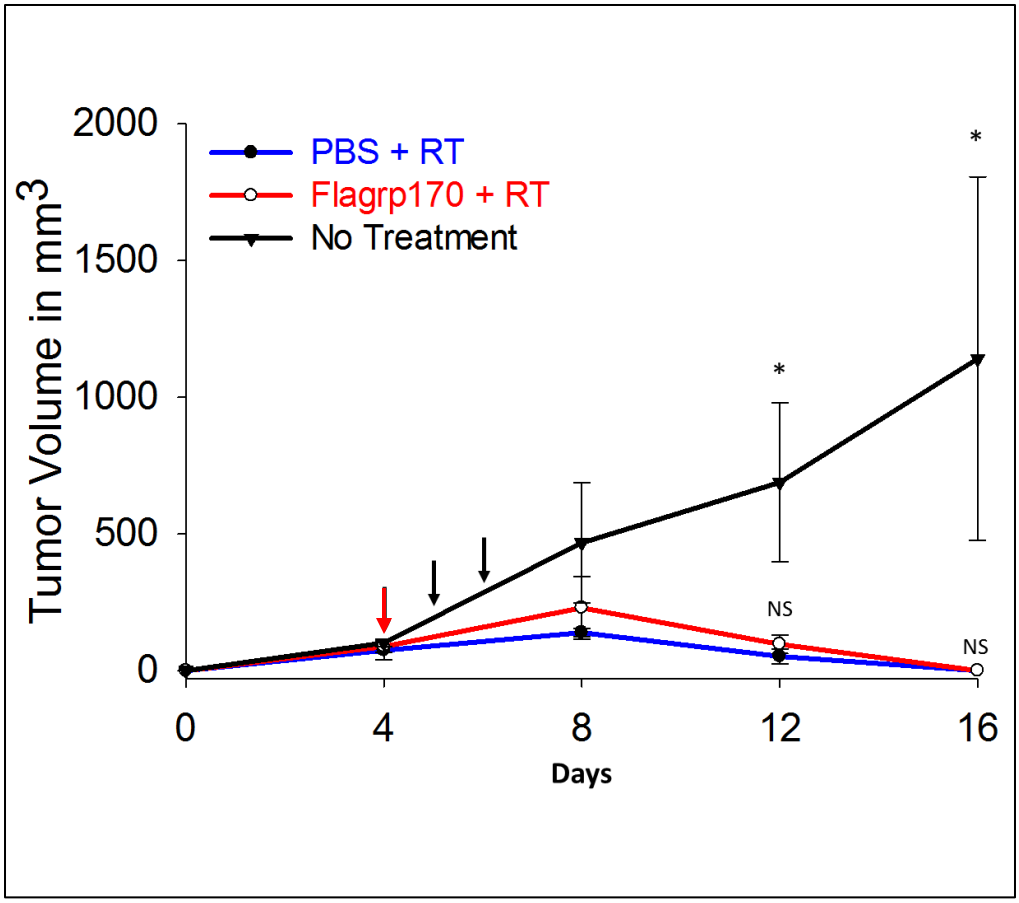
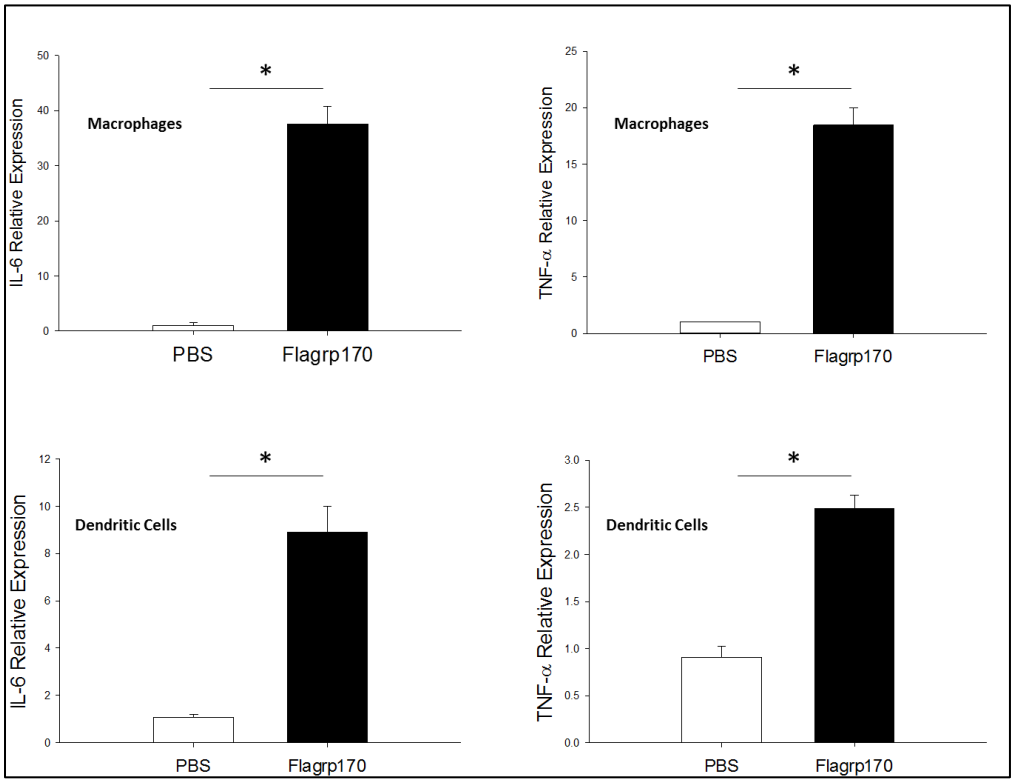


Figure 4. Flagrp170 does not alter tumor response to radiation therapy. Balb/c mice (n =3) were s.c. injected with 5 x 10^6 CT-26 cells to induce tumor growth on the right leg. Tumor growth was recorded every 4 days. When tumor size reached 4-5 mm in diameter (indicated with red arrow), 2 groups of mice were s.c. injected in the area of the tumor with either 50 µg of Flagrp170 or PBS. 1 hour after treatment, mice underwent 10 Gy of local radiation therapy where the tumor resided. The injections and the irradiation treatment was repeated twice more in the following 2 days (indicated by black arrows). 1 group of mice was left untreated as a negative control. Results show there is no significant difference in tumor response to radiation therapy in the PBS treatment group when compared to Flagrp170 treatment group. *, $P < 0.05$; NS, not significant

A


IL-6 Relative Expression
Macrophages
*
PBS
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*
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Flagrp170
IL-6 Relative Expression
Dendritic Cells
*
PBS
Flagrp170
TNF-α Relative Expression
Dendritic Cells
*
PBS
Flagrp170

B

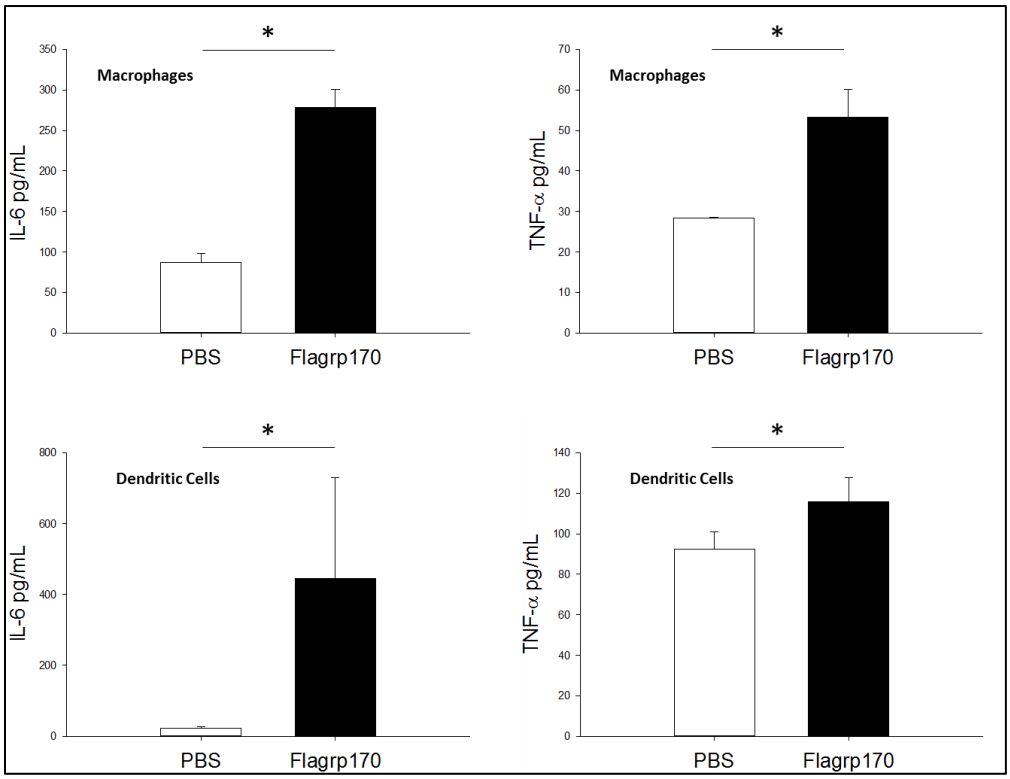

IL-6 pg/mL
Macrophages
*
PBS
Flagrp170
TNF-α pg/mL
Macrophages
*
PBS
Flagrp170
IL-6 pg/mL
Dendritic Cells
*
PBS
Flagrp170
TNF-α pg/mL
Dendritic Cells
*
PBS
Flagrp170

Figure 5. Flagrp170 induces the transcription and production of radioprotective cytokines IL-6 and TNF-α in bone marrow derived macrophages (BMDM) and dendritic cells (BMDC). (A) BMDMs and BMDCs were treated with Flagrp170 (20 μg/mL) and vehicle (PBS). After 12 hours BMDMs and BMDCs were collected and RNA was extracted. qPCR analysis determined that there were significantly more IL-6 and TNF-α mRNA in BMDMs and BMDCs treated with Flagrp170 when compared to PBS treated BMDMs and BDMCs. (B) BMDMs and BMDCs were treated with Flagrp170 (20 μg/mL) or vehicle (PBS). After 24 hours BMDMs and BMDCs culture media was collected. ELISA determined there was a significantly higher concentration of IL-6 and TNF-α cytokine in the culture media of BMDMs and BMDCs treated with Flagrp170 when compared with the culture media from BMDMs and BMDCs treated with PBS. *, $P < 0.05$

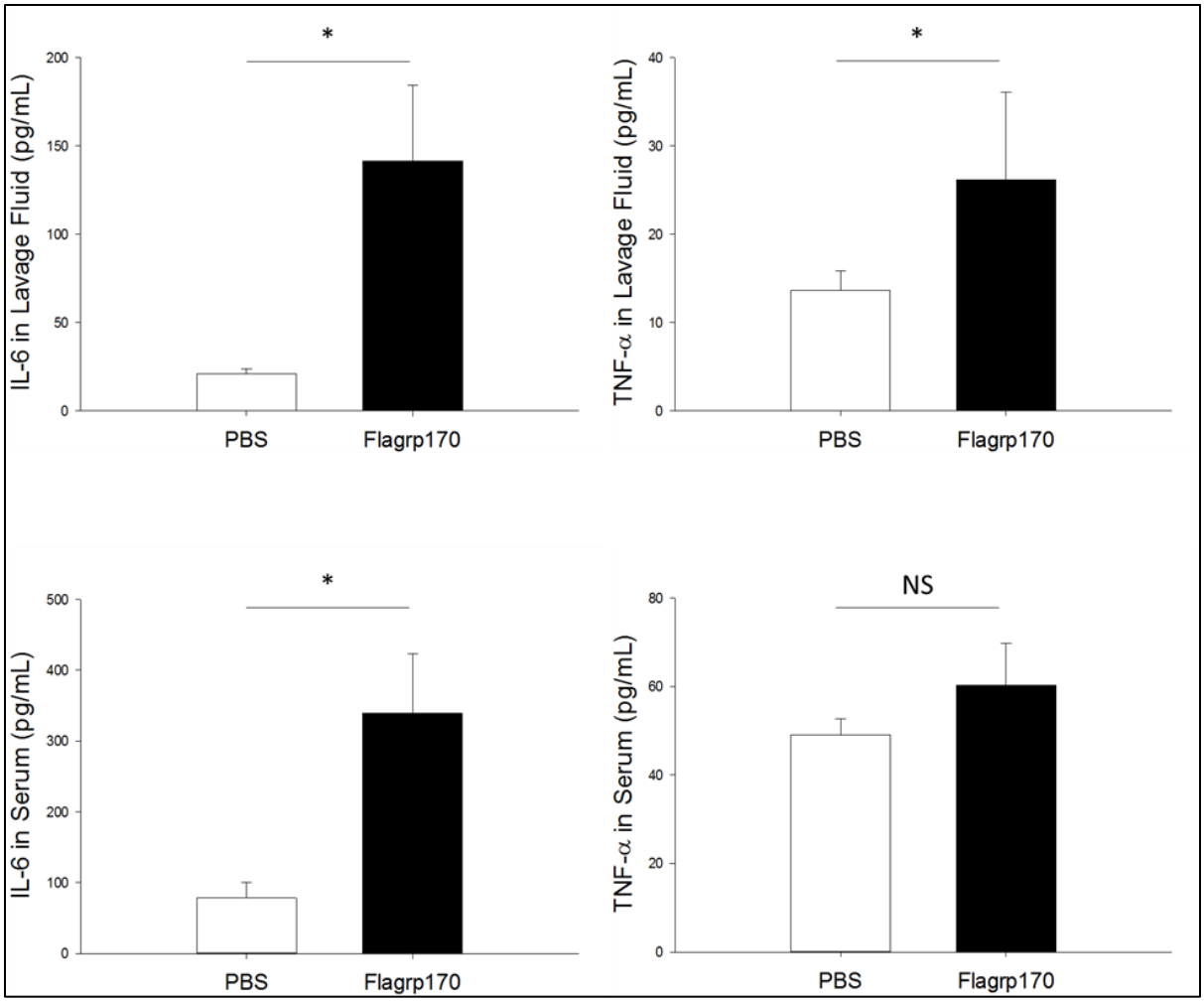
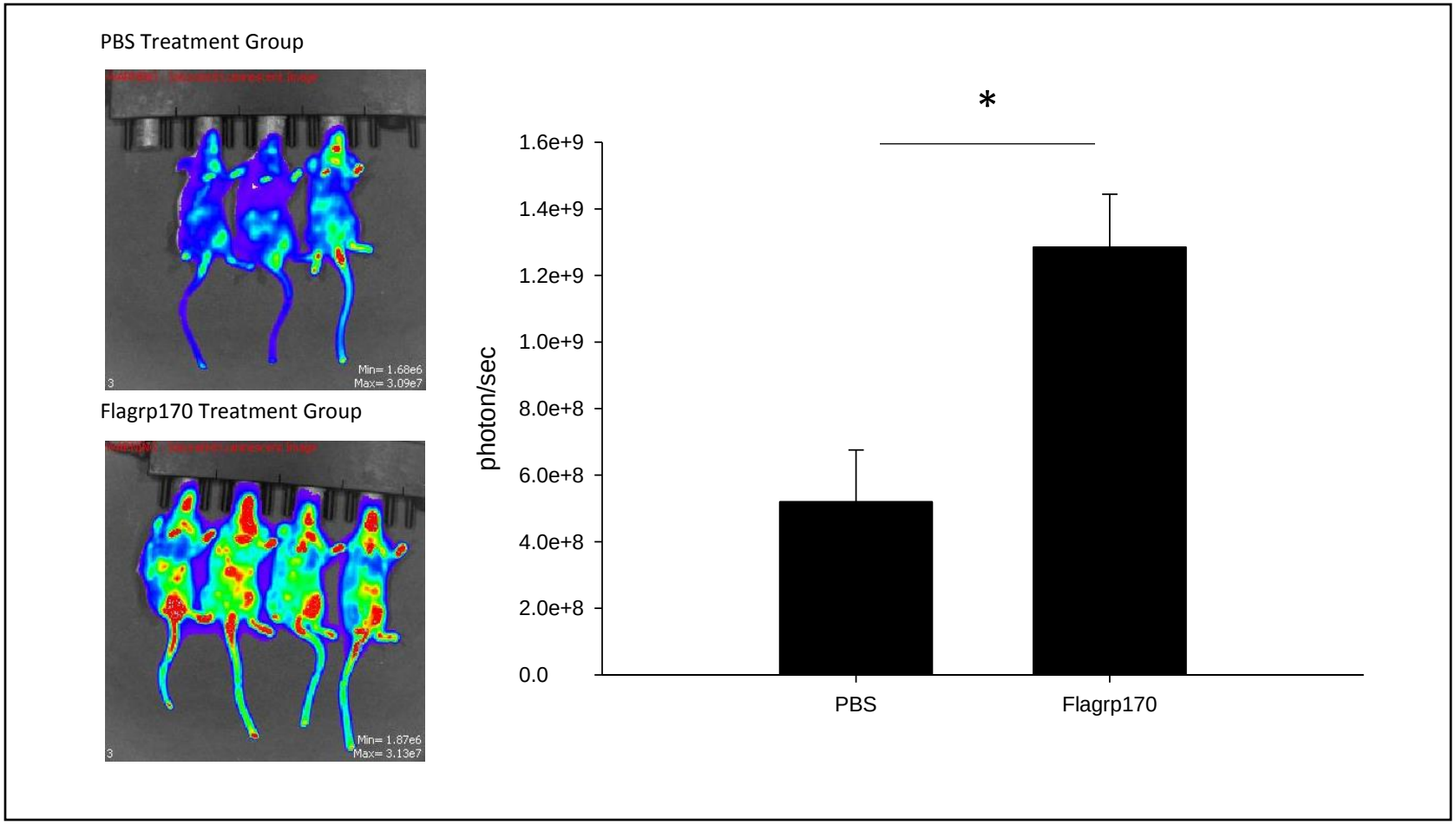


Figure 6. Flagrp170 induces IL-6 and TNF-α *In vivo*. Balb/c mice (n=2) were i.p. injected with 50 µg of Flagrp170 or vehicle (PBS). Peritoneal lavage fluid and serum was collected 4 hours after injection. The concentration of IL-6 and TNF-α in peritoneal lavage fluid and serum was determined via ELISA. The concentration of IL-6 cytokines was significantly higher peritoneal lavage fluid and serum from the Flagrp170 treatment group compared to PBS treatment group. The concentration of TNF-α was significantly higher in peritoneal lavage from the Flagrp170 treatment group compared to PBS treatment group, however there was no significant difference in serum concentration of TNF-α. Experiment repeated 2 additional times with similar results, above data is representative. *, $P < 0.05$; NS, not significant

A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Max= 3.09e7
Flagrp170 Treatment Group
Min= 1.87e6
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*
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1.4e+9
1.2e+9
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4.0e+8
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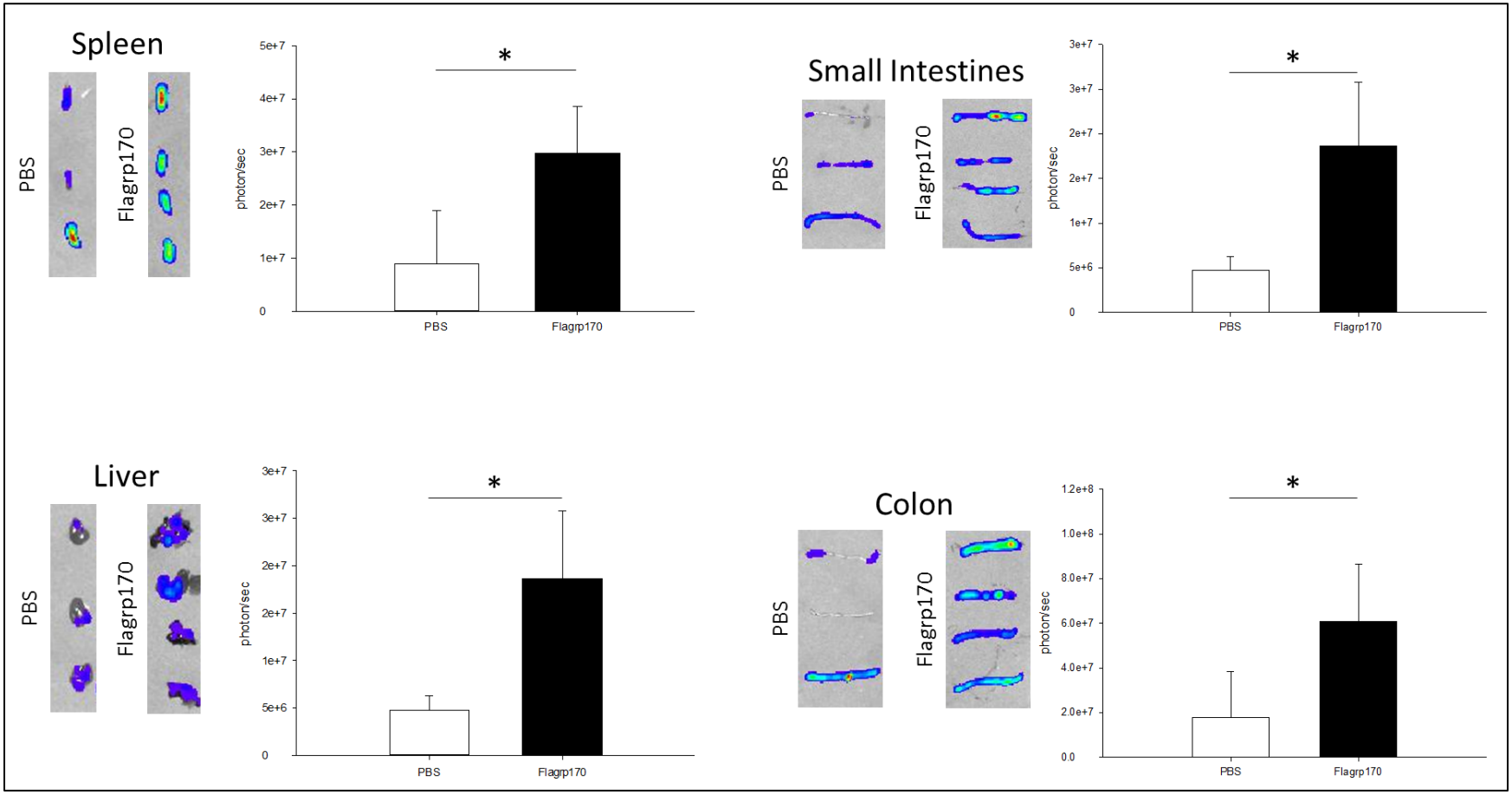
0.0
PBS
Flagrp170

B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Flagrp170
photon/sec
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*
PBS
Flagrp170
Small Intestines
PBS
Flagrp170
photon/sec
3e+7
3e+7
2e+7
2e+7
1e+7
5e+6
0
*
PBS
Flagrp170
Liver
PBS
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photon/sec
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2e+7
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photon/sec
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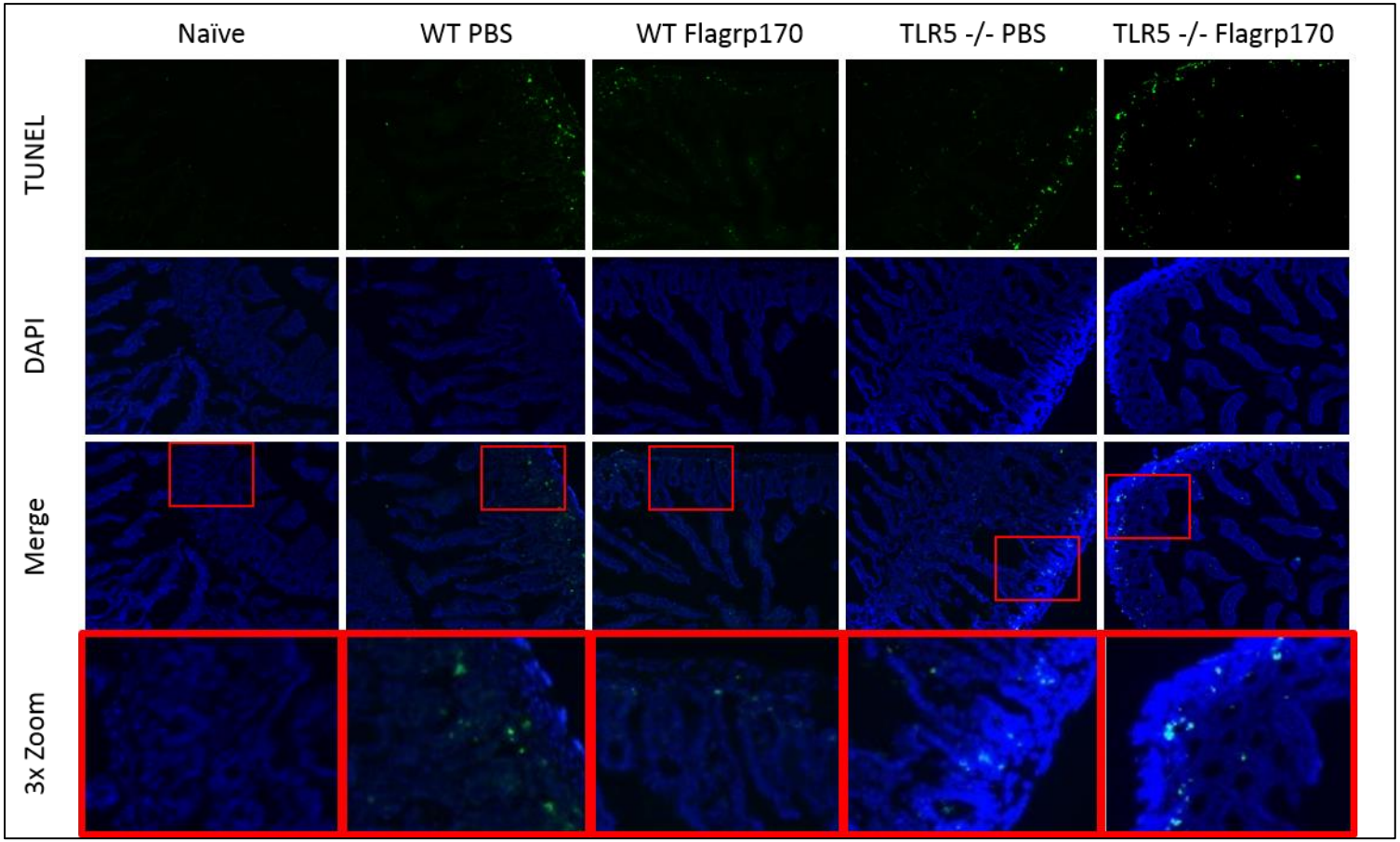
1.0e+8
8.0e+7
6.0e+7
4.0e+7
2.0e+7
0.0
*
PBS
Flagrp170

Figure 7. Flagrp170 induces systemic activation of NF-κB. (A) NF-κB-luc-tg reporter mice (n = 3) were i.p. injected with 50 μg of Flagrp170 or vehicle (PBS). 90 minutes after injection, mice were injected with luciferin (30mg/mL) and imaged using an IVIS imaging instrument. Fluorescence was determined by Living Image software. Results show Flagrp170 treatment strongly activates NF-κB systemically when compared to PBS treatment in NF-κB-luc-tg reporter mice. (B) Immediately after imaging, NF-κB-luc-tg reporter mice (n = 3) were sacrificed and organs samples were collected. Spleen, liver, small intestines and colon samples from all mice were imaged using the IVIS. Fluorescence was determined by living image software. Results show that organ samples from the Flagrp170 treatment group had much stronger fluorescent signals when compared to the PBS group. Flagrp170 treatment group had stronger activation of NF-κB compared to PBS treatment group *, $P < 0.05$

A1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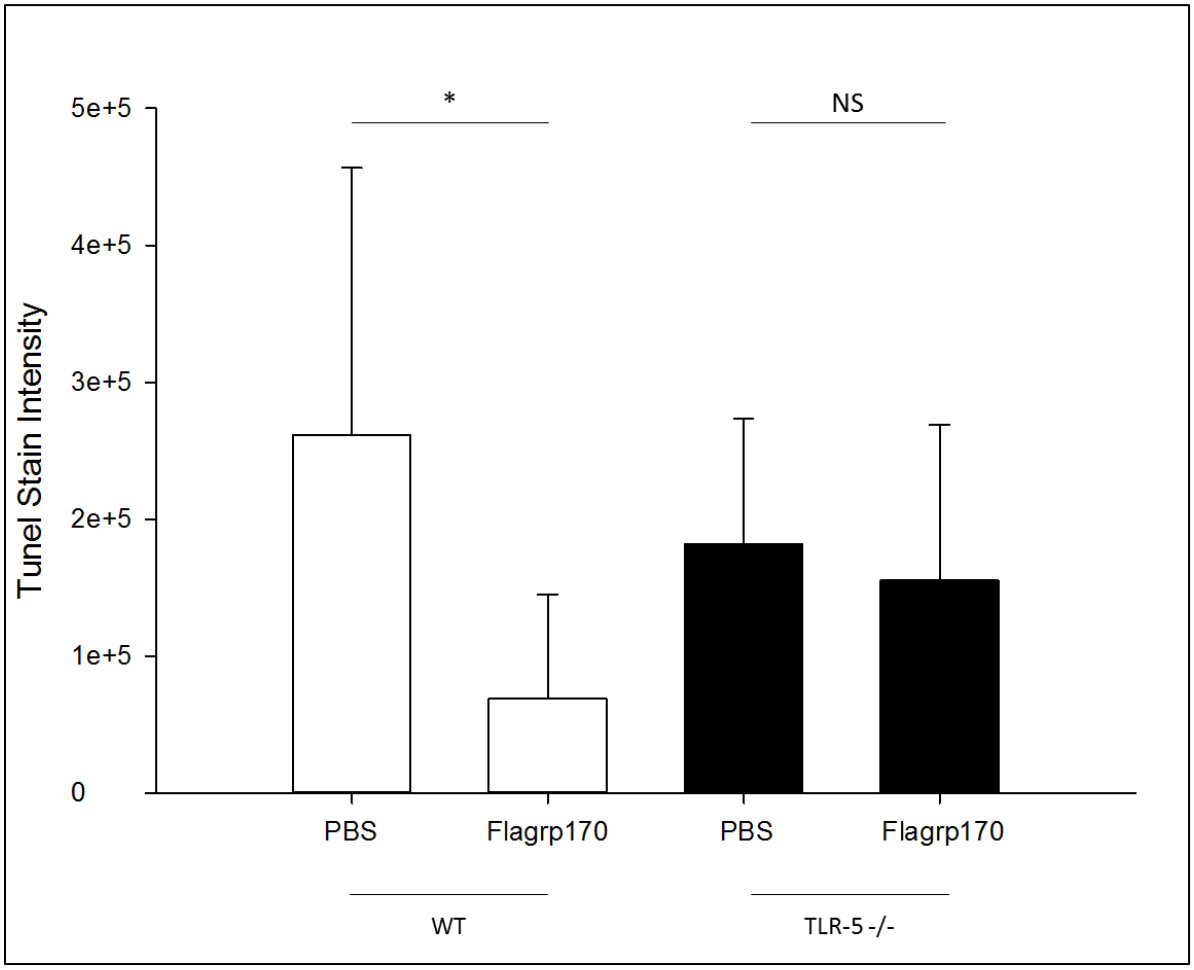
WT PBS
WT Flagrp170
TLR5 -/- PBS
TLR5 -/- Flagrp170
TUNEL
DAPI
Merge
3x Zoom

A2


*
NS
5e+5
4e+5
3e+5
2e+5
1e+5
0
Tunel Stain Intensity
PBS
Flagrp170
PBS
Flagrp170
WT
TLR-5 -/-

B

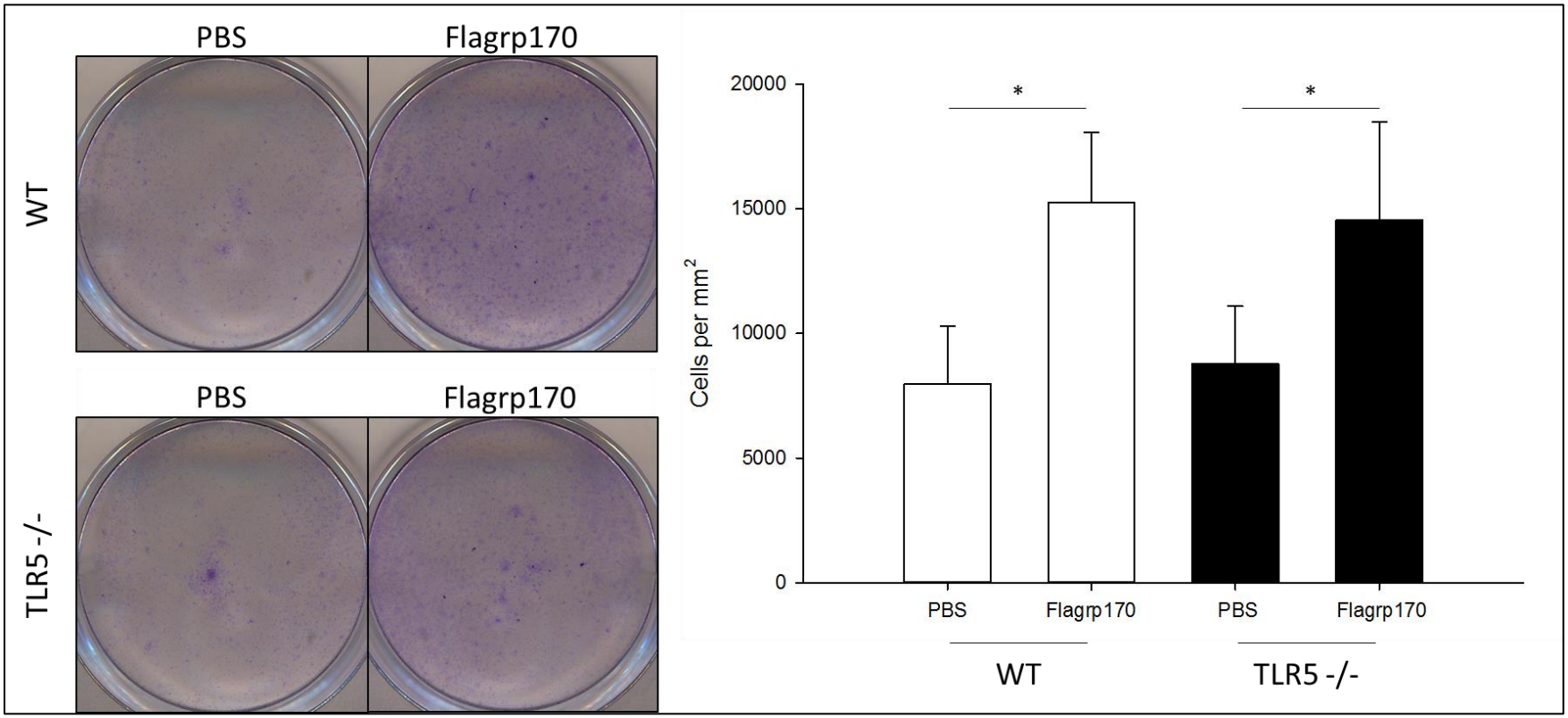

PBS
Flagrp170
WT
PBS
Flagrp170
TLR5 -/-
Cells per mm2
20000
15000
10000
5000
0
*
*
PBS
Flagrp170
PBS
Flagrp170
WT
TLR5 -/-

Figure 8. Flagrp170 is dependent on TLR5 to alleviate radiation induced damage in the intestinal compartment, however Flagrp170 does not require TLR5 to improve bone marrow cell viability after irradiation. (A) C57BL6 (WT) and TLR5 -/- mice (n=3) were i.p. injected with Flagrp170 or vehicle (PBS). 1 hour after injection, all mice underwent a lethal dose (9 Gy) of whole body irradiation. 4 hours after irradiation small intestines were harvested and frozen for sectioning. Tissue selections from all irradiated mice was stained with TUNEL/DAPI. Intestinal tissue from untreated and un-irradiated WT mice was stained as a negative control. Intensity of TUNEL staining was determined by ImageJ. Flagrp170 treated WT mice had significantly less apoptotic cells after irradiation when compared to PBS treated WT mice. There was no significant reduction of apoptotic cells after irradiation between Flagrp170 treated and PBS treated TLR5 -/- mice. Flagrp170 in dependent TLR5 to reduce normal tissue damage after irradiation. (B) BMCs were harvested 5 hours after irradiation from the femora of all mice groups for clonogenic assay. BMCs were plated at 1 x 10^6 cells per well. All BMC samples were cultured with in complete RPMI1640 media with GM-CSF in 12 well plates. BMC were cultured for 10 days. On the 10^{th} day, each plate was stained with crystal violet dye and cell count was estimated via image processing software, ImageJ. Results indicate there is a significantly higher cell per mm^2 volume in the plates cultured with BMC's from Flagrp170 treated mice when compared to BMC's from PBS treated mice regardless of phenotype. *, $P < 0.05$; NS, not significant

Results[DS31]

Protein purified from SF-21 cells infected baculovirus encoding Flagrp170 sequence.

Prior to the experiments in which Flagrp170 was characterized as a radioprotectant, we first purified a stock of the protein. The baculovirus encoding Flagrp170 was used to infect SF-21 cells which produced the protein. The cells were lysed and the recombinant Flagrp170 was purified via nickel affinity chromatography. The purified protein was ran through a SDS-page gel and purity was determined to be above 80% via ImageJ (Figure 1). To confirm that the purified protein was indeed Flagrp170, the protein elute was ran through a polyacrylamide gel, transferred to a nitrocellulose membrane, and detected using anti-Grp170 antibody (Figure 1). The antibody recognized Flagrp170 at 150 kDa with two bands. The two bands[DS32] are likely due to post-transcriptional modification, glycosylation.

Flagrp170 protects the gastrointestinal and the bone marrow compartment from irradiation.

Gastrointestinal acute radiation syndrome (ARS) is defined by the destruction of clonogenic crypt cells.[1] To provide evidence of Flagrp170 induced radioprotection we examined the number[DS33] of apoptotic cells in the gastrointestinal compartment of mice after irradiation. Our studies showed that Flagrp170 reduced the number of apoptotic cells in the gastrointestinal tract when compared to the PBS treated group. This effect was specific to Flagrp170 and we did not see a dose dependent effect in current experimental settings. In addition, it is likely that the NF-κB activating domain of flagellin is responsible for this effect as there is a significant reduction of apoptosis in small intestines of mice from Flagrp170 groups when compared to Grp170 groups (Figure 2A). In this experiment, we also examined the bone marrow compartment after

irradiation. We collected and cultured bone marrow cells (BMC) from all groups. After 10 days, the clonogenic assay showed that there was a significantly increased BMC density in plates cultured from mice that received Flagrp170 injection prior to irradiation when compared to PBS treatment group. Our results indicate an increased BMC viability[DS34] from animals treated either dosage of Flagrp170 when compared to PBS groups. However, there is no significant difference in BMC viability between Grp170 and Flagrp170 groups (Figure 2B). Though there is not a significant difference between Grp170 treatment and Flagrp170, this result is likely artificial as Grp170 should not elicit an immune response by itself and should not have any mechanisms which can improve BMC viability.

Flagrp170 induced radioprotection is specific to normal tissue.

In order for Flagrp170 treatment to improve the therapeutic index of radiotherapy, the protein's radioprotective effect must be specific to normal tissue. Aberrant NF-κB activation is already a naturally occurring mechanism by which tumor cells escape the death pathway. If Flagrp170-induced[DS35] radioprotection does depend on NF-κB activation, we predict that it be effective only in normal tissue and not in tumor tissue. To test our hypothesis, we established tumors by injecting CT-26 cells (5×10^5 per injection), a colon cancer cell line s.c. at the abdominal area of Balb/c mice. Once the tumor grew to size (5 mm in diameter), mice were treated with Flagrp170 or PBS and underwent partial body irradiation. Consistent with our previous findings (Figure 2A), Flagrp170 treated mice had a significant reduction of apoptosis in small intestinal tissue in the context of radiotherapy. In addition, we also conducted a tumor growth study to examine the effect of Flagrp170 on tumor response to radiation treatment over time. Our studies

showed that there was no significant difference in tumor response between the Flagrp170 treatment and the PBS treatment group after irradiation (Figure 4). Our findings indicate that Flagrp170 has radioprotective effect specific to normal tissue.

Flagrp170 induces radioprotective cytokines both in vitro and in vivo.

In order to explore the possible mechanisms responsible for the radioprotective capabilities of Flagrp170, we first determined the biological effects of the protein without radiation. Given that Flagrp170 has been initially described as an effective immune adjuvant[13] we used two types of immune cells, bone marrow derived macrophages and bone marrow derived dendritic cells as experimental models. Our qPCR results demonstrated that the transcription of IL-6 and TNF-α was significantly higher in Flagrp170 treatment groups of both macrophages and dendritic cells in comparison to PBS treatment groups (Figure 5A).

Next we examined radioprotective cytokine concentrations in the culture media of dendritic cells and macrophages to ensure there is an elevated production of IL-6 and TNF-α after treatment. Consistent with our qPCR results, the culture media from cells treated with Flagrp170 had significantly higher concentrations of IL-6 and TNF-α when compared to culture media from PBS treated cells (Figure 5B). Despite the statistical significance, the Flagrp170 induced production of TNF-α in dendritic cells may not be biologically significant.

After we established a statistically significant result from *in vitro* studies, we moved on to examine the effect of Flagrp170 *in vivo*. ELISA results shows that the concentration of IL-6 and TNF-α was significantly higher in peritoneal lavage fluid from Flagrp170 treated mice when compared to PBS treated mice (Figure 6). Furthermore, our results showed a significant increase

of IL-6 concentration in serum from Flagrp170 treated mice when compared to PBS treated mice. However, despite the increased concentration of IL-6 in serum after Flagrp170 treatment, the difference in concentration of TNF-α in serum between PBS treated mice and Flagrp170 was non-significant (Figure 6). Overall our *in vivo* studies determined that Flagrp170 delivered intraperitoneally has both a significant local and systematic effect.

Flagrp170 induces the activation of NF-κB in vivo.

It has been previously shown that NF-κB activation can plays a significant role in radioprotection.[1,30,31] Since a NF-κB activating domain is incorporated into Flagrp170, we predicted that NF-κB activation may be the principle mechanism by which Flagrp170 provides radioprotection to normal tissue. To ensure that Flagrp170[DS36] treatment does induce the of NF-κB, we examined the protein's effect in NF-κB-luc-tg mice, a transgenic NF-κB reporter model. Factors that activate NF-κB can induce the expression of luciferase which correlates with NF-κB activation. Flagrp170 treatment groups were shown to have a significantly higher signal of luciferase compared to PBS treatment group (Figure 7A). In addition, our studies indicate that Flagrp170 induces the activation of NF-κB strongly in the spleen, liver, small intestines, and colon when compared to organs harvested from PBS treated groups (Figure 7B).

Flagrp170 induced gastrointestinal radioprotection requires Toll like receptor 5 (TLR5).

As previously mentioned[DS37], we have predicted the principle mechanism behind the radioprotective effect of Flagrp170 is caused by the flagellin-derived[DS38] NF-κB activating conjugated to the protein. TLR5 is the innate receptor responsible for the sensing of flagellin and

initiates the signaling pathway which results in the activation of NF-κB.[15] Thus[DS39], to explore mechanism behind Flagrp170 induced radiation protection, we examined the effect of Flagrp170 in TLR5 -/- mice and compared to C57BL6 (WT) with normal functional TLR5 expression. Consistent with our previous results, Flagrp170 significantly reduced apoptosis after irradiation in intestinal tissue from WT mice when compared to PBS treated WT mice. However, Flagrp170 had seemingly no significant effect on the gastrointestinal compartment in TLR5 -/- mice after irradiation (Figure 8A).

Flagrp170 does not require TLR5 to increase bone marrow cell viability.

After examining the gastrointestinal compartment in TLR5 -/- mice we preceded to examine the bone marrow compartment. Unexpectedly, Flagrp170 treatment improved the viability of the bone marrow cells from both WT and TLR5 -/- mice. Consistent with our previous clonogenic assay (Figure 2B), BMCs from Flagrp170 treated WT mice had increased viability after irradiation when compared to BMCs from PBS treated mice (Figure 5B). Interestingly, Flagrp170 also had a similar effect on BMCs from TLR5 -/- mice. BMCs derived from Flagrp170 treated TLR5 -/- mice had significantly increased cell density after a 10 day culture, when compared to BMCs from PBS treated mice (Figure 8B). Our results indicate that Flagrp170 is dependent on TLR5 to protect gastrointestinal compartment from radiation induced injury, however Flagrp170 does not require TLR5 to improve BMC viability after radiation.

Discussion

Exposure to radiation can result in varying biological responses associated with tissue injury. Ionizing radiation induces the production of free radicals which if unregulated can adversely affect lipids, proteins, and DNA.[38] Oxidative stress induced[DS40] by free radicals can lipid peroxidation which has been implicated in neurodegenerative diseases, ischemic reperfusion injury, kidney damage, and many other serious disease states.[39] Oxidative damage to proteins can incur adverse changes to amino acid structures, peptide cleavage, and cause protein crosslinks[DS41]. Any of these modifications to protein structure can alter cellular such as signal transduction, enzyme activity, and various other protein function important to homeostasis.[38,40]

On a genetic level, radiation-induced[DS42] free radicals can break DNA to a point beyond leading to cell death by mitotic death,[DS43] or apoptosis.[2] The resulting cell death may lead to toxicity in which proliferative progenitor cells (epithelial and hematopoietic) are quickly depleted and unable to replenish terminal functional cells. At high enough radiation dose, acute radiation syndrome or radiation sickness is life threatening. At lower radiation doses[DS44], secondary early toxicity occurs in which cell populations with low turnover rates (endothelium, fibroblast, neural, muscle, etc.) are unable to function. Such an event leads to late radiation toxicities such chronic tissue dysfunction and tissue disorganization. Late reactions to radiation exposure is incredibly detrimental to quality of life and may even be life threatening. Though some cells are able to self-repair DNA damage after radiation, they may still retain permanent genetic mutations which can lead to radiation induced carcinogenesis.[1]

To avoid these disease states researchers have explored several preventative measures against radiation toxicity. Detoxifying free radicals with scavengers is one of the most effective method of radioprotection.[1,11,41-43] Mitochondrial manganese superoxide dismutase (MnSOD) is a well-researched ROS scavengers and has a very potent effect, converting oxygen radicals to hydrogen peroxide.[41,43] The only FDA approved radioprotectant Amifostine is a ROS scavenger that is specific to normal tissue.[11]

Outside of preventative measures, agents that enhance the DNA repair process after oxidative damage also has the potential to serve as radioprotectant. Agents such as Resveratrol, Oxoguanine DNA glycosylase, and PARP-1, [DS45], help alleviate oxidative damage and enhancing DNA repair.[1] Resveratrol reduces chromosomal aberrations by expression of sirtuins (silent information regulator 2), proteins which accelerates DNA repair by histone deacetylation or mono-ribosyltransferase activity.[44,45] Oxoguanine DNA glycosylase (OGG1) directly enhance repair by interacting with multiple enzyme involved in the repair process, accelerating restoration.[46] PARP-1 is a nuclear enzyme that is involved in the suppression of imprecise repair and possibly preventing mutagenesis.[47,48]

DNA damage due to radiation exposure triggers pro-apoptotic responses starting with the upregulation of the p53 gene, the guardian of genomic integrity. Blockers of p53 such as Sodium orthovanadate and pifithrin-α can significantly enhance survival and protect the gastrointestinal compartment from damage.[49] The Bcl-2 family of proteins are important regulators of apoptosis. Bcl-2 and Bcl-xl are anti-apoptotic, while Bax and Bcl-2 homologous antagonist are pro- Treatment with glycogen synthase kinase 3β, SB216763, and SB415286 can inhibit Bax and upregulate Bcl-2 in the intestinal crypt. These agents have been shown to reduce the apotosis in

crypt cells and protect mice against gastrointestinal radiation syndrome.[50] NF-κB is an important transcription factor which regulates a host of genes including the Bcl-2 family and other inhibitors of apoptosis. Flagellin, a PAMP, has been shown to induce a radioprotective effect through the activation of NF-κB. Treatment with flagellin with prior to radiation significantly improves survival in mouse models.[31] CBLB502, an engineered derivative of NF-κB activating domain of flagellin, has great potency as a radioprotectant. CBLB502 administered prior to irradiation preserved the normal crypt cell proliferation in mice and significantly improves survival even more effectively than Amifostine in mouse models.[30]

Our examination of gut epithelial cells from lethally irradiated mice show that Flagrp170 could significantly reduce apoptosis in the radiosensitive gastrointestinal compartment in both radiosensitive and radioresistant[DS47] mice strains, Balb/c and C57BL6 mice, respectively.[51] The reduction of apoptosis is likely due to Flagrp170 induction of NF-κB activation. The data from our NF-κB reporter imaging indicates that Flagrp170 injection significantly activates NF-κB in the small intestines and colon tissue. Though the entirety of the mechanisms behind NF-κB induced anti-apoptotic effect has not been completely elucidated, it is understood that NF-κB induces the transcription of anti-apoptotic members of the Bcl-2 family and certain IAPs.[52] IAPs induced by NF-κB, c-IAP1 and c-IAP2, can either directly block caspase functions or indirectly induce their degradation.[28] C-Flip is another major anti-apoptotic gene that is under NF-κB control. C-Flip acts directly at the DISC, it blocks CD95-mediated apoptosis.[53,54] Bcl-2 family members, Bcl-2, Bcl-xl, and Bfl-1[DS48], prevent cytochrome-C release and subsequent caspase 9 activation.[27]

Our *in vitro* and *in vivo* studies showed that treatment with Flagrp170 induced a significant increase of IL-6 and TNF-α concentration, which are both radioprotective. Research

has shown that administration of IL-6 can accelerate hematopoietic recovery after irradiation.[21] IL-6 induced hematopoietic recovery may be an essential mechanism for which [DS49]Flagrp170 provides radioprotection in the hematopoietic compartment. Despite accelerating hematopoietic recovery, IL-6 alone does not significantly improve recovery from lethal radiation damage. Administration of IL-6, however, does improve TNF-α and IL-1-induced[DS50] recovery lethal radiation.[55] IL-6 likely requires interactions with TNF-α or IL-1 for a prominent radioprotective effect. Studies have demonstrated anti-TNF-α antibodies reduce survival of mice after irradiation, suggesting that TNF-α is involved in the natural response to radiation damage. Furthermore, TNF-α administration can reduce lethality after whole body irradiation by exert a radioprotective effect on bone marrow precursor cells.[56-58] TNF-α has also been found to induce the transcription of MnSOD, an effective ROS scavenger effective in radioprotection.[59,60] Though Flagrp170 induces a significant amount of IL-6 both *in vitro* and *in vivo*, TNF-α is only strongly induced significantly *in vitro*. Flagrp170 does induce a modest production of TNF-α, detected locally at site of injection in the lavage fluid. However, TNF-α is highly toxic so a high induction of the inflammatory cytokine would be detrimental. The modest induction of TNF-α may be adequate for synergistic radioprotective effect with IL-6. In addition, studies have indicated that only a minute concentration of TNF-α is required for transcription of MnSOD.[59]

Though we have yet to compare Flagrp170 to other NF-κB activating agents, Flagrp170 may be more effective in activating NF-κB due to its molecular chaperone functions. Flagrp170, as molecular chaperone function, could stabilize the structure and conformation of the NF-κB activating sequence conjugated to it.[13,32] In addition, Flagrp170 may also be able to facilitate the sensing of NF-κB activating PAMP. Previous studies have indicated that Grp170, which Flagrp170

is derived from, can amplify TLR9 activation during pathogen recognition.[37] Several studies have shown that uptake of chaperone proteins by antigen-presenting cells is mediated by receptors, such as SR-A, CD91, and LOx1.[33,34,61] Flagrp170 may be more efficiently endocytosed[DS51] by presenting cells (APCs) and efficiently induce the production of radioprotective cytokines which is consistent with our *in vitro* studies.

In this study, we did not delve deeply into the mechanisms by which Flagrp170 induces radioprotection. Our initial hypothesis was that Flagrp170[DS52]-induced radioprotection was TLR5 dependent because Flagrp170 contains the NF-κB activating stimuli derived from flagellin, a TLR5 ligand. From our mechanistic studies, we demonstrated that Flagrp170 does require TLR5 to reduce occurrence of apoptosis in the gastrointestinal compartment, supported by the observation that there was no difference in the number of apoptotic epithelial cells between PBS treated and Flagrp170 treated TLR5 -/- mice. Surprisingly however, our clonogenic assay indicated that Flagrp170 could improve the viability of BMCs derived from TLR5 -/- mice. This implies that there may be another mechanism responsible for Flagrp170-enhanced BMC viability. Supplementary figures from Dr. Yu revealed that TLR5 expression does not appear to mediate the activation of NF-κB in BMDC with adenovirus mediated delivery of Flagrp170.[13] Our unpublished data also indicated that Flagrp170-induced[DS53] antitumor immunity may involve inflammasomes. It possible that NF-κB activation may be induced by another PRR.

Though [DS54]we have characterized some aspects of Flagrp170's potential as a there is still much left to be explored. In this study, we have mostly focused on Flagrp170's effect in respects to acute early reactions to RT and thus are mostly short term. The duration, as well as the peak of Flagrp170's radioprotective effect has yet to be defined. In addition, we have only

explored two methods of drug delivery for Flagrp170, intraperitoneal and subcutaneous. the Flagrp170 can be administrated orally or via intravenous injection as those are convenient methods of drug delivery for human treatment

We have shown that Flagrp170 can substantially reduce apoptosis, but it is still unknown if this radioprotective effect is substantial. It is important to establish that Flagrp170 treatment does significantly improve the lifespan of mice after a lethal dose of ionizing radiation. A long term survival study should be conducted with lethally irradiated[DS56] mice to truly confirm efficacy as a radioprotectant. In addition, survival studies would be a straight forward approach of comparing Flagrp170 to other radioprotective agents.

Even if Flagrp170[DS57]-inducedradioprotection can drastically improve lifespan[DS58] after radiation, inhibitors of apoptosis signaling may inadvertently increase the risk of mutagenesis by allowing genetically compromised cells to the escape death. Essentially, inhibitors of apoptosis [DS59]reduces early radiation toxicity[DS60], but at the cost of increased risk for radiation-induced carcinogenesis. However, Flagrp170 has distinct and effective immune modulating capabilities and the enhanced immune detection of tumor antigen may decrease the risk of recurring tumor regardless. Future studies should examine on the long-term effects of sub lethal radiation and explore the balance between the risk of radiation[DS61]-induced carcinogenesis and the lethality radiation or CLE. Cells lines and mice strains that are sensitive to DNA damage or have ineffective DNA repair mechanism would serve as good models.

To this date, there has been no FDA-approved therapeutic mitigators for radiation damage. In the chance[DS62] occurrence of a nuclear incidence, there is no viable method of

radiation damage. CBLB502, an agent with similar attributes to Flagrp170, has been shown to be moderately effective as radiation mitigator taken after radiation exposure[DS63].[30] In this study, have only observed Flarp170 induced radioprotection when delivered prior to radiation exposure, which supports its potential use in a preventative setting in clinical RT. There is[DS64] no evidence to exclude the possible mitigative effect of Flagrp170 if treatment occurs after radiation.

E~~Though~~[DS65] ~~e~~early toxicity may be reduced by Flagrp170, however it is unclear if has any effect on long term consequential damage. Acute reactions that fail to heal can result in damage that extends to late radiation toxicities.[2] Since the processes that results in later radiation sequelae are extremely complex, it was out of the scope [DS66]of the current study, future studies should examine if Flagrp170 affects vascular endothelial cells and fibroblasts. Future *in vivo* studies should examine whether Flagrp170 can reduces fibrosis and ulcers which are common symptoms among late effects of radiation.

The dual role of Flagrp170 as an immune modulator and, potentially, as a radioprotectant is an exciting[DS67][DS68] and unexplored prospect. Flagrp170 has already been described as an immune modulator and could induce therapeutic antitumor immunity against multiple lines of cancers. We predicted in our tumor study that Flagrp170 via s.c. delivery would be able to load tumor antigens after radiation treatment and to prime the immune system against the Counter to our predictions, Flagrp170 seemly had little positive effects on the reduction of the tumor size after radiation. Nevertheless, the tumor study did provide evidence that Flagrp170 does not impact tumor response to RT, the combined immune-modulating[DS70] and effect of Flagrp170 should be further investigated in the setting of radiotherapy. Perhaps

adenoviral delivery of Flagrp170 can still effectively induce a radioprotective effect as previous studies suggested that there is still NF-κB activation with a viral delivery method.

If applied as a therapeutic adjuvant in the setting of RT, Flagrp170[DS71] could potentially reduce common side effects of RT. Nausea, vomiting, and diarrhea results in part from damaged gut epithelial surfaces and an insufficient amount of function epithelial cells. Flagrp170 could quicken recovery since it can protect intestinal crypt cells from apoptosis and increase BMC viability. The quicken[DS72] recovery prevents extended acute toxicities thus lowering the risk of Opportunistic infections, which are a well-known phenomenon in patients severely damaged by radiation, could be prevented by Flagrp170 treatment. Flagrp170[DS73] by improving BMC viability inducing radioprotective IL-6 could accelerate hematopoietic recovery. Taken together our results indicate that Flagrp170 may be potentially exploited as a radioprotective agent in the clinical setting of RT.

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