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# FSL-1: A Synthetic Peptide Increases Survival in a Murine Model of Hematopoietic Acute Radiation Syndrome

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In the current geopolitical climate there is an unmet need to identify and develop prophylactic radiation countermeasures, particularly to ensure the well-being of warfighters and first responders that may be required to perform on radiation-contaminated fields for operational or rescue missions. Currently, no countermeasures have been approved by the U.S. FDA for prophylactic administration. Here we report on the efficacious nature of FSL-1 (toll-like receptor 2/6 agonist) and the protection from acute radiation syndrome (ARS) in a murine total-body irradiation (TBI) model. A single dose of FSL-1 was administered subcutaneously in mice. The safety of the compound was assessed in non-irradiated animals, the efficacy of the compound was assessed in animals exposed to TBI in the AFRRI Co-60 facility, the dose of FSL-1 was optimized, and common hematological parameters [complete blood cell (CBC), cytokines, and bone marrow progenitor cells] were assessed. Animals were monitored up to 60 days after exposure and radiation-induced damage was evaluated. FSL-1 was shown to be non-toxic when administered to non-irradiated mice at doses up to 3 mg/kg. The window of efficacy was determined to be 24 h prior to 24 h after TBI. FSL-1 administration resulted in significantly increased survival when administered either 24 h prior to or 24 h after exposure to supra-lethal doses of TBI. The optimal dose of FSL-1 administration was determined to be 1.5 mg/kg when administered prior to irradiation. Finally, FSL-1 protected the hematopoietic system (recovery of CBC and bone marrow CFU). Taken together, the effects of increased survival and accelerated recovery of hematological parameters suggests that FSL-1 should be developed as a novel radiation countermeasure for soldiers and civilians, which can be used either before or after irradiation in the aftermath of a radiological or nuclear event. © 2024 by Radiation Research Society

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## INTRODUCTION

The threat of nuclear exposure either through intentional or accidental means is heightened in the current geopolitical

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climate. Such exposure could be disruptive to military personnel and operations in a multitude of ways: 1. personnel exposed to ionizing radiation would be expected to develop symptoms such as nausea, vomiting and diarrhea, and ultimately death depending on the exposed dose; 2. non-lethal exposure could lead to long-term health effects such as cataracts, pulmonary fibrosis, and cancer; and 3. fear and panic without proper contingencies could lead to a public response that could impede the ability of war fighters or first responders to properly execute their orders.

Military service members and first responders are often confronted with working in hostile regions and conditions. Services members are likely to encounter improvised explosive devices or radiological dispersal devices (i.e., dirty bombs), a threat that is elevated by the rise in prevalence of terrorist groups. Current geopolitical events such as the Russian invasion of Ukraine have elevated the concern of damage to nuclear sites and the potential release of radioactive materials. In addition, the U.S. military has a wide range of radiological devices and materials in use, underscoring the potential for accidental exposure. For these reasons, protection of warfighters and first responders deployed in a radiation-contaminated field for rescue or military operations, as well as exposed civilian populations is an urgent need. The paucity of non-toxic prophylactic radiological medical countermeasures is a serious capability shortfall.

To date, the United States Food and Drug Administration (FDA) has approved five countermeasures for H-ARS that can be administered as mitigators 24 h after radiation exposure: Neupogen<sup>®</sup> (granulocyte colony-stimulating factor (G-CSF), filgrastim) (1), Neulasta<sup>®</sup> (PEGylated G-CSF, pegfilgrastim) (2), Leukine<sup>®</sup> (granulocyte macrophage colony-stimulating factor (GM-CSF), sargramostim) (3), Nplate<sup>®</sup> (thrombopoietin (TPO) analog, romiplostim) (4), Udenyca<sup>®</sup> and Stimufend<sup>®</sup> (pegfilgrastim biosimilars) (5); Neupogen, Neulasta, Udenyca, Stimufend and Leukine stimulate white blood cell production and Nplate stimulates platelet production. However, to date no prophylactic countermeasure has been approved by the FDA. Hence, it is a national priority to develop radiation countermeasures that are effective when administered to our soldiers prior to radiation exposure.

There are a few promising radioprotectors of various classes that have been evaluated in pre-clinical animal

models following the FDA animal rule. One class of compounds are toll-like receptor (TLR) agonists, which are the key sensor elements of innate immunity. The role of TLRs was first identified in 1991 (6) when a previously unidentified molecule was shown to interact with the toll receptor and modulate immune function; the identity of this class of molecules was described as TLRs (7, 8) in 1994. Humans express a total of 10 TLRs (TLR1-10), but 13 family members (TLR1-13) have been identified in mammalian species (9, 10). TLRs are widely expressed in a variety of cells including mononuclear cells, macrophages, and lymphocytes (11, 12). TLRs can also be divided into three types according to their ligands: 1. TLRs that recognize lipid species (e.g., TLR1, TLR2, TLR4, TLR6) (13), among which TLR4 recognizes lipopolysaccharide (LPS); 2. TLRs that recognize pathogen proteins, for example TLR5 recognizes Flagellin (14); and 3. TLRs that recognize nucleic acid from cells or viruses (e.g., TLR3, TLR7, TLR8, TLR9) (15, 16).

Among TLRs, TLR2 complexes are widely expressed on different types of cells and are stimulated by numerous ligands, including bacterial lipoproteins (BLP) which induce innate immune responses in mammals by activating heterodimeric receptor complexes containing TLR2. TLR2 signaling results in nuclear factor-kappaB (NF-kB)-dependent upregulation of anti-apoptotic factors, anti-oxidants and cytokines; these factors have been implicated in conferring radioprotection as published earlier (17, 18). It has been demonstrated that synthetic lipopeptides (sLP) that resemble the structure of a naturally occurring mycoplasmal BLP significantly increase survival in mice exposed to lethal total-body irradiation (TBI) when administered between 48 h before and 24 h after irradiation (19). It was found that sLP treatment accelerated recovery of bone marrow (BM) and spleen cellularity, and ameliorated thrombocytopenia in irradiated mice. sLP did not improve survival of irradiated TLR2-knockout mice, confirming that sLP-mediated radioprotection requires TLR2. Another pharmacological agent, CBLB613, which is a naturally occurring Mycoplasma-derived lipopeptide ligand for TLR2/6, has been developed as a novel radiation countermeasure in mice. CBLB613 significantly protected mice from dying after exposure to a lethal dose of gamma radiation. CBLB613 was found to protect animals from hematopoietic damage by reducing radiation-induced cytopenia and increasing bone marrow cellularity in irradiated mice. In addition, CBLB613 was not immunogenic in mice (20).

Fibroblast-stimulating lipopeptide (FSL-1, Pam2CGDP KHPKSF) is a synthetic diacylated lipoprotein, (S,R)-(2,3-bispalmitoyloxypropyl)-Cys-Gly-Asp-Pro-Lys-His-Pro-Lys-Ser-Phe, originally derived from *Mycoplasma salivarium*, and is available from a commercial source (Invivogen). The mycoplasmal lipopeptide FSL-1 contains a diacylated cysteine residue and is recognized by the TLR2/TLR6 heterodimer. The TLR2/6 heterodimer has established roles in immune response including

sensing of pathogen-associated molecular patterns and recognition of gram-positive bacteria and mycobacteria (21–23). FSL-1 binding to TLR2/6 activates signaling to induce biological responses such as the expression of NF-kB-induced-cytokines, chemokines, and cell proliferative growth factors (24, 25). Biologic pathways mediated by myeloid differentiation primary response gene 88 (MyD88), the common adaptor for TLR and Interleukin-1 receptor signaling, are critical for radioprotection. Preliminary studies conducted by Ting and coworkers (26) demonstrated that FSL-1 substantially prolongs survival in both male and female mice when administered as a single dose 24 h after irradiation and showed MyD88-dependent function. FSL-1 treatment resulted in accelerated recovery of hematopoietic injury including bone marrow and spleen (26).

Here we describe our efforts to demonstrate dose-dependent prophylactic efficacy of FSL-1 in mice exposed to a 100% lethal dose of gamma radiation. These studies support the development of FSL-1 as a very potent medical countermeasure that can be developed for warfighters or first responders, to be given before sending them into harm's way for rescue or clean-up operations in a radiation-contaminated field.

## MATERIALS AND METHODS

### *Total Body Irradiation Studies*

Since clinical trials in which humans are exposed to lethal doses of TBI cannot ethically be conducted, the approval process for radiation countermeasures is governed by guidance issued by the FDA known as the Animal Rule (27). Under this guidance, preclinical data in one or more species along with safety and dosing data in humans is used to support the approval of drugs for use in this indication. The mouse is an accepted model for development of radiation countermeasures and it was therefore used to evaluate FSL-1.

### *Animal Procurement and Care*

Pathogen free male and female C57BL/6 mice (12–14 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were housed in the Uniformed Services University of the Health Sciences (USUHS) Department of Laboratory Animal Resources (DLAR) facility [accredited by the Association for Assessment and Accreditation of Laboratory Animal Care- (AAALAC) International]. After receipt, animals were acclimated for a minimum of 5 days prior to use in the experimental study. Mice were housed in groups of up to 5 in individually ventilated cages with a total floor space of 500 cm<sup>2</sup>. The cages utilize an external water bottle and a dual feeder rack. These cages were stored in the Allentown Inc. storage rack which provides temperature, humidity, and ventilation control to each individual cage. Sani-Chips (P.J Murphy Forest Products Corp., distributed by Harlan Teklad) were used as bedding material in each cage. Animals were identified by ear tags that were applied immediately after irradiation. Both room and cage humidity were maintained between 30–70%. Each hour, 10–15 air changes occurred in the room housing the racks with the cages. In addition, 35 air changes occurred each hour for the cages. An automated lighting system was used providing a 12-h light, 12-h dark cycle. The light cycle began at 0600 and terminated at 1800 each day. The dark cycle began at 1800 and terminated at 0600 on the following day. Mice were provided Harlan Teklad Global Rodent Diet 8604 ad libitum from the feeder rack within the cage. During critical periods of peak mortality, food pellets were added directly to the floor of the cage in addition to

being distributed from the feeder rack. This is a means of providing ease of access to animals that might struggle to obtain the food otherwise. Water was made available to animals ad libitum from bottles attached to individual cages. The water provided was acidified (pH~2.5) from an Edstrom water bottle filling station. The pH was monitored each time the bottles were filled. Sterile and pathogen-free Nestlets (Ancare) and enrichment lofts (NexGen Mouse Enrichment Loft, Allentown Inc.) were provided as a means of enrichment. All procedures pertaining to animals were reviewed and approved by the USUHS Institutional Animal Care and Use Committee (IACUC) using the principles outlined in the National Research Council's Guide for the Care and Use of Laboratory Animals and performed in accordance with relevant guidelines and regulations (28).

Animal studies were conducted in compliance with Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. Handling of the animals was conducted in accordance with the USUHS IACUC and included the use of personal protective equipment including hair bonnets, facemasks, lab coat, gloves, and shoe covers. Manipulations involving the animals were conducted inside a fume hood with a HEPA filtration system. For tissues collected in formalin buffer, appropriate PPE and fume hood/ventilation was used. For manipulations involving the formulation of FSL-1 or its vehicle, minimum PPE requirements of gloves and a lab coat were met. Animals were weighed and randomly assigned into different groups based on the study design. Veterinary care was available throughout the course of the study. Animals were examined by the veterinary staff at a minimum of once per day, with increased frequency as warranted by clinical signs or changes in appearance (28, 29).

#### *Irradiation Conditions and Dosimetry*

For irradiation, animals were transported to the Armed Forces Radiobiology Research Institute (AFRRI) animal handling facility. Animals were transported in a climate-controlled van to the AFRRI facility. Animals were transported in the same cages that they were housed in; however, cages were transported within a secondary container to limit exposure when outside of the housing rooms. The total time of transportation was less than 10 min. After animals were received at the AFRRI, they were rested for a minimum of 60 min prior to the start of any procedures. After this period, animals were placed in custom restraint boxes constructed from Lucite and built such that 8 animals can be contained in a single box. The boxes feature a sliding lid and adequate ventilation for the animals via holes in the sides of the boxes. Animals were restrained in these boxes for the minimum time necessary to complete the irradiation (less than 30 min). After irradiation, animals were returned to their cages and ultimately returned to the DLAR facility via the climate-controlled van.

Individual restraint boxes measured  $6.5 \times 3$  inches with Lucite dividers, which created a total of  $8 \sim 1 \times 3$  inch compartments in each corner of the cuboid. This box is part of a greater grid of boxes, which were arranged linearly perpendicular to the bilateral Cobalt-60 sources in 7 wide by 8 high grid pattern. Animals were placed in the center of this grid to achieve highest field uniformity, with remaining positions occupied by acrylic phantoms. Animals were irradiated bilaterally (simultaneously) at an estimated dose rate of  $\sim 0.6$  Gy/min at the centerline of the grid of boxes. The dosimetry of the facility is frequently ( $\sim$ yearly) checked using National Institute of Standards and Technology (NIST) traceable ion chambers to measure the dose rates in the cores of acrylic phantoms (3 inches long and 1 inch in diameter) at each position within the Lucite restraint boxes. The corrections applied to the measured dose rates in phantoms were for decay of the Co-60 source and for a small difference in mass-energy absorption coefficients for water and soft tissue at the Co-60 energy level. The radiation field was previously reported to be uniform within  $\pm 2\%$  (30). Additionally, calibrated ion chambers were used as real-time dosimeters in each run for confirmation of delivered dose.

#### *Veterinary Care after Irradiation*

After irradiation, animals were monitored three to four times daily. Animals that have been irradiated experience a period of peak mortality. This period, which we refer to as the critical period, is typically 14 continuous days from approximately day 12 of the study through day 26 of the study in this strain of mice. For weekdays during this period the animals were checked by the research team three times each day (24 h period) with an additional check performed by the veterinary team each day. For weekends during this period, the animals were checked 2 times per day (over a 24 h period) by the research team with an additional check performed by the veterinary team. Animals that were found dead in the course of the study were documented and removed from the cage. Pain and distress were monitored using several predetermined criteria refined from Koch et al. (31) including unresponsiveness, abnormal posture, unkempt appearance, immobility, and lack of coordination. Mice were considered moribund when they showed an inability to remain upright, were cold, unresponsive or displayed decreased or labored respiration. Morbid animals were monitored very closely according to their health in accordance with pre-defined criteria described and approved in the IACUC protocol. Animals were weighed prior to the start of the experiment. During the course of the study, animals that lost more than 35% of their initial body weight were euthanized. Moribund mice were euthanized according to American Veterinary Medical Association (AVMA) guidelines.

#### *Drug and Drug Preparation*

The lyophilized FSL-1 is dissolved in sterile 1x PBS in a tissue culture hood. Then single injection aliquots are pulled into 0.5 cc "insulin" syringes at the time of administration in an animal hood. Dissolved FSL-1 was light-shielded by covering with aluminum foil and kept on ice or at 4°C. Since it tends to "stick" to surfaces, the number of transfers or manipulations between original vial and administration to animal was kept to a minimum. Sterile PBS was used as a vehicle control. Either FSL-1 or the vehicle was injected subcutaneously (SC) at the nape of the neck 24 h before exposure to total-body gamma irradiation.

#### *Fourteen-day Acute Toxicity Study*

C57BL/6 male mice were weighed and distributed into two groups (vehicle control and FSL-1,  $n = 5$ /group). A third untreated control (naïve control,  $n = 5$ ) was used to account for average growth of animals in the batch. Animals were injected SC at the nape with a single 3 mg/kg dose of FSL-1 or PBS (vehicle) in a volume of 0.1 ml.

On the day of test or control article administration, animals were continuously monitored for the first hour for acute toxicity signs such as decreased activity, squinting eyes, hunching, labored breathing or injection site swelling (28). Clinical observations were made and recorded during the first 1 h and 4 h after administration on the days of dosing and then daily for the following 14 days (28). Animals were observed twice daily for morbidity (signs of toxicity such as weight loss, decreased activity, hunched posture, labored breathing or any other abnormal clinical signs of toxicity). During the study, animals were weighed at various times to monitor body weight change [0 (1 h prior to dosing), 3, 7, 11, and 14 days]. On days of weight collection, approximately 20  $\mu$ L of blood was collected into tubes containing EDTA and blood was continually rotated until CBC/differential analysis of white blood cell (WBC), absolute neutrophil (NEU), monocytes (MON), lymphocyte (LYM), and platelet (PLT) counts with the HESKA Element HT<sup>TM</sup> 5 Analyzer system (HESKA Corporation, Loveland, CO) were conducted. At the end of the study, blood was collected via cardiac stick under isoflurane anesthesia and then animals were humanely euthanized by cervical dislocation and standard necropsy performed to identify any abnormalities in major tissues and organs. Serum was separated from whole blood by collecting in serum collection tubes (BD Microtainer tubes reference number 36596), centrifugation at  $2,400 \times g$  for



10 min and used to evaluate hepatic and renal panel chemistry (BUN, creatinine, ALT, AST, ALKP, total protein) with a Heska Element DC5X Veterinary Chemistry analyzer.

#### *Radioprotective Efficacy Studies with FSL-1 in C57BL/6 Male and Female Mice*

To investigate the radioprotective efficacy of FSL-1, mice in all studies were weighed prior to the start of the study; animals outside  $\pm 10\%$  of the mean weight were excluded, and mice were randomly assigned to two groups (vehicle control and FSL-1) with five animals per cage. Mice were administered a single dose of 0.25 mg/kg dose of FSL-1 or vehicle (sterile PBS) by SC injection at the nape using a 23-G needle 24 h prior to radiation exposure. The selection of the 0.25 mg/kg dose in this study was based on the previously published mitigation study (26). Each treatment group for drug and its vehicle (PBS) contained 25 animals and the radiation dose for males was 8.1 Gy ( $\sim LD_{70/30}$ , 70% mortality over a 30 day period). Since females were found to be more radioresistant than males (unpublished data from probit analysis of the dose response curve), they were irradiated at a higher dose of 8.7 Gy ( $\sim LD_{70/30}$ ). Survival was monitored up to four times a day for 30 days and surviving animals were euthanized at the completion of the study. Survival data was plotted as Kaplan-Meier plots and statistical significance of the survival differences was determined by log-rank and Fisher's exact tests using GraphPad Prism 9 software.

#### *Dose Optimization Study with FSL-1 in C57BL/6 Male Mice*

For "dose response study 1 at 8.5 Gy," three doses of FSL-1, one lower and one higher than 0.25 mg/kg (0.125, 0.25, 0.75 mg/kg) were selected to determine the optimum single dose of FSL-1 to achieve maximum efficacy at 24 h pre-TBI in C57BL/6 mice. Mice were administered one of the doses listed above or the equivalent volume of the vehicle ( $n = 25$  mice/cohort) and were irradiated at 8.5 Gy [a supralethal ( $LD_{100/30}$ ) dose without treatment]. For "dose response study 2 at 9.5 Gy," a second dose optimization study was conducted with the same three dose levels (0.125, 0.25, 0.75 mg/kg) at 24 h prior to TBI with drug and vehicle cohorts ( $n = 25$  mice/cohort) exposed to a supralethal dose of 9.5 Gy. For "dose response study 3 at 10 Gy," to determine the optimum dose of FSL-1, another study was performed with two higher drug doses above the last one (0.75 mg/kg) tested in study 2. The study was conducted with three dose levels (0.75, 1.5, 3.0 mg/kg) at 24 h prior to TBI with drug and vehicle cohorts ( $n = 25$  mice/cohort) exposed to an even higher radiation dose than study 2 (10 Gy). Animals were monitored for 30 days in the same way as described previously for all survival studies.

#### *Colony Forming Unit (CFU) Assay from Femoral Bone Marrow*

After euthanasia, femurs were collected and the bone marrow was extracted by flushing with IMDM with 2% FBS, and then plated ( $2 \times 10^5$  cells/plate) following protocols from the manufacturer (Mouse Colony-Forming Cell Assays Using MethoCult, Stem Cell Technologies, Cambridge, MA). Cultures were incubated for 14 days after plating and Granulocyte-macrophage colony forming units (CFU-GM), granulocyte-erythrocyte-monocyte-macrophage CFU (CFU-GEMM), colony-forming unit-erythroid (CFU-E) and erythroid burst-forming units (BFU-E) were identified and quantified using a Nikon TS100F microscope. Fifty or more cells were considered to be a colony. Data are shown as mean  $\pm$  standard error of mean (SEM) and statistical significance was determined between irradiated vehicle-treated and drug-treated groups (29).

#### *Determination of Dose Response Relationship of FSL-1 at 0.75 mg/kg Dose*

Male C57BL/6 mice from the various studies mentioned above that were irradiated at various doses were compiled into a dose response analysis. This was coupled with both results from this study as well as historical data for the control animals to determine the dose reduction

factor (unpublished data). Animals were monitored as mentioned above with the primary endpoint being 30-day survival. Probit analysis was conducted with IBM SPSS statistics pack.

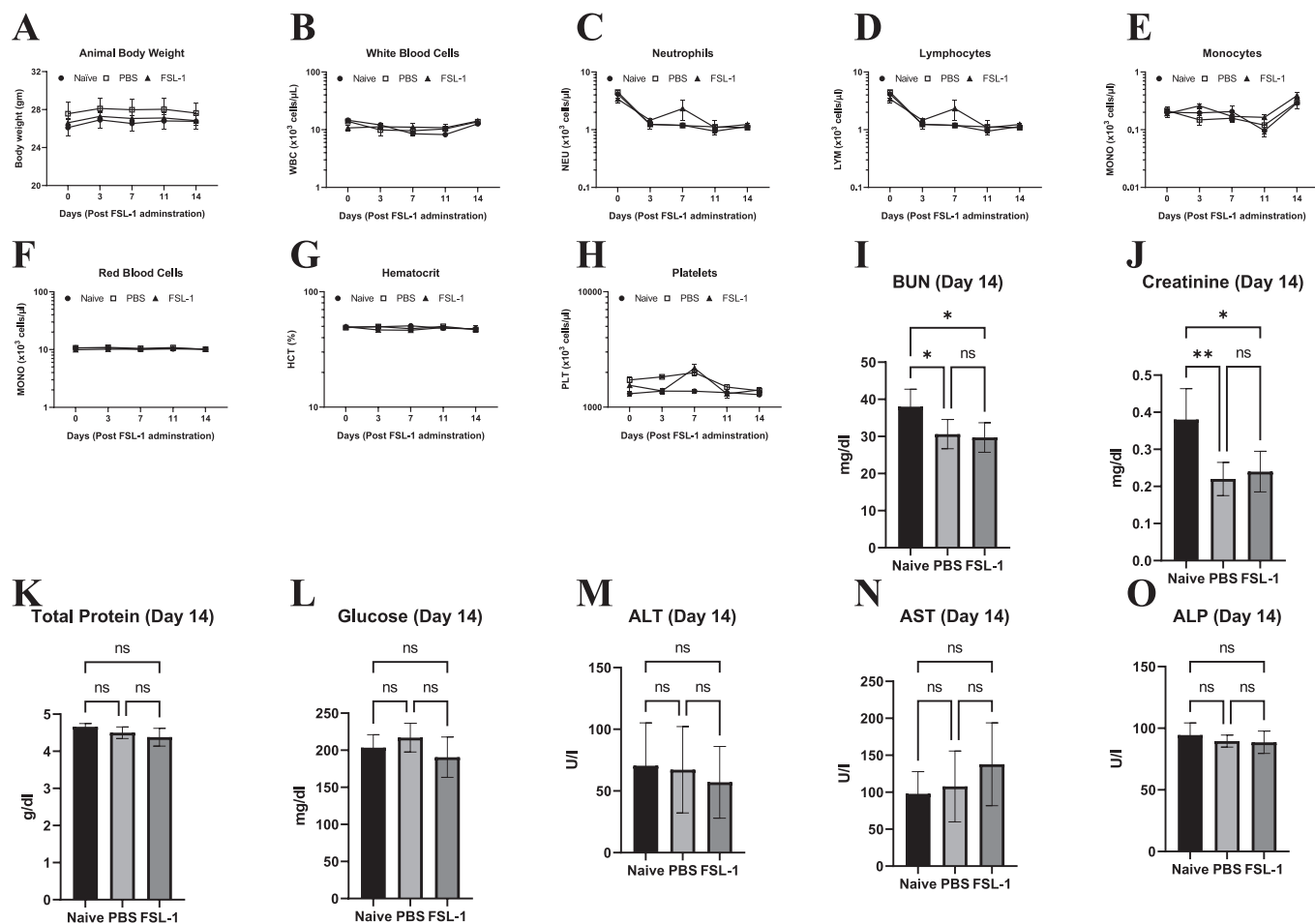
#### *Statistical Analysis*

Survival data were plotted as Kaplan-Meier plots; GraphPad Prism 9 software was utilized to perform Fisher's exact test to compare survival at 30 days and a log-rank test to compare survival curves. Probit analysis was conducted with IBM SPSS Statistics 25.0 and a student's t-test was used to assess differences between groups. Averages were reported  $\pm$  standard error of the mean (SEM).

## RESULTS

### *Non-Clinical Toxicology*

During the course of the study, no clinical observations were made that were indicative of any toxicity associated with FSL-1. After administration of FSL-1, animals exhibited normal behavior both in the short term (first 4 h after administration) and throughout the entirety of the study. At the conclusion of the study, a gross necropsy was performed by the lab staff and no abnormalities were noted in any of the major organs (liver, kidneys, spleen, heart, etc.). Over the course of the 14-day study there was no significant change in body weight amongst any of the days for the PBS (vehicle) group or the FSL-1 group when compared with the naïve group (Fig. 1A). Complete blood cell (CBC) counts were collected on days 0 (1h post injection) 3, 7, 11, and 14 (Fig. 1B–H). Among all analyzed parameters including white blood cells, neutrophils, lymphocytes, monocytes, red blood cells, hematocrit, and platelets, the only significant difference was noted for platelet counts. When the vehicle group was compared to the naïve group, counts were elevated on days 0, 3, and 7 ( $P = 0.0064$ ,  $P = 0.0001$ , and  $P = 0.0029$ , respectively), and for the FSL-1 group compared to the Naïve group, counts were elevated on day 7 only ( $P = 0.0018$ ). Finally, comparing vehicle and FSL-1 treatments, there were higher counts in the vehicle group on day 3 only ( $P = 0.0010$ ). On the final day of the study, day 14, a terminal blood draw was collected, serum separated and a panel of serum chemistry analytes measured (Fig. 1I–O) including blood urea nitrogen (BUN), creatinine, total protein, glucose, alanine transaminase (ALT), alkaline phosphatase (ALP), and aspartate transaminase (AST). Amongst these parameters, BUN was elevated in the naïve group compared to both the vehicle and FSL-1 groups ( $P = 0.0406$ , and  $P = 0.0222$ , respectively) and creatinine was elevated in the naïve group compared to both the vehicle and FSL-1 groups ( $P = 0.0046$ , and  $P = 0.0113$ , respectively). The alterations in platelets, BUN, and creatinine were within normal healthy ranges for mice. Taken together, these results indicate a lack of toxicity after administration of a single dose of FSL-1 at 3 mg/kg. This dose is  $>10$  times higher than the first dose tested (0.25 mg/kg) in mouse survival efficacy studies. However, we tested the 3 mg/kg dose in dose response study 3.



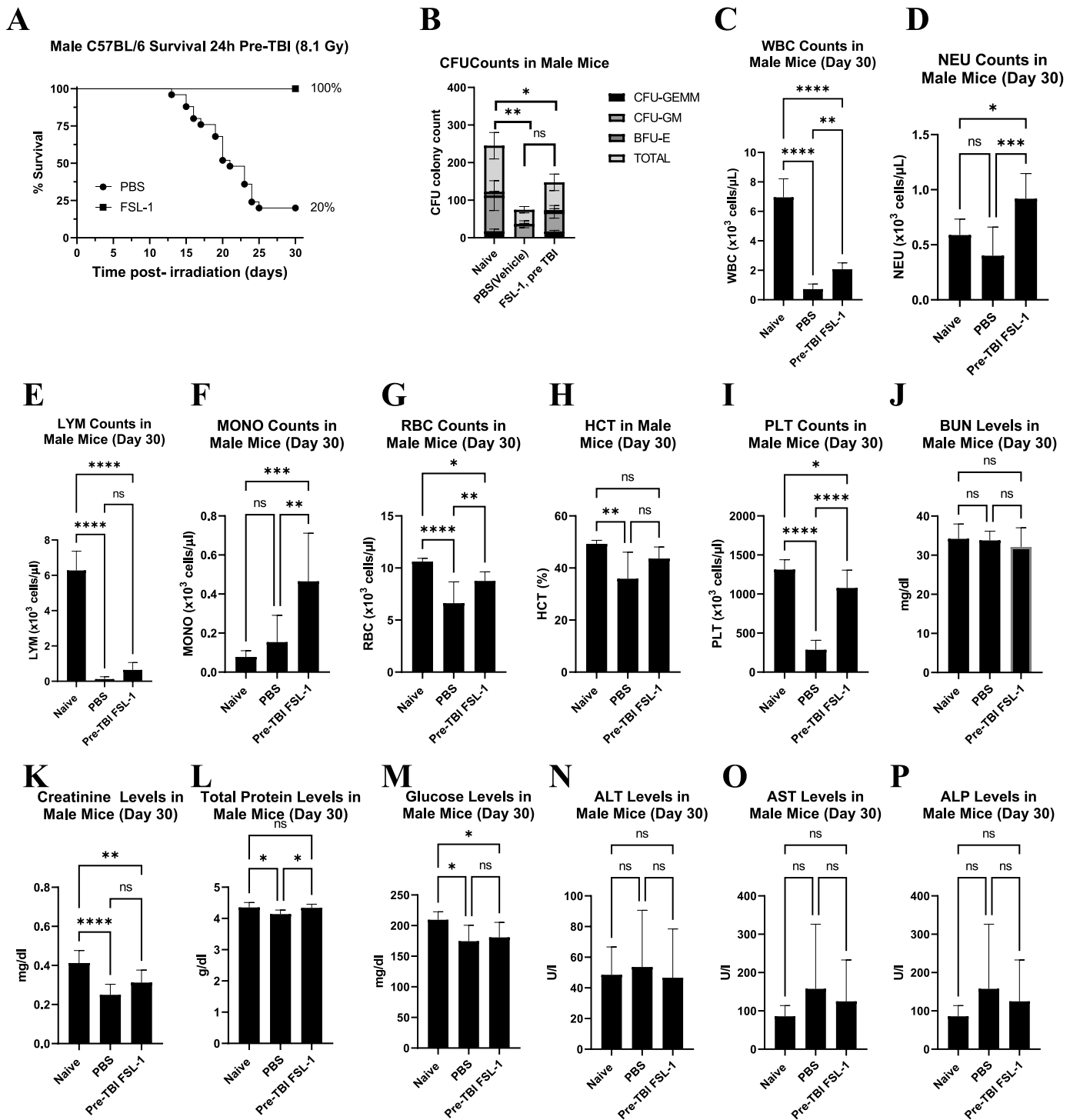
**FIG. 1.** Fourteen Day acute toxicity study. Male C57BL/6 mice were administered 3.0 mg/kg FSL-1 and observed for 14 days. Body weights (panel A) and complete blood cell counts (panels B–H) from serially sampled blood from the submandibular vein were collected on days 0, 3, 7, 11, and 14, and serum chemistry (panels I–O) was measured on day 14. N = 5 animals per group; \*P < 0.05, ns-not significant.

*Prophylactic Administration of FSL-1 Significantly Improved Survival in Male and Female C57BL/6 Mice and Protected Animals from Radiation-induced Hematological Damage*

**Male survival.** Finding no evidence of toxicity after treatment with the compound, FSL-1 was next tested for its ability to increase survival after exposure to gamma radiation. For this study, we utilized the dosing strategy that was shown to be effective in the mitigator studies (26), specifically 0.25 mg/kg administered SC 24 h prior to exposure. We investigated male mice exposed to a dose normally lethal to ~80% of animals (8.1 Gy) and observed 100% (25 out of 25) survival in the animals administered FSL-1 compared to 20% (5 out of 25) survival for animals administered the vehicle. Survival was plotted as a Kaplan-Meier curve (Figure 2A) and the two curves were compared via log-rank analysis and found to be significantly different (P < 0.0001); furthermore, survival of mice on day 30 was compared, and the increase in survival was found to be significantly higher in the animals treated with FSL-1 (P < 0.0001).

**Male CFU.** At the conclusion of the 30-day study we collected bone marrow from the femurs (Fig. 2B), which we cultured to enumerate the number of viable colonies. Both irradiated groups that received FSL-1 or vehicle treatment showed an expected decline in the number of colonies formed. However, the FSL-1 treatment showed a trend toward an increase over the vehicle treatment.

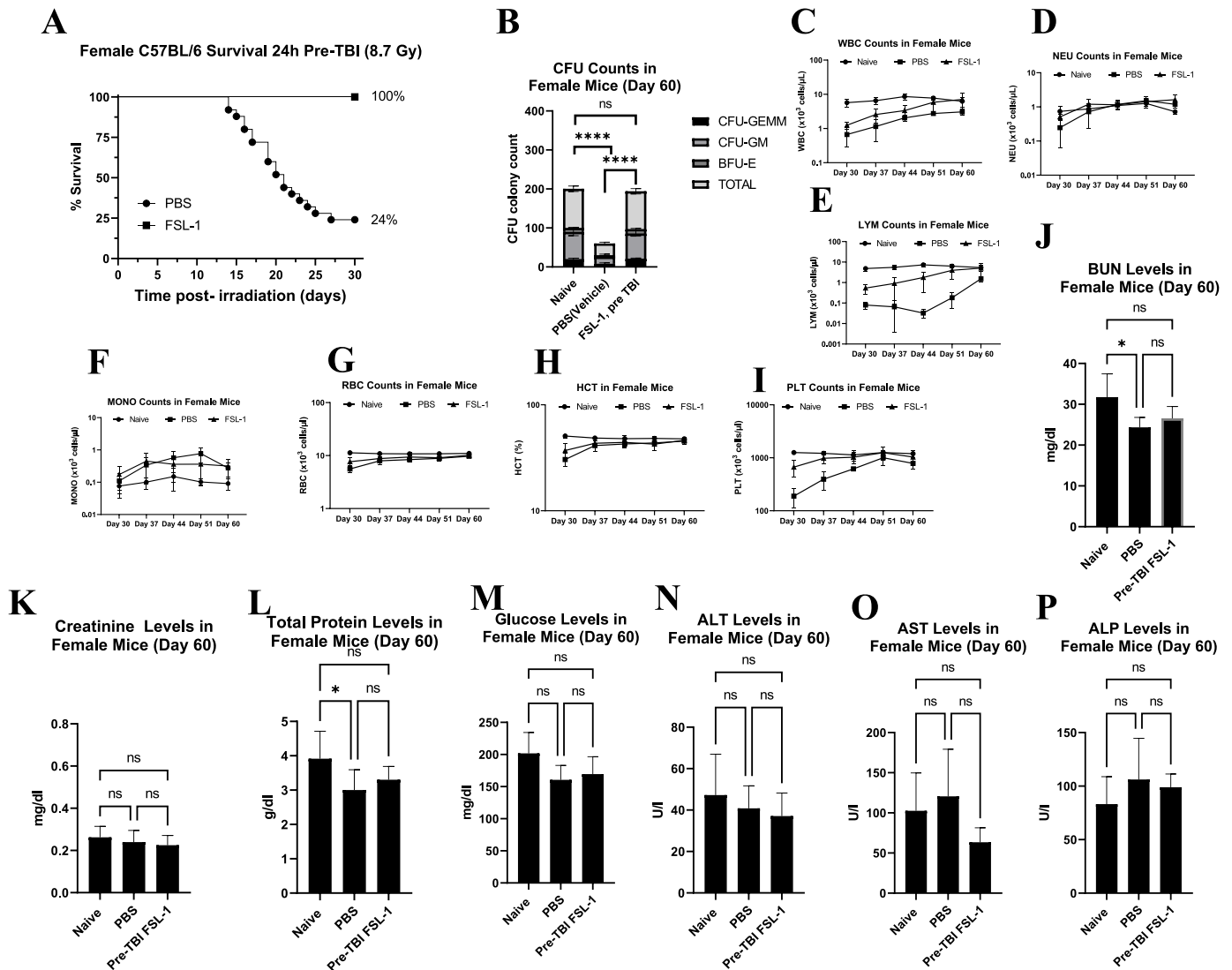
**Male CBC.** On day 30, blood was collected for CBC analyses (Fig. 2C–I). WBC levels were lower in both irradiated groups (P < 0.0001) compared to the naïve group, however FSL-1 levels were elevated relative to the vehicle group (P = 0.0070). For neutrophils, the FSL-1 counts were higher than both the naïve (P = 0.0164) and the vehicle-treated animals (P = 0.0003). Lymphocytes counts were lower in both irradiated groups (P < 0.0001) compared to the naïve group, but FSL-1 levels were higher than the vehicle group although this difference was not significant. For monocytes, the FSL-1 counts were higher than both the naïve (P = 0.0003) and vehicle-treated animals (P = 0.0029). RBC levels were lower in both irradiated groups (P < 0.0001 for vehicle and P = 0.0235 for FSL-1)



**FIG. 2.** Thirty day survival study in males. Male C57BL/6 mice were administered 0.25 mg/kg SC 24 h prior to TBI (8.1 Gy). Panel A: Survival curves of the 30 day study (n = 25/group); Panel B: Colony-forming units of the femoral bone marrow on day 30; Panels C–I: Complete blood cell counts on day 30; Panels J–P: serum chemistry parameters on day 30. For Panels B–I, n = 5–10 animals per group; \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001, and \*\*\*\*P < 0.0001, ns-not significant.

compared to naïve animals, however for FSL-1 treated animals, levels were elevated relative to the vehicle group (P = 0.0096). Hematocrit levels were lower in the vehicle group compared to naïve (P = 0.0013) and FSL-1 (P = 0.0666) groups; however, for FSL-1 treated animals, levels were not

significantly different from naïve animals. Finally, platelet levels were lower in both irradiated groups (P < 0.0001 for vehicle and P = 0.0252 for FSL-1) compared to naïve animals, but FSL-1 levels were elevated relative to animals treated with vehicle (P < 0.0001).

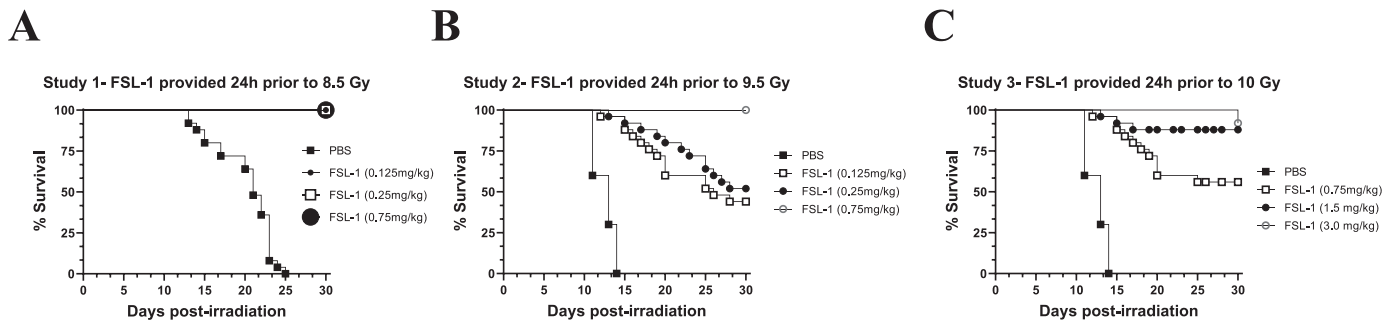


**FIG. 3.** Thirty day survival (60 day observation) study in females. Female C57BL/6 mice were administered 0.25 mg/kg SC 24 h prior to TBI (8.7 Gy). Panel A: Survival curves of the 30 day study ( $n = 25/\text{group}$ ); Panel B: Colony-forming units of the femoral bone marrow on day 60; Panels C–I: Complete blood cell counts from blood serially collected on days 30, 37, 44, 51 and 60; Panels J–P: Serum chemistry parameters on day 60. For Panels B–I,  $n = 5\text{--}10$  animals per group; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , and \*\*\*\* $P < 0.0001$ , ns-not significant.

**Male serum chemistry.** On day 30, serum chemistry panels were analyzed (Fig. 2J–P). Alterations were observed in creatinine, total protein, and glucose levels. For creatinine, levels were lower in both irradiated groups ( $P < 0.0001$  for vehicle and  $P = 0.0093$  for FSL-1) compared to the naïve group, and a trend towards increased levels in the FSL-1 group compared to the vehicle group was observed, although this was not significant. For total protein, lower levels were observed in the irradiated vehicle group compared to both the naïve and FSL-1 groups ( $P = 0.0147$  and  $P = 0.0220$ , respectively); however there was not a significant difference in the levels between naïve and FSL-1 treated animals. For glucose, levels were lower in both irradiated groups ( $P = 0.0122$  and  $P = 0.0429$  for vehicle and FSL-1, respectively) compared to the naïve group.

**Female Survival.** Since we observed significant increases in survival amongst the males, we next wanted to validate the same result in females. The same dose of FSL-1 (0.25mg/kg) was administered SC 24h prior to exposure. As with the males, females were irradiated with a dose that normally results in  $\sim 80\%$  lethality (8.7 Gy), and on day 30 after this exposure, we observed 100% (25 out of 25) survival in the animals administered FSL-1 compared to 24% (6 out of 25) survival for animals administered the vehicle. Animal survival was plotted as a Kaplan-Meier curve (Figure 3A) and the two curves were compared via log-rank analysis and found to be significantly different from one another ( $P < 0.0001$ ). Furthermore, survival on day 30 was compared, and the increase in survival was found to be significantly higher in the animals treated with FSL-1 ( $P < 0.0001$ ). Since we had not observed complete recovery





**FIG. 4.** Optimum dose determination study. Male C57BL/6 mice were administered escalating doses of FSL-1 24 h prior to TBI with various radiation doses. Panel A: Animals were administered vehicle (PBS), 0.125, 0.25, or 0.75 mg/kg 24 h prior to TBI with 8.5 Gy ( $n = 25/\text{group}$ ). Panel B: Animals were administered vehicle ( $n = 10$ ), 0.125, 0.25, or 0.75 mg/kg 24 h prior to TBI with 9.5 Gy ( $n = 25/\text{group}$ ); Panel C: Animals were administered vehicle ( $n = 10$ ), 0.75, 1.5, or 3.0 mg/kg 24 h prior to TBI with 10 Gy ( $n = 25/\text{group}$ ).

of CBC parameters in the 30-day study in males, we monitored females through 60 days. At the completion of the 60 day period, all females treated with FSL-1 had survived, and there was one additional mortality in the group treated with vehicle (5 out 25, 20% survival).

**Female CFU.** Since the study was extended to 60 days in an effort to see increased recovery of CBC, bone marrow was collected from the femurs at this time point (Fig. 3B), which we cultured to enumerate the number of viable colonies. At this 60-day time point, the levels of CFU in the naïve animals and animals treated with FSL-1 were not significantly different from one another; however the levels of the group treated with vehicle were significantly lower ( $P < 0.0001$ ) than levels in both the naïve and FSL-1 groups.

**Female CBC.** Blood ( $\sim 20 \mu\text{l}$ ) was collected from the submandibular vein on days 30, 37, 44, 51, and 60 to monitor the recovery of the CBC lineages. This sampling method is non-terminal; thus, the same animals were followed throughout the study (Fig. 3C–I). On day 30, nearly all lineages (WBC, NEU, LYM, RBC, HCT, and PLT) were lower than naïve animals in both irradiated groups. However, over the course of the following 30 days, through day 60, an accelerated recovery to naïve levels was observed in the FSL-1 group compared to the vehicle group. For instance, WBC recovered to naïve levels by day 51 for animals treated with FSL-1, whereas the animals treated with vehicle were still slightly lower on day 60. For neutrophils, the vehicle group appeared to recover concurrently with the FSL-1 group by day 37; however, the levels decreased again on day 60. Lymphocyte counts remained lower for groups treated with vehicle compared to the naïve group, with recovery observed in the group treated with FSL-1 by day 51. Monocyte counts were elevated in the irradiated groups but the FSL-1 group recovered quicker. RBC and HCT were both recovered in the irradiated groups by day 37. Finally, the PLT counts recovered to naïve levels in animals treated with FSL-1 by day 37; however, this recovery was not observed until day 51 in the vehicle group.

**Female serum chemistry.** On day 60, serum chemistry panels were analyzed (Fig. 3J–P). Among all analytes there were no statistically significant changes between Naïve and FSL-1 groups. Additionally, there were no statistically significant differences between animals treated with FSL-1 or vehicle. Statistically significant differences were observed between the vehicle and naïve groups for BUN ( $P = 0.0175$ ), and total protein ( $P = 0.0447$ ).

#### *Dose-dependent Increase in Survival Efficacy Demonstrated Ability of FSL-1 to be an Extremely Potent Radiation Prophylactic Agent*

In this series of studies, we attempted to determine the dose of FSL-1 that provides optimal survival. Male mice were administered 0.125, 0.25, 0.75, 1.5 or 3.0 mg/kg FSL-1; this range included a dose that was lower than the 0.25 mg/kg used in the previously published mitigator studies (26) and the initial studies reported here, as well doses escalated up to the highest tolerated dose in this study (3.0 mg/kg). Our initial studies in both males and females (Figs. 2A and 3A) demonstrated 100% survival under conditions that resulted in  $\sim 80\%$  lethality in the animals treated with vehicle.

#### *First Study (8.5 Gy)*

We first tested the survival of male mice treated with 0.125, 0.25, or 0.75 mg/kg 24 h prior to TBI at a lethal dose (8.5 Gy,  $n = 25/\text{group}$ ). In this study (Fig. 4A), all animals administered FSL-1 survived, whereas as predicted, all animals in the vehicle group died. This resulted in a highly significant difference in survival curves (log-rank analysis,  $P < 0.0001$ ) for all FSL-1 groups compared to the vehicle group. Further the Fisher's exact test was also highly significant ( $P < 0.0001$ ) for all FSL-1 groups compared to the vehicle group. However, the survival curves and survival proportions for the groups administered FSL-1 were statistically indistinguishable from one another.

### Second Study (9.5 Gy)

We next increased the radiation challenge to a higher dose (9.5 Gy) and utilized the same drug dosing (0.125, 0.25, and 0.75 mg/kg) in male mice. In this study (Fig. 4B), we observed 100% lethality in the control group ( $n = 10$ ), 44% mortality in group that received the lowest FSL-1 dose (0.125 mg/kg,  $n = 25$ ), 52% mortality in the group that received the middle dose (0.25 mg/kg,  $n = 25$ ), and 100% survival in the group that received the highest dose (0.75 mg/kg,  $n = 25$ ). The log-rank analysis for all groups receiving FSL-1 compared to those receiving vehicle were highly significant ( $P < 0.0001$ ), and the Fisher's exact test revealed significant increases in 30-day survival ( $P = 0.0146$ ,  $P = 0.0052$ , and  $P < 0.0001$  for 0.125, 0.25, and 0.75 mg/kg, respectively). Comparing the survival curves (log-rank analysis) of the groups receiving FSL-1, the group receiving 0.75 mg/kg was statistically different than both groups receiving 0.25 and 0.125mg/kg ( $P < 0.0001$ ) (which were not significantly different from one another). The 30-day survival (Fisher's exact test) revealed similar findings with the survival significantly increased ( $P < 0.0001$ ) for the highest dose compared to both lower doses, and the two lower doses being indistinguishable from one another.

### Third Study (10 Gy)

In the final study in this series, the dose of radiation was increased further (10 Gy) and the doses of FSL-1 were escalated to include 0.75, 1.5, and 3.0 mg/kg in male mice. In this study (Fig. 4C) we once again observed 100% lethality in the control group ( $n = 10$ ), 56% mortality in the group receiving the lowest FSL-1 dose (0.75 mg/kg,  $n = 25$ ), 88% mortality in the group receiving the middle dose (1.5 mg/kg,  $n = 25$ ), and 92% survival in the group receiving the highest dose (3.0 mg/kg,  $n = 25$ ). Once again, log-rank and Fisher's exact tests of all groups receiving FSL-1 showed high statistical significance compared to the vehicle group ( $P < 0.0001$ ). Among the groups receiving FSL-1, there was not a statistically significant difference in the survival curves for the animals receiving 1.5 and 3.0 mg/kg, both of which showed a statistical advantage over the lowest dose (0.75mg/kg). Thus, the optimum dose of FSL-1 was determined to be 1.5 mg/kg, as this represented the lowest dose to produce the same survival outcome.

## DISCUSSION

Here we report the first use of FSL-1 as a prophylactic radiation countermeasure. FSL-1 is TLR2/6 agonist which activates NF- $\kappa$ B, a pathway which has a well-established role in various cancers (32). Further NF- $\kappa$ B activation is implicated in response to various stressors including radiation exposure (33, 34). In perhaps a related role, NF- $\kappa$ B regulates innate and adaptive immune functions including the inflammatory response (35). The activation of NF- $\kappa$ B can occur through a MyD88-dependent interaction. TLRs

activate MyD88, which promotes a cascade of events including polyubiquitination of target proteins (36) and ultimately NF- $\kappa$ B activation. TLRs 2, 5, 7, and 9 exhibit this MyD88-dependent activation. However, TLR2 specifically requires the additional adapter protein TIRAP (TIR-containing adapter protein) for this function (37).

A series of TLR agonists have been studied for their roles as radiation countermeasures. Entolimod (CBLB502), a TLR5 agonist that is being developed by Cleveland Biolabs is derived from Salmonella flagellin and has shown protective effects when administered to mice and nonhuman primates (NHP) 30 min prior to radiation exposure (38). Additionally, CBLB612 is being developed as an agonist of TLR2 (19) and CBLB613 is being developed as an agonist of TLR2/6 (20), both of which have shown activity as radiation countermeasures in mice. FSL-1, as mentioned, is also a TLR2/6 agonist with the key difference between FSL-1 and CBLB613 being that the former is a synthetic peptide while the latter is a naturally derived Mycoplasma protein. The use of the synthetic peptide offers potential advantages over the naturally-derived compound, including the high level of characterization of synthetic compounds, potential to customize the synthetic compound, and defined function (39).

In the studies reported here, we first demonstrate that subcutaneous FSL-1 administration at doses up to 3.0 mg/kg showed no toxicity in male C57BL/6 mice. Further, we demonstrated that FSL-1 treatment (0.25 mg/kg,) administered 24 h prior to a  $\sim$ LD<sub>80/30</sub> dose of radiation enhanced survival of male and female C57BL/6 to 100%. In both sexes, FSL-1 treatment increased the number of white blood cells, neutrophils, lymphocytes, and platelets. In female mice, we demonstrated that FSL-1 treatment accelerated the recovery of these lineages to non-irradiated naïve levels quicker than vehicle treatment alone. Slight (although significant) alterations in serum chemistry parameters were observed in irradiated animals (in males, creatinine, total protein, and glucose; in females, BUN and total protein). In females, the serum chemistry parameters were at naïve levels in animals treated with FSL-1 and in males an increase toward naïve levels was observed. This near-complete recovery in females is likely the result of the extended period of observation in the females (60 days) compared to males (30 days).

We next determined the optimum dose of FSL-1 administration when it is administered 24 h prior to TBI. Previous work had utilized a dose of 0.25 mg/kg when FSL-1 is administered as a mitigator 24 h after TBI (26), which we confirmed in our own studies (data not shown). However, when FSL-1 is provided as a prophylaxis, the optimum dose required increases. In the current study, we have shown that 1.5 mg/kg provides the optimum survival benefit. It is our hypothesis that the compound is metabolized or cleared in the ensuing time after administration, thus requiring a higher dose for action when administered at 24 h prior to TBI. In our dose optimization studies, we were able to determine that treatment with 0.75 mg/kg FSL-1 resulted in 100% survival for animals exposed to 9.5 Gy or

below, and only by increasing the dose of radiation did we begin to see mortality at this dose. Specifically, at 10 Gy we saw 56% survival, and while our future work will include a dedicated dose reduction factor (DRF) to quantify the effectiveness at a 1.5 mg/kg dose, the results presented here indicate that the DRF for FSL-1 will be in excess of 1.27. This analysis is based on the ~50% survival of the 0.75 mg/kg group at 10 Gy and our historical data on this mouse strain indicating an LD<sub>50/30</sub> of ~7.9 Gy without intervention. At the optimal dosing, it is expected that this DRF estimate would be higher.

#### *Future Work, Study Limitations, and Conclusions*

While the current work has demonstrated the excellent prophylactic efficacy of FSL-1 in a murine model, there are still some limitations, which will be addressed in future studies. For instance, the dose optimization studies have been limited to a single sex, and it would be optimal to confirm these dosing strategies in female mice. Further this work was conducted in a single mouse strain. The confirmation of efficacy in additional strains that are inherently more radiosensitive (e.g. C3H/HeN) or more radioresistant (e.g. CD2F1) would provide more insight into the universal protection afforded to murine models. Enhancing this dosing information as well as demonstrating the pharmacokinetics and pharmacodynamics will be studied in future work to optimize the dose for large animal studies which would ultimately be required for FDA approval.

As with all countermeasures developed under the FDA animal rule, ultimate approval is contingent on a well-understood mechanism of action for the drug, a reliable means to demonstrate a bridging efficacy in humans, determination of the optimal dose and safety in humans, and commonly demonstrating efficacy in one or more species. These guidelines set the future work for this compound, namely, to establish that the mechanism of action is the same as the mitigator mechanism of action through additional studies focused on sample collection and analysis, as well testing the efficacy of the compound in additional species, and ultimately establishing safety and dosing in human subjects. Despite the work ahead, these results reveal the promise of FSL-1 as a prophylaxis for radiation exposure. This promising countermeasure, FSL-1, will be of great benefit to military personnel and first responders that are likely to encounter radiation-contaminated areas in the execution of their duties.

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