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SHORT COMMUNICATION

Re-Examination of the Exacerbating Effect of Inflammasome Components during Radiation Injury

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Radiation can be applied for therapeutic benefit against cancer or may result in devastating harm due to accidental or intentional release of nuclear energy. In all cases, radiation exposure causes molecular and cellular damage, resulting in the production of inflammatory factors and danger signals. Several classes of innate immune receptors sense the released damage associated molecules and activate cellular response pathways, including the induction of inflammasome signaling that impacts IL-1β/IL-18 maturation and cell death. A previous report indicated inflammasomes aggravate acute radiation syndrome. In contrast, here we find that inflammasome components do not exacerbate gamma-radiation-induced injury by examining heterozygous and gene-deletion littermate controls in addition to wild-type mice. Absence of some inflammasome genes, such as *caspase-1/11* and *Nlrp3*, enhance susceptibility of treated mice to acute radiation injury, indicating importance of the inflammasome pathway in radioprotection. Surprisingly, we discover that the survival outcome may be sex-dependent as more inflammasome-deficient male mice are susceptible to radiation-induced injury. We discuss parameters that may influence the role of inflammasomes as radioprotective or radioexacerbating factors in recovery from radiation injury including the use of littermate controls, the sex of the animals, differences in microbiota within the colonies and other experimental conditions. Under the conditions tested, inflammasome components do not exacerbate radiation injury, but rather provide protective benefit.

INTRODUCTION

Radiation can be applied for therapeutic benefit to destroy cancer cells or may cause devastating harm due to accidental or intentional release of nuclear energy. Radiation exposure causes DNA and cellular damage mediated through the production of damage associated molecular patterns (DAMPs) (2, 3). These molecular danger signals, including nucleic acid molecules, ATP, HMGB1, heat shock proteins, oxidized protein and lipid fragments and metabolic factors, alert local and systemic immune responses to address radiation-induced injury and promote repair processes. Pattern recognition receptors (PRR) sense the released DAMPs and activate cellular response pathways. Triggering these signaling pathways impacts subsequent production of inflammatory factors and activation of cell survival processes. To employ these pathways to mitigate radiation-induced damage, research has focused on characterizing biomolecules that engage PRR to provide radioprotection (4, 5).

The aim of this report is to examine the role of the inflammasome in responding to radiation damage. Inflammasome components are induced by radiation and regulate cell death and inflammatory factors (6). The inflammasome is a protein complex comprised of a receptor/sensor (such as NLRP3 or AIM2), the ASC common adaptor molecule and the executioner caspases (1 and 11 in mice). This leads to caspase-1 activation, resulting in pro-IL-1β, pro-IL-18 and pro-Gasdermin D (GSDMD) cleavage to mature IL-1β, IL-18 and GSDMD, causing inflammation and pyroptotic cell death (7). Due to the pivotal role of inflammasomes in orchestrating molecular and cellular responses to injury, we explored the impact of inflammasomes loss in animals exposed to radiation.

The published report by Hu et al. (1) described the role of AIM2, ASC and caspase-1/11 in exacerbating subtotal-body irradiation (SBI) induced gastrointestinal syndrome and total-body irradiation (TBI) induced hematopoietic (H) failure resulting from X-ray irradiation. In their study,

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wild-type (WT) C57BL/6N or C57BL/6J sub-strains were purchased from the National Cancer Institute or Jackson Laboratories and cohoused with *Casp1/11*$^{-/-}$, *Casp1*$^{-/-}$, *Asc*$^{-/-}$ (backcrossed to C57BL/6N) or *Aim2*$^{-/-}$ (backcrossed to C57BL/6J), and in each case, mice lacking the inflammasome gene showed a significant survival advantage over wild-type controls.

To clarify the complex responses of inflammasomes to environmental insults, we investigated inflammasome components in an in vivo irradiation model. In contrast to Hu et al. (1), we examined if inflammasomes exacerbate or protect against acute radiation syndrome (ARS) induced by TBI by comparing survival of gene-deletion to gene-competent littermates to minimize genomic background and microbiota differences and to WT controls. We also separately studied males and females. Here, mice were subjected to cesium-sourced TBI (7.5–8.2Gy) and monitored for 30 days. We found that rather than exacerbating injury, inflammasome components are needed for protecting against the development of lethal ARS from exposure to high-dose gamma radiation.

MATERIALS AND METHODS

Mice

Gene-deletion mice were backcrossed to C57BL/6J for more than 12 generations, housed in AAALAC-accredited specific pathogen free facilities and treated under protocols approved by the Institutional Animal Care and Use Committee at University of North Carolina at Chapel Hill. *Asc*$^{-/-}$ were obtained from V. Dixit (Genentech, San Francisco, CA) (*8*). *Casp1/11*$^{-/-}$ and *Nlrp3*$^{-/-}$ were as described elsewhere (9). *Aim2*$^{-/-}$ were generated at UNC as described elsewhere (10). The C57BL/6 controls, referred to as either wild-type or WT, were handled under similar conditions, but bred in separate cages from gene-deficient strains. Male and female gene-deficient, heterozygous littermates and WT control mice (8–24 weeks of age) were age- and sex-matched, subjected to TBI and provided autoclaved, extruded chow (LabDiet, Purina 5V0F/G) and autoclaved, non-acidified water *ad libitum* for 30 days of monitoring. Euthanasia criteria included lethality, 25% weight loss, severe dehydration, agonal respirations, lying in prone position, or combinations of milder symptoms such as lesions, diminished activity, hunched posture or ruffled fur.

Irradiation

Conscious mice were placed in 12-compartment pie containers. The lucite cages were placed in 137-cesium Irradiator (Gammacell 40 Exactor, Best Theratronics, Ontario, Canada) for TBI with 7.5, 8.0 or 8.2 Gy gamma rays at a ~0.94 Gy/min dose rate. Dosimetry with phantoms conducted by Dr. Keith Kunugi (University of Wisconsin) confirmed that 101.9% of target dose was delivered.

Statistics

Survival distributions were estimated using the Kaplan Meier method with differences analyzed using the log-rank (Mantel-Cox) test based on the corresponding asymptotic *P* value, employing a two-sided unadjusted significance level of 0.05. These estimates were performed using GraphPad Prism v9. Results were derived from combining two to three replicate studies according to genotype. Additionally, we employed the stratified log-rank test implemented by the survdiff function of the R survival package. No sample size power calculations were performed prior to the experiments as all available age- and sex-matched gene-deletion, gene-competent and WT mice were compared during the study.

RESULTS

We investigated the role of inflammasomes in exacerbating or protecting against radiation-induced injury by subjecting mice lacking expression of inflammasome components to total-body irradiation. First, we analyzed *Aim2*$^{-/-}$ mice, a focus of the published article by Hu et al. (1). AIM2 is a receptor of double-stranded DNA and associates with ASC and caspases to perform its inflammasome function (11–14). *Aim2*$^{+/-}$ male mice presented enhanced survival over *Aim2*$^{-/-}$ littermates at both 8.0 and 8.2 Gy TBI (Fig. 1A and C), while no survival difference was seen among female *Aim2*$^{-/-}$ and *Aim2*$^{+/-}$ littermates given 8.0 or 8.2 Gy TBI (Fig. 1B and D). In addition, we compared WT mice that were from different cages to the *Aim2*$^{-/-}$ mice. *Aim2*$^{-/-}$ males were more significantly susceptible to ARS at both 8.0 and 8.2 Gy TBI with no animals surviving more than 17 days, while *Aim2*$^{-/-}$ females showed significantly less survival than WT females at 8.0 Gy TBI.

We also analyzed NLRP3, which is also important for DNA-activated inflammasomes in both human and mice (15, 16). Male *Nlrp3*$^{+/-}$ mice had significant survival advantage over male *Nlrp3*$^{-/-}$ littermates at 8.0 and 8.2 Gy TBI (Fig. 1E and G), while no statistical difference in survival was observed between *Nlrp3*$^{+/-}$ and *Nlrp3*$^{-/-}$ female littermates at 8.0 and 8.2 Gy TBI (Fig. 1F and H). *Nlrp3*$^{-/-}$ males were more susceptible to radiation-induced lethality with no males surviving past 16 days in contrast to WT males. *Nlrp3*$^{-/-}$ females showed modest, but significantly greater susceptibility to ARS than WT females at both radiation doses. Unlike the littermates, these two groups of mice were not from the same parents or the same cage.

Next, we investigated the impact of loss of the commonly shared activating partners of the inflammasome complex, namely caspase-1/11 and ASC. *Casp1/11*$^{-/-}$ male mice revealed significantly reduced survival compared to *Casp1/11*$^{+/-}$ male littermates, while females showed no difference at 8.2 Gy TBI (Fig. 2A and B). Similarly, *Asc*$^{+/-}$ displayed a statistically significant survival advantage over *Asc*$^{-/-}$ male littermates when exposed to 7.5 Gy TBI, approximating reported whole-body irradiation (1), while females showed no difference (Fig. 2C and D). Similar outcomes were evident when comparing gene-deficient animals to WT controls: *Asc*$^{-/-}$ males were significantly less protected than WT males, while *Asc*$^{-/-}$ females showed no survival difference from WT females. Overall, using a stringent *P* value of 0.05/9 or 0.0056 when comparing all genotypes, we found that Nlrp3, caspase-1/11 and Asc in males were significantly protective against H-ARS.

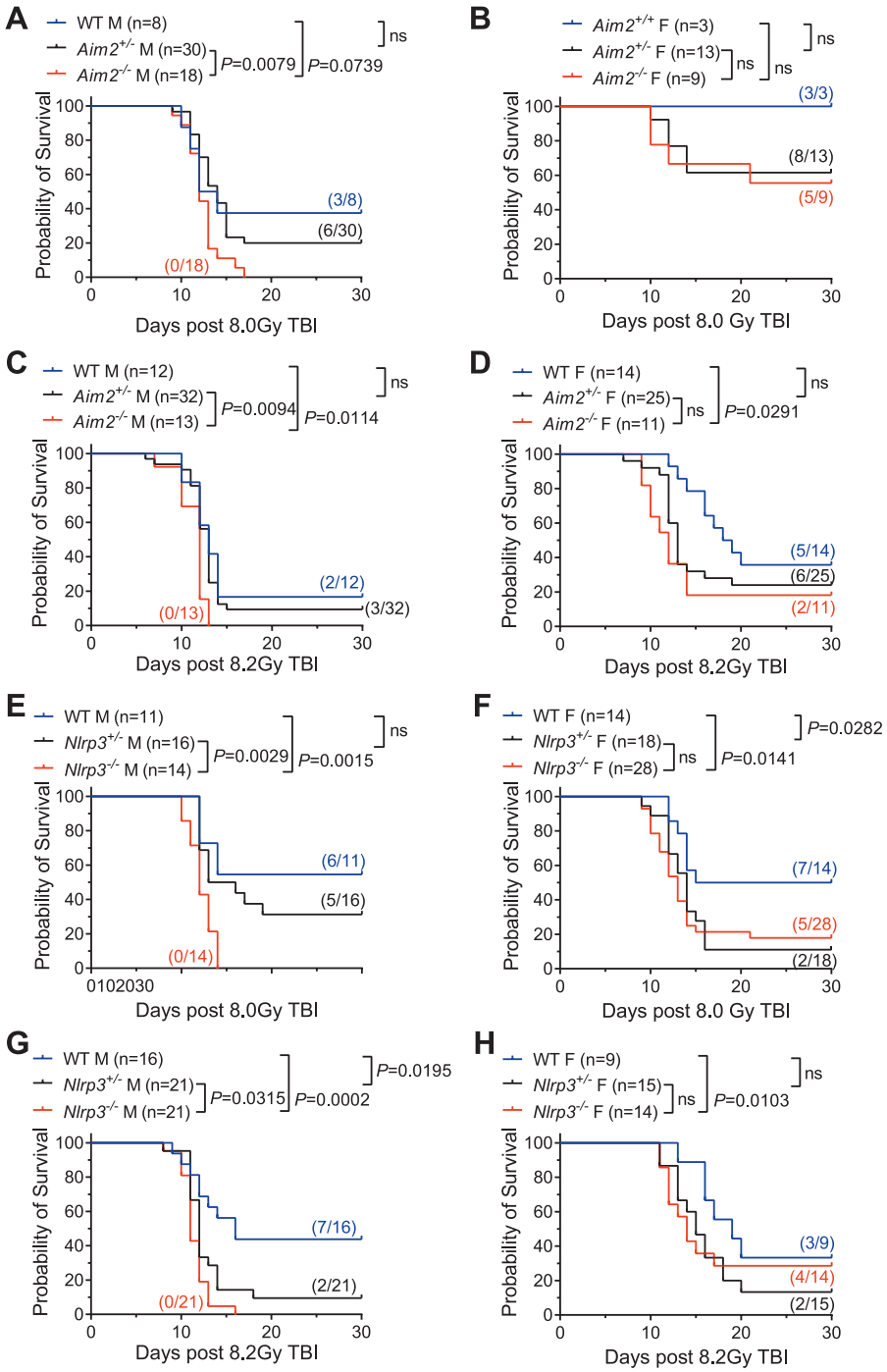

FIG. 1. Deletion of inflammasome components Aim2 and Nlrp3 do not protect against ARS. The following animals were subjected to total-body irradiation (TBI): WT (not littermates of the next two groups), $Aim2^{+/-}$ and $Aim2^{-/-}$ littermate males (panel A) and females (panel B) at 8.0 or 8.2 Gy TBI (panels C and D); WT (not littermates), $Nlrp3^{+/-}$ and $Nlrp3^{-/-}$ littermate males (panel E) and females (panel F) at 8.0 or 8.2 Gy TBI (panels G and H). Mice were monitored for 30 days postirradiation. Survival distributions were estimated using the Kaplan Meier method where differences were examined using the log-rank (Mantel-Cox) test based on the corresponding asymptotic P value, employing a two-sided unadjusted significance level of 0.05. Multiple replicate studies with age- and sex-matched mice were combined with total animals (n) and 30-day survivors per total animals tested indicated within survival plots.

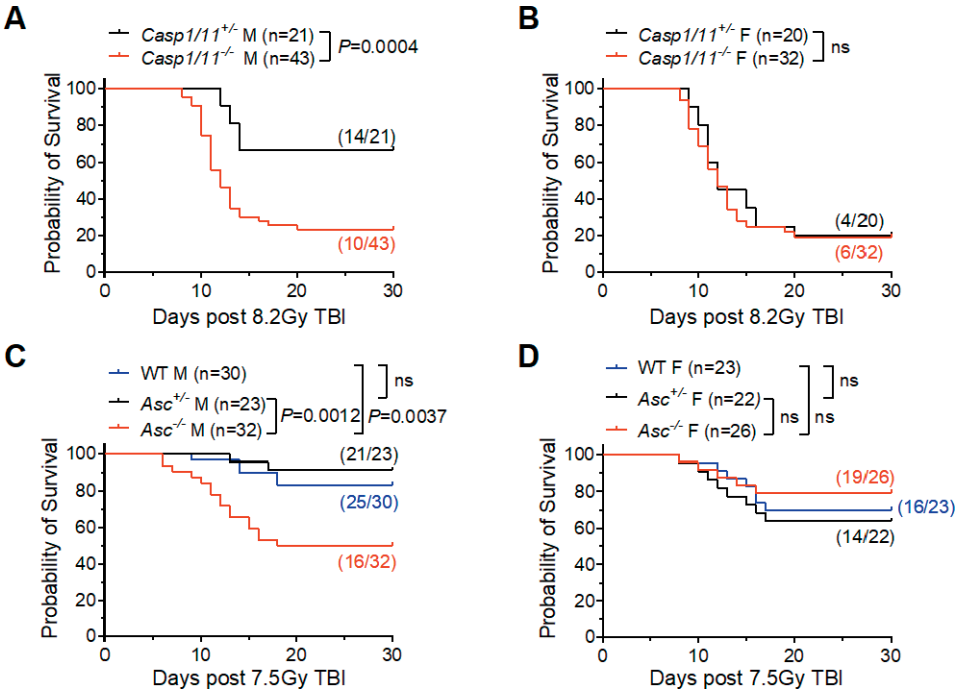

FIG. 2. Deletion of inflammasome components caspase-1/11 and Asc do not protect against ARS. The following animals were subjected to TBI: C*asp1/11*$^{+/-}$ vs. *Casp1/11*$^{-/-}$ littermate males (panel A) or females (panel B) at 8.2 Gy TBI; WT (not littermates of the next two groups), *Asc*$^{+/-}$ vs. *Asc*$^{-/-}$ littermate males (panel C) or females (panel D) at 7.5 Gy TBI. Mice were monitored for 30 days postirradiation. Survival distributions were estimated using the Kaplan Meier method where differences were examined using the log-rank (Mantel-Cox) test based on the corresponding asymptotic *P* value, employing a two-sided unadjusted significance level of 0.05. Multiple replicate studies with age- and sex-matched mice were combined with total animals (n) and 30-day survivors per total animals tested indicated within survival plots.

DISCUSSION

In summary, these results show that the absence of inflammasome does not protect against ARS, but the presence of inflammasome is correlated with a survival advantage, especially prominent in male mice. Many links of inflammasome activity to the control of inflammation and metabolism as well as to pathogenesis related to pathogen infection or immune dysregulation (autoimmunity) have been reported (17–19). In this study highlighting use of littermate controls, we find that the inflammasome protects against radiation injury in male mice. It is possible that inflammasome sensing of danger signals resulting from gamma irradiation stimulates immunity and directs responses toward recovery from, rather than aggravation of injury. These observations suggest the important potential for engaging the inflammasome pathway to induce protective mechanisms and factors against the damaging effects of radiation.

In contrast to a previous report (*1*), our results do not indicate an exacerbating role for *Casp1/11, Asc, Nlrp3* or *Aim2* upon exposure to lethal doses of TBI using a cesium source. Instead, all exhibited a protective role in males, but not in females, with radioprotection varying with gene-deletion target. *Casp1/11* and *Asc,* which are shared by all or most inflammasome pathways, exhibited significant radioprotective effects, while the effects of *Aim2* and *Nlrp3* were much less prominent. In a companion report, Daniel et al. (20) also found a non-exacerbating role for *Casp1/11* deficient mice using X-ray irradiation approaches in both TBI and partial-body irradiation models, but they did not observe the sex-dependent protective effect.

Several explanations can be considered for the differences between our results and the published report by Hu et al. (1). In this study, sex-specific survival outcomes were reported, with gene-deficient males showing greater lethality than gene-competent counterparts whereas modest differences in survival were only evident for *Nlrp3* females. In contrast, Hu et al. (1) reported survival curves combining both sexes. Sex differences impacting immunity have been noted in mice subjected to viral infection and vaccination conditions, with females showing greater antibody production, B cell maturation and transcriptional activation stimulating inflammatory responses (21, 22). Although the effect of sex on mitigating responses to radiation has been demonstrated previously (23, 24), further investigation is required to fully understand the underlying mechanisms that cause differential responses in males and females.

Another possibility is that we used cesium-based gamma-radiation, while Hu et al. (1) used X-ray radiation with the caveat that gamma radiation is more homogeneous than X-ray radiation. Although considered to perform comparably (25, 26), a cesium source delivers a more consistent

radiation beam, thereby promoting reproducibility in outcomes in contrast to the less consistent and scattering beams emanating from an X-ray source. However, the study by Daniel et al. (20) also used X-ray radiation and did not observe radioprotection by caspases-1/11. Genetic drift is a less likely possibility because Hu et al. (1) observed the same phenotype across multiple genetically distinct strains obtained from different sources.

Another likely source of variation may be attributed to microbiota, which is significantly altered by radiation. Previously, we reported that radiation induces protective microbiota composition that emerges by 7 days after lethal irradiation and supports long-term survival from radiation injury (26). Hu et al. (1) used C57BL/6N and C57BL/6J controls obtained from commercial sources, whereas we and Daniel et al. (20) used littermate controls to minimize differences in background genetics and microbiota. In addition to harboring distinct microbiome profiles, commercially sourced vs. in-bred mice differ in metabolomic profiles. While Hu et al. (1) conditioned their mice with two-week co-housing prior to testing, this duration may be insufficient to equalize the microbiota composition. Littermates originating from a common source should possess similar microbiota and alleviate concerns about maternal effects. However, authors of the Hu et al. report (1) communicated to us that preliminary studies using a limited number of littermates replicated their earlier results, showing an exacerbating role for inflammasomes in X-ray radiation. Thus, an expanded, well-powered littermate study would be useful to elucidate answers to address contributions of microbiome and to address questions related to how sex and genotype differences impact microbiota (28, 29). Due to the focused scope of this study on the impact of gene-deletion on survival from TBI, analyses of microbiome differences between sexes and genotypes were not conducted.

Finally, the pathogen containment or cleanliness status may differ between facilities and between institutions such that the endemic microbiota in each animal facility (30) may uniquely and differentially influence the biologic impact of the inflammasome in response to radiation (27). Cross testing of institutionally derived microbiota would provide an opportunity to determine differential contributions of microbiota to responding to radiation injury. Additional remaining factors related to age of subjects, body weight or fat content related to metabolic parameters, time of day of radiation exposure and radiation doses (i.e., LD50/30 dose appropriate to male or female mice) may contribute to variable responses to radiation and should be considered in future investigations. Careful consideration of variables in well-controlled studies using genetically defined intact organisms (both male and female) is critical for validating cellular studies and elucidating the molecules, pathways and kinetics of injury and recovery responses to radiation.

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