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Radiation exposure induces cell and tissue damage, causing local and systemic inflammatory responses. Because the inflammasome pathway is triggered by cell death and danger-associated molecular patterns, we hypothesized that the inflammasome may signal acute and chronic immune responses to radiation. Using a mouse radiation model, we show that radiation induces a dose-dependent increase in inflammasome activation in macrophages, dendritic cells, NK cells, T cells, and B cells as judged by cleaved caspase-1 detection in cells. Time course analysis showed the appearance of cleaved caspase-1 in cells by day 1 and sustained expression until day 7 after radiation. Also, cells showing inflammasome activation coexpressed the cell surface apoptosis marker annexin V. The role of caspase-1 as a trigger for hematopoietic cell losses after radiation was studied in caspase-1−/− mice. We found less radiation-induced cell apoptosis and immune cell loss in caspase-1−/− mice than in control mice. Next, we tested whether uric acid might mediate inflammasome activation in cells by treating mice with allopurinol and discovered that allopurinol treatment completely blocked caspase-1 activation in cells. Finally, we demonstrate that radiation-induced caspase-1 activation occurs by a Nod-like receptor family protein 3–independent mechanism because radiation-exposed Nlrp3−/− mice showed caspase-1 activation profiles that were indistinguishable from those of wild-type mice. In summary, our data demonstrate that inflammasome activation occurs in many immune cell types following radiation exposure and that allopurinol prevented radiation-induced inflammasome activation. These results suggest that targeting the inflammasome may help control radiation-induced inflammation. The Journal of Immunology, 2015, 194: 1178–1189.

It is well known that radiation exposure occurring by intentional or unintentional ways causes cell death and damage in a dose-dependent fashion. In mammals, radiation does the most damage to actively dividing cell populations such as hematopoietic cells and cells lining mucosal surfaces. Individuals exposed to radiation show significant loss of peripheral immune cells, which predispose them to a wide range of microbial opportunistic infections. For this reason, a clearer understanding of the cellular and molecular events following radiation injury in humans is of paramount importance if a radiounerential event ever happens. Although radiation therapy is used to treat various types of cancer or for bone marrow transplants in patients, when used medically, radiation exposures are tightly controlled and directed to localized body areas to minimize the damage to healthy tissue or they are given at a minimal effective dose for bone marrow transplants. Nevertheless, unwanted side effects of radiation therapy do occur, including tissue injury, inflammation, and suppressed immune function, resulting in sickness and higher susceptibility to infections in these patients (1). Thus, a better understanding of how radiation exposure influences immune cell activation and function will advance the development of ways to mitigate undesirable effects of radiation exposure on the body.

Cell death after radiation occurs by mitotic catastrophe and by apoptosis. Another cell death modality described after radiation is cell senescence. Cell fate after radiation is mainly determined by the level of DNA damage and by the cell type affected (2). Whereas apoptosis is generally thought to be a relatively silent way for cells to die, dead cells do release molecules that have been shown to activate the immune system (3). These molecules act as endogenous danger signals to alert the immune system and are subsumed under the term alarmins (4, 5). Alarmins and microbial pathogen-associated molecular patterns are together categorized as danger-associated molecular patterns (DAMPs) (4). DAMPs are sensed by pattern recognition receptors (PRRs) (4). Currently, four types of PRRs are known and have been classified into the TLRs and the C-type lectin receptors, which are both located on the cell surface, and the NOD-like receptors (NLRs) and RIG-I-like receptors, which are intracellular PRRs (6).

Although many signaling pathways can be triggered when cells respond to DAMPs, a protein signaling complex called the inflammasome represents a highly controlled signaling mechanism for acute and chronic inflammatory changes in cells (7). Inflammasome complexes can contain one of the NLRs, that is, NLR protein family (NLRP1, NLRP3, or NLRP6), IPAF; or the HIN-200 family member AIM2 complexed with the adaptor protein called ASC (7, 8). Formation of the inflammasome results in the activation of the caspase-1 enzyme, which results in the formation of the IL-1–converting enzyme. The IL-1–converting enzyme is a homodimer enzyme complex with catalytic domains consisting of two p10 and p20 subunits that mediate the cleavage of the pro–IL-1β and pro–IL-18 into their respective active forms (9).

Caspase-1 activation can also result in caspase-1–dependent cell death in immune cells. This process is also called pyroptosis (10).
Pyroptosis can be triggered by a variety of stimuli. These include pathogens such as Salmonella and Listeria, but also noninfectious pathologies such as stroke and myocardial infarction can induce caspase-1-mediated pyroptosis (11–14). Interestingly, stroke and myocardial infarction involve a large degree of tissue destruction and cell death, which will result in the release of alarmins. Because radiation also leads to widespread cell death with presumed release of alarmins, we hypothesized that: 1) inflammasome activation occurs after radiation; 2) inflammasome activation leads to caspase-1–dependent effects of whole-body radiation exposure on inflammasome activation and pyroptosis; and 3) inflammasome activation after radiation is mediated by the release of alarmins from dead and damaged cells. To test our hypotheses, we exposed mice to total body irradiation from a $^{137} \text{CsCl}$ source irradiator and assessed the state of inflammasome activation in spleen immune cells by intracellular flow cytometry staining for the p10 subunit of activated caspase-1. This flow cytometry method for measuring caspase-1 activation in immune cell subsets has recently been used by our group to demonstrate inflammasome activation after traumatic injury (15). We also tested the influence of caspase-1 deficiency on cell death and immune cell changes after radiation. Moreover, we sought to identify a potential alarmin that might trigger radiation-induced inflammasome activation in immune cells. We found that allopurinol, a drug that inhibits uric acid synthesis, significantly reduced radiation-induced caspase-1 activation in mice. This finding suggests that uric acid or uric acid crystal formation may be a central trigger for radiation-induced inflammasome pathway activation in immune cells (16). Because NLRP3 is a primary pathway for caspase-1 pathway activation for DAMPs, we tested for radiation-induced caspase-1 activation in Nlrp3<sup>−/−</sup> mice and discovered that radiation-induced caspase-1 activation in immune cells does not depend on the NLRP3 signaling pathway. In this study, we describe the kinetics and dose-dependent effects of whole-body radiation exposure on inflammasome pathway activation in mice and provide evidence to suggest that treating mice with allopurinol, a drug that inhibits the xanthine oxidase enzyme and uric acid synthesis, reduces radiation-induced caspase-1 pathway activation. Additionally, we demonstrate that allopurinol treatment reduces radiation-induced inflammation.

**Materials and Methods**

**Mice**

Male 5- to 7-wk-old CD-1 mice were purchased from Charles River Laboratories (Wilmington, MA). Male caspase-1 gene–deficient (caspase-1<sup>−/−</sup>) mice (strain NOD.129S2(B6)-Casp1tm1Sesh<sup>−/−</sup> and NOD.129S2(B6)-Casp1tm1Sesh<sup>+/−</sup>) and strain-matched controls (strain NOD/ShiLtJ) were purchased from The Jackson Laboratory (Bar Harbor, ME). Male 7-wk-old C57BL/6J mice were purchased from The Jackson Laboratory, and Nlrp3<sup>−/−</sup> mice on the C57BL/6J genetic background were from Dr. Vishva Dixit (Genentech, San Francisco, CA). All mice were acclimated for at least 5 d in our full-barrier animal facility under a 12-h light/dark cycle and were given access to mouse chow and water ad libitum. All animal procedures used in this study were approved by the Harvard Medical School Institutional Animal Care and Use Committee and were performed following National Institutes of Health guidelines.

**Reagents**

Fluorescently-labeled Abs for flow cytometry were purchased from BioLegend (San Diego, CA) or eBioscience (San Diego, CA). The Abs used in these experiments were as follows: CD3 (145-2C11), CD11c (N418), CD19 (6D5), CD49b (DX5), and F4/80 (BM8). The caspase-1 p10–specific polyclonal Ab (m315) and the PE-conjugated dog anti-goat Ab (sc3857) used in these studies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Culture medium, called complete 5 (C5), was RPMI 1640 containing 5% heat-inactivated FBS, HEPES, glutamine, nonessential amino acids, antibiotic/antimycotic, and 2-ME.

**Radiation injury**

Mice were exposed to whole-body irradiation by timed exposure to $^{137} \text{CsCl}$ radiation delivered by a Mark I irradiator (J.L. Shepherd & Associates, San Fernando, CA). The dose rate for these experiments ranged from 1.63 to 1.65 Gy/min and doses were delivered by timed exposures to attain the desired radiation dose of 0.5, 1, 2, or 4 Gy.

**Allopurinol treatment**

Allopurinol (Cayman Chemical, Ann Arbor, MI) was dissolved in DMSO and injected i.p. (20 mg/kg) 2 h before and 6 h after radiation exposure. Control-treated mice were given DMSO diluted in saline at identical concentrations used for allopurinol delivery.

**Uric acid detection assay**

Uric acid concentrations in heparinized plasma were measured using a colorimetric assay as described by the manufacturer (BioAssay Systems, Hayward, CA). In brief, blood was harvested by cardiac puncture at 2 h, 6 h, or 1 d after 4 Gy radiation from individual male CD-1 mice, immediately following sacrifice by CO<sub>2</sub>. Blood was centrifuged at slow speed (100 × g) for 15 min and plasma was collected for the uric acid assay. The assay was performed using uric acid to generate a standard curve to estimate uric acid levels in mouse plasma samples.

**Cell preparations**

Mice were killed by CO<sub>2</sub> asphyxiation. Blood was harvested into heparinized 1-cc syringes by cardiac puncture. Spleens were harvested and homogenized under sterile conditions by mincing on sterile 70-μm nylon cell strainers ( Falcon, Franklin Lakes, NJ) in C5 medium. Cells were centrifuged at 300 × g for 10 min and the pellet was resuspended in 5 ml RBC lysis buffer for 5 min followed by neutralization with 5 ml C5 medium. Following an additional wash by centrifugation, cells were resuspended in C5 medium and filtered through nylon cell strainers (BD Falcon, Bedford, MA) to remove cell debris.

**Flow cytometry procedures**

Cell suspensions (50 μl) were plated in round-bottom 96-well plates (Costar, Corning, Corning, NY) for flow cytometry (FACS) staining. All stains were performed in PBS with 1% BSA and 0.1% sodium azide. Non-specific binding of staining Abs was blocked by preincubation for 10 min with TruStain FcX reagent (BioLegend, San Diego, CA). Splenocytes were surface-stained in separate wells with mixtures of fluorescence-tagged anti-F4/80 (macrophages), anti-CD11c (dendritic cells), anti-CD19 (B cells), anti-CD3 (T cells), or anti-CD49b Abs (NK cells) (all purchased from BioLegend). To measure cell apoptosis and pyroptosis by flow cytometry, cells were incubated at 37˚C with propidium iodide (PI). After 30 min, cells were pelleted by centrifugation at 300 × g for 6 min and surface-stained with FITC-labeled annexin V (BioLegend) to identify dead and apoptotic cell populations. When analyzed, cells gated as annexin V<sup>−</sup> were classified as apoptotic cells, cells gated as annexin V<sup>+</sup> and PI<sup>−</sup> were identified as pyroptotic cells, and those gated as only PI<sup>−</sup> were classified as necrotic (dead) cells. To stain for caspase-1 activation in cells, spleen cells were added to wells of a round-bottom 96-well plate cells and pelleted by centrifugation at 300 × g for 6 min. Cells were fixed with 100 μl 0.4% paraformaldehyde at room temperature. After 10 min, cells were pelleted by centrifugation at 300 × g for 6 min and then fixed with 200 μl ice-cold 100% methanol for 20 min on ice. After washing cells twice by centrifugation at 300 × g for 6 min in PBS with 1% BSA and 0.1% sodium azide, cells were stained with mixtures of cell subset marker Abs and Abs specific for activated caspase-1 (p10 subunit) (Santa Cruz Biotechnology). After overnight incubation at 4˚C, cells were washed once by centrifugation at 300 × g for 6 min and cells were stained with PE-conjugated donkey anti-goat Ab (Santa Cruz Biotechnology). After 30 min staining, cells were washed twice by centrifugation at 300 × g for 6 min and the cells were fixed again with 0.4% paraformaldehyde. All flow cytometry was performed using a MACSQuant analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany). The *.FCS files from flow cytometry were analyzed using FlowJo 7.6.5 software (Tree Star, Ashland, OR). Cells were gated as F4/80<sup>−</sup>, CD11c<sup>−</sup>, CD49b<sup>−</sup>, CD3<sup>−</sup>, and CD19<sup>−</sup> subsets for analysis. Mean fluorescence intensity (MFI) of cleaved caspase-1 p10 staining was calculated for each gated cell subset, including annexin V<sup>+</sup> and annexin V<sup>−</sup> fractions of each subset. Control stains without anti-p10 Ab addition were used to determine background stain levels, and MFI values were calculated by subtracting background MFI from caspase p10-stained samples.

**Cytokine assays**

Blood was collected in EDTA anticoagulant by cardiac puncture, and plasma was prepared by centrifugation at 12,000 × g for 10 min. Spleenocytes were prepared from mice and equal cell numbers (5 × 10<sup>6</sup> cells/well) were plated in round-bottom 96-well tissue culture–treated plates. Cells were cultured for 48 h at 37˚C in the presence of Escherichia coli–derived LPS (LPS 026:B6;...
Sigma-Aldrich, St. Louis, MO). Plasma and culture supernatants were tested for IL-1α, IL-1β, IL-6, IL-12p40, IL-18, TNF-α, and MCP-1 levels using bead-based Luminex assays. Recombinant cytokine standards were used to estimate cytokine concentrations. Data acquisition was performed on a Luminex 200 instrument (Luminex, Austin, TX) with StarStation software (Applied Cytometry, Sheffield, U.K.).

**Statistical analysis**

Data were analyzed for statistical significance using GraphPad Prism 5.0.4 software (GraphPad Software, La Jolla, CA). An unpaired Student t test or one-way ANOVA with Bonferroni multiple comparisons test was used when appropriate, and p values ≤0.05 were considered statistically significant.

**FIGURE 1.** Inflammasome pathway activation in spleen cell subsets following radiation with increasing whole-body radiation doses. Inflammasome pathway activation was measured by intracellular staining for the p10 subunit of cleaved caspase-1 in spleen immune cell subsets prepared from groups of CD-1 mice (n = 5) at 1 d after radiation exposure at the indicated doses. The FACS histograms in (A) are representative of the caspase-1 p10 staining levels seen at 1 d after 1 or 4 Gy whole-body radiation exposure by this method. Immune cell subsets were identified by flow cytometry with fluorescently labeled cell subset–specific Abs as follows: macrophages, F4/80+ cells; dendritic cells, CD11c+ cells; NK cells, NK1.1+ cells, T cells, CD3+ cells; B cells, CD19+ cells. The data plotted in (B) show caspase-1 p10 staining in the indicated spleen immune cell subsets at 1 d after radiation injury with increasing whole-body radiation doses. The levels of caspase-1 p10 staining in cells are plotted as MFI. The results indicate a dose-dependent increase in inflammasome pathway activation in all immune cell subsets examined. *p ≤ 0.05 by one-way ANOVA with Bonferroni multiple comparisons test.
Results

Inflammasome activation in response to radiation is dose-dependent

Groups of male CD-1 outbred mice were exposed to whole-body radiation by timed exposure to $^{137}$CsCl. At 1 d after radiation, spleen cells were prepared and stained for caspase-1 activation by measuring the intracellular levels of cleaved caspase-1 p10 subunit by flow cytometry. Specific immune cell subsets showing increased cleaved caspase-1 p10 were also identified by flow cytometry. The FACS histograms shown in Fig. 1A illustrate a detectable increase in caspase-1 p10 expression in spleen cells and all major immune cell subsets examined, including macrophages, dendritic cells, T cells, B cells, and NK cells, from mice exposed to 1 or 4 Gy radiation. We next performed dose response radiation studies to determine a dose response curve for radiation-induced inflammasome activation. The data plotted in Fig. 1B demonstrate that caspase-1 p10 expression is linear and reaches high levels by 4 Gy whole-body radiation. A clear dose-dependent increase in inflammasome activation was observed in all immune cell subsets that were analyzed. However, macrophages and dendritic cells showed peak activation by 2 Gy radiation that was sustained at 4 Gy, whereas NK cells, T cells, and B cells showed peak activation at 4 Gy radiation. This observation suggests that macrophages and

![FACS histograms showing caspase-1 p10 expression in spleen cells and immune cell subsets.](image)

FIGURE 2. Radiation injury induces a dose-dependent increase in immune cell apoptosis. In (A), spleen cells were prepared from mice ($n = 5$) at 1 d after 0, 0.5, 1, 2, or 4 Gy whole-body radiation injury. Cells were stained with fluorescently labeled immune cell subset markers and apoptotic cells were identified by annexin V staining. The data are plotted as means ± SEM of the percentage of annexin V$^+$ cells in gated immune cell subsets. *$p ≤ 0.05$ for annexin V$^+$ staining for 0 Gy versus radiation exposure by one-way ANOVA with Bonferroni multiple comparisons test. The data plots in (B) show inflammasome activation measured by caspase-1 p10 levels in apoptotic cells (annexin V$^+$) versus nonapoptotic (annexin V$^-$) spleen cell subsets. Increased inflammasome activation was found in apoptotic cells of all spleen cell subsets. *$p ≤ 0.05$ by unpaired t test, annexin V$^+$ versus annexin V$^-$. 
dendritic cells are comparatively more sensitive to initiators of inflammasome activation following radiation injury.

Because radiation is known to induce cell damage and death by apoptosis, we assessed whether cells showing high levels of caspase-1 p10 were also showing signs of apoptosis by measuring caspase-1 p10 expression levels in annexin V+ versus annexin V− cells. As expected, we observed that the percentage of apoptotic cells increased in direct relationship to whole-body radiation dose (Fig. 2A). Macrophages and dendritic cells showed high apoptosis at lower dose radiation exposure than did T cells, B cells, and NK cells, similar to what we observed for cleaved caspase-1 p10 expression. When cells were FACS-gated on annexin V+ cells, we found higher caspase-1 p10 expression in annexin V+ cells than in annexin V− cells (Fig. 2B). This result indicates that cells showing inflammasome activation are also undergoing apoptosis.

**Inflammasome pathway activation and cell death are delayed events after radiation injury**

The kinetics of radiation-induced inflammasome activation were defined by exposing mice to 2 Gy whole-body radiation and measuring caspase-1 p10 levels in cells at 1 h, 4 h, and 1, 3, 7, and 14 d after radiation. We were surprised to find that inflammasome activation was not significantly induced at 1 or 4 h after radiation injury, but was easily detected at day 1 after radiation in all immune cell types. This suggests that inflammasome activation and cell death are delayed events after radiation injury.

**FIGURE 3.** Time course of radiation-induced inflammasome pathway activation and immune cell pyroptosis. Groups of mice (n = 5) were exposed to 2 Gy whole-body radiation and spleen cells were harvested at 1 h, 4 h, and 1, 3, 7, or 14 d after radiation. In (A), spleen cells were stained with immune cell subset–specific Abs and counterstained for caspase-1 p10 to measure inflammasome pathway activation. In (B), the same cell preparations were stained for annexin V and PI to measure cells undergoing cell death by pyroptosis. *p \leq 0.05 by one-way ANOVA with Bonferroni test for multiple comparisons.
cell subsets, indicating that inflammasome activation by radiation injury is a delayed host response (Fig. 3A). Moreover, we found that inflammasome activation was sustained for 7 d after radiation injury in all immune cell subsets and returned to baseline levels by 14 d after radiation. Cell death as measured by annexin V expression on cells followed the exact kinetics of inflammasome activation (Fig. 3B) for most immune cell subsets examined. The exceptions were that T cells showed increased apoptosis at 4 h after radiation and that B cells did not show signs of apoptosis until 3 d after radiation. These results suggest that radiation injury causes prolonged inflammasome activation in immune cells and that the kinetics of inflammasome activation coincide with increased annexin V expression on most immune cell types.

Enhanced immune cell survival following radiation injury in caspase-1 gene–deficient mice

To identify a functional role for inflammasome activation in radiation injury, we tested the effects of radiation injury on cell death and apoptosis in caspase-1 gene–deficient (caspase-1<sup>-/-</sup>) mice. Caspase-1<sup>-/-</sup> and wild-type (WT) mice were exposed to 2 Gy whole-body radiation and spleen cells were compared at 1 d after radiation for changes in cell numbers, and cell surface annexin V/PI staining was used as a measure for pyroptosis. As shown in

![Figure 4](http://www.jimmunol.org/Downloadedfrom)

**FIGURE 4.** Reduced radiation-induced immune cell loss in caspase-1<sup>-/-</sup> mice. Groups of WT and caspase-1<sup>-/-</sup> mice (n = 5/group) were exposed to 2 Gy whole-body radiation. One day later, spleen cells were prepared and tested for radiation-induced immune cell subset losses by FACS (A). *p ≤ 0.05 for WT versus caspase-1<sup>-/-</sup> by a one-tailed unpaired t test. In (B), the indicated immune cell subsets were stained with annexin V and PI to measure the percentages of cells undergoing pyroptosis. *p ≤ 0.05 for 2 Gy WT versus 2 Gy caspase-1<sup>-/-</sup> by a one-tailed unpaired t test.
radiation-induced inflammasome pathway activation in immune cells, caspase-1 activation suggested to us that uric acid synthesis, uric acid production (Fig. 7). These data support that uric acid generation might act to control the inflammatory behavior of the immune system following radiation.

Uric acid synthesis plays a role in caspase-1 activation following radiation

Uric acid is known to potentially activate the inflammasome pathway in cells by acting as a DAMP molecule (16, 17). Given the high level of cell death caused by radiation injury, we suspected that uric acid might be a trigger for inflammasome activation in cells. To test this idea, we first measured systemic levels in uric acid in mice that were exposed to 4 Gy whole-body radiation. As shown in Fig. 5, we observed increased levels of uric acid in the plasma of mice at 2 h, 6 h, and 1 d after radiation exposure. Next, we tested the functional effects of blocking uric acid generation by treating mice with the xanthine oxidase inhibitor drug allopurinol before and after 2 Gy whole-body radiation. We observed that allopurinol treatment significantly decreased inflammasome activation in macrophages, dendritic cells, and NK cells to levels observed in sham mice (Fig. 6A). Allopurinol treatment also reduced inflammasome activation in T and B cells, but the reduction was not statistically significant. However, allopurinol treatment did not have a major effect on immune cell losses from radiation injury, but it did cause significant increases in spleen cell numbers in sham mice (Fig. 6B). Importantly, allopurinol treatment did significantly suppress radiation-induced increases in LPS-stimulated IL-1β production by spleen cells and also had a generalized suppressive effect on LPS-induced inflammatory cytokine production as shown by reduced IL-1α, IL-6, IL-12p40, MCP-1, and TNF-α production (Fig. 7). These data support that uric acid generation may be a primary stimulus for inflammasome pathway activation and might act to control the inflammatory behavior of the immune system following radiation.

The NLRP3 signaling pathway does not influence radiation-induced caspase-1 activation in immune cells

The observation that allopurinol could block radiation-induced caspase-1 activation suggested to us that uric acid synthesis, uric acid, or uric acid crystal formation in tissue could be mediating radiation-induced inflammasome pathway activation in immune cells. Although multiple pathways can trigger assembly of the inflammasome and caspase-1 activation, the NLRP3 signaling pathway is a promiscuous sensor of DAMPs and has been shown to be involved in uric acid-mediated caspase-1 activation (18, 19). Thus, we tested whether Nlrp3 gene–deficient (Nlrp3−/−) mice might show differences in radiation-induced inflammasome pathway activation and cell loss. Nlrp3−/− or C57BL/6J (WT) mice were exposed to 2 Gy total body irradiation and caspase-1 activation was measured in spleen immune cell subsets by p10 staining using flow cytometry at 1 d after radiation. Changes in immune cell numbers were also measured by flow cytometry. As shown in Fig. 8A, immune cell subsets from Nlrp3−/− mice showed levels of caspase-1 activation that were indistinguishable from WT mice. Additionally, radiation-induced immune cell loss in Nlrp3−/− mice was not significantly different from WT mice (Fig. 8B). However, we did observe that Nlrp3−/− mice intrinsically have higher numbers of total spleen cells and macrophages than do age- and sex-matched WT mice. Given that caspase-1−/− and Nlrp3−/− mice showed differences in radiation responses, we compared their inflammatory response to radiation exposure by measuring circulating cytokine levels at 1 d after 2 Gy radiation exposure using multiplex Luminesx bead cytokine assays. The plasma cytokine data shown in Fig. 9 demonstrate that caspase-1−/− mice produce significantly lower levels of circulating IL-1β, IL-18, IL-6, and IL-12p40 than did WT mice. Interestingly, Nlrp3−/− mice also showed significantly lower levels of plasma IL-1β, IL-18, and IL-6 than did WT mice, but IL-18 and IL-12p40 levels were higher than those observed in caspase-1−/− mice. Thus, both caspase-1−/− and Nlrp3−/− mice showed lower inflammatory-type responses to radiation exposure than did genetically normal mice, suggesting that these pathways are involved in triggering the host response to radiation injuries. However, it appears that radiation-induced caspase-1 activation does not depend on the NLRP3 inflammasome activation pathway.

Discussion

A primary goal of this study was to contribute an improved basic understanding of how radiation exposure activates the immune system. Although it is known that radiation causes a dose-dependent reduction in hematopoietic and peripheral immune cells, less is known about the specific phenotypic changes in the cellular and molecular pathways in immune cells that remain after radiation injury. In this study, we focused our efforts on studying the influence of radiation on activating the inflammasome activation pathway in immune cells for several reasons. First, we think that the extensive cell death and damage that occurs after radiation exposure might provide an early stimulus for inflammasome pathway activation by the immune system. Second, the physiological consequences of radiation injury suggest that radiation injury induces clinical symptoms similar to severe infections and trauma, which include fever, fatigue, loss of appetite, vomiting, and diarrhea. Third, radiation injury promotes chronic inflammatory conditions that can predispose exposed individuals to inflammatory diseases such as pulmonary fibrosis and cancer. The inflammasome pathway has been shown to be a central feature of these diseases (20–23).

The results of the present study demonstrate that whole-body radiation, also referred to as total body irradiation, induces substantial inflammasome pathway activation in immune cell populations, especially macrophages, dendritic cells, and NK cells. The activation was found to be radiation dose–dependent, sustained and partially inhibited by treatment with allopurinol, a xanthine oxidase inhibitor drug that inhibits uric acid synthesis by altering purine metabolism. To our knowledge, this may be the first study to demonstrate that whole-body ionizing radiation exposure...
causes significant inflammasome pathway activation in immune cells. Although not investigated in this study, it is also possible that the inflammasome pathway is activated in nonimmune cell types by radiation exposure, which could contribute to prolonged tissue inflammation or damage. The results in the present study were greatly facilitated by a flow cytometry technique that detects p10 cleaved caspase-1 levels as a measure for caspase-1 activation in immune cells. Because caspase-1 activation is the final step of inflammasome pathway activation, we made the assumption that increased levels of cleaved caspase-1 p10 detection in cells should indicate assembly of the inflammasome signaling complex (7). When this approach was developed, we compared the p10 staining profiles in cells to those measured using a fluorescent caspase-1 detection reagent called fluorochrome-labeled inhibitor of caspases (FLICA), a FAM-tagged cleaved caspase-1 inhibitor, FAM-YVAD-fmk (15). Comparative flow cytometry analysis showed

FIGURE 6. Allopurinol treatment prevents radiation-induced inflammasome pathway activation in immune cells. Mice were given 20 mg/kg allopurinol or vehicle by i.p. injection at 2 h before and 6 h after 2 Gy whole-body radiation. Spleen cells were prepared and FACS analysis was performed to detect p10 cleaved caspase-1 levels in the indicated FACS-gated immune cell subsets (A). In (B), spleen cells were analyzed by FACS to determine allopurinol treatment effects on the indicated immune cell subset numbers. The data are plotted as the means ± SEM for n = 6 mice/group. *p ≤ 0.05 for allopurinol-treated versus vehicle-treated mice by one-way ANOVA with a Bonferroni multiple comparisons test.
that the p10 staining results in cells were directly comparable to those measured by FLICA (15). The primary advantage of using flow cytometry to measure caspase-1 activation was that it allowed us to specifically identify which cell subsets showed the highest levels of inflammasome pathway activation. Furthermore, this technique allowed us to efficiently follow the kinetics of inflammasome pathway activation in immune cells. We also used anti-p10 Ab staining to measure caspase-1 activation because staining cells for caspase activation by flow cytometry using FLICA has been criticized as being nonspecific (24). Accordingly, we discovered an anticipated dose-dependent effect of radiation exposure on inflammasome pathway activation. However, we did not foresee that radiation exposure would induce sustained inflammasome pathway activation in immune cells. This prolonged effect of radiation on the immune system suggests that radiation has both acute and chronic proinflammatory influences on the immune system that are mediated in part by the inflammasome. The sustained activation observed in these experiments also supports the idea that inflammasome-activating molecules remain active in the body for long periods of time after radiation injury.

The sustained inflammasome pathway activation raises the question of whether radiation directly activates the inflammasome pathway, which has been described in response to UV radiation in the skin, or whether alarmins released by damaged cells may be the trigger for activation (25). One way to answer this question was to assess the time course for inflammasome activation after radiation. Because we found that radiation-induced inflammasome activation was delayed until day 1, direct activation of the inflammasome by radiation seems unlikely. Furthermore, the activation was detectable in cells even at 7 d after radiation exposure, suggesting that inflammasome activation occurs secondarily to radiation injuries. Based on these results, we favor the hypothesis that inflammasome activation is induced by the release of DAMPs in response to radiation-induced cell death or damage. This hypothesis is supported by findings in other related studies, which showed that trauma, osteoarthritis, experimental autoimmune encephalitis, and lung injury induced by ventilation or anthrax could induce delayed and sustained inflammasome pathway activation (15, 26–28).

Because inflammasome activation can lead to caspase-1–mediated cell death, referred to as pyroptosis, we wanted to determine whether immune cell types in mice showing inflammasome activation by radiation also show signs of undergoing cell death by pyroptosis (10). Using a combination of cell-surface annexin V binding as a marker to detect phosphatidylserine residues that appear on the outer membrane leaflet of stressed or dying cells and PI DNA intercalation staining, we discovered that cells showing high caspase-1 activation showed evidence of dying by pyroptosis. Based on this observation, it appears that caspase-1 activation after radiation is linked to cell death and establishes that many types of immune cells can undergo caspase-1–dependent cell death following radiation. Additionally, although the early cell loss that is seen after radiation may occur by apoptosis and mitotic catastrophe, caspase-1–mediated cell death by pyroptosis appears to be an ongoing process after radiation exposure (2). In relationship to this observation, links between activation of tumor suppressor p53 and the activation of caspases in cells have been described (29–31). Moreover, it is known that radiation exposure increases p53 expression in cells that undergo radiation-induced apoptosis, but p53 can also protect cells from death by acting as a DNA repair enzyme (32). The overlapping activation pattern between p53 and caspases suggest that there may also be a link between p53 and caspase-1. If so, then modulating caspase-1 or p53 activation may have similar effects on cell survival and inflammation after radiation exposure. Given the importance of tumor suppressor p53 for regulating radiation-induced cell death, future studies could address whether allopurinol might have cell-protective effects by influencing p53 expression in cells showing inflammasome activation.

To further test the idea that cells undergo caspase-1–initiated cell death following radiation exposure, we tested the influence of caspase-1 gene deficiency on radiation-induced cell death. We discovered that caspase-1 gene deficiency results in higher cell numbers and decreased percentages of dying cells in the spleen.

FIGURE 7. Allopurinol treatment blocks the radiation-induced proinflammatory phenotype of immune cells. Spleen cells were prepared from mice exposed to 2 Gy radiation and treated with 20 mg/kg allopurinol or vehicle at 2 h before and 6 h after radiation or control mice that were not exposed to radiation. Spleen cells were cultured with 1 μg/ml LPS and 48-h culture supernatants were tested for cytokine levels using a Luminex multiplex cytokine assay platform. The data are plotted as the means ± SEM for n = 6 mice/group. *p ≤ 0.05 for control versus 2 Gy plus vehicle, #p ≤ 0.05 for 2 Gy plus vehicle versus 2 Gy plus allopurinol by one-way ANOVA with Bonferroni multiple comparisons test.
These findings further support the hypothesis that the caspase-1 pathway contributes to some of the cell death after whole-body radiation exposure. This finding is line with a host of other publications demonstrating that cell death by caspase-1–mediated pyroptosis occurs in sterile inflammatory conditions such as stroke or hypoxia-induced renal damage (33, 34). Other findings also suggest that caspase-1 activation plays a direct role in cell death that goes beyond initiating pyroptosis (35). Nevertheless, we found that ablating caspase-1 activity in vivo rescued only a fraction of the cells dying after radiation, which supports the conclusion that radiation-induced cell death is not wholly caspase-1–dependent. However, in contrast, we found that Nlrp3−/− mice did not show any difference in radiation-induced cell loss as compared with WT mice. This finding is novel and further supports that caspase-1 and inflammasome pathway activation may occur independent of the NLRP3 signaling pathway. Several other reports also demonstrated caspase-1–independent NLRP3 signaling (36, 37).

Next, we sought to identify possible signaling triggers for inflammasome activation following radiation. We suspected that
uric acid or uric acid synthesis may be involved owing to the widespread cell destruction that occurs after radiation exposure (38). This is because cells contain intracellular uric acid and extracellular uric acid levels rise significantly due to the release and degradation of DNA and RNA from dying cells (39). Extracellular uric acid, especially uric acid crystals, is recognized by the immune system as DAMPs and triggers activation of the inflammasome (18). Accordingly, we examined circulating levels of uric acid in mice exposed to 4 Gy whole-body radiation and found significantly higher uric acid levels as compared with control mice. Thus, we suspected that targeting uric acid might attenuate radiation-induced inflammasome activation. To test this hypothesis, mice were treated with the xanthine oxidase inhibitor drug allopurinol to investigate whether reducing in vivo uric acid synthesis might reduce inflammasome pathway activation in immune cells. We discovered that allopurinol treatment blocked radiation-induced inflammasome activation in immune cells and also increased immune cell numbers in the spleen. Thus, it appears that uric acid or uric acid crystals may act as a central trigger for radiation-induced inflammasome pathway activation by the immune system. Allopurinol is a xanthine oxidase inhibitor and may have other effects that are independent of reducing uric acid production that could contribute to its effects on suppressing inflammasome activation during radiation exposure. For example, allopurinol could affect reactive oxygen and nitrogen generation by acting as a xanthine oxidase enzyme inhibitor (40–42).

We think our finding that uric acid or uric acid synthesis may mediate radiation-induced inflammasome activation in vivo is clinically significant for several reasons. First, we demonstrate that the inflammasome pathway is activated in immune cells for up to 7 d following radiation exposure. Sustained inflammatory conditions are thought to promote cancer development in tissues (43). Thus, it is possible that blunting uric acid production in tissues following radiation may reduce the risk of developing inflammatory complications or cancer after accidental or therapeutic radiation exposure. Second, our findings suggest that reducing uric acid production by allopurinol or other uric acid–lowering drugs such as rasburicase, a uricase drug, may improve immune cell survival and recovery. We suspect that modulating uric acid levels or reducing uric acid crystal formation could in turn improve immune function in people after radiation exposure and reduce the development of opportunistic infections, a common cause of morbidity and mortality following radiation injuries. We speculate that radiation effects on antimicrobial immunity might be mediated in part by chronic stimulation of the immune system by uric acid or uric acid crystals, which may promote immune system exhaustion in a manner similar to what has been reported to occur in chronic diseases such as cancer and HIV infection (44, 45). In support of this idea, a recent report showed that UV radiation increased uric acid levels in the skin (46). The increased uric acid levels in the skin were found to be associated with decreased contact hypersensitivity responsiveness because allopurinol treatment reduced skin uric acid levels and restored contact hypersensitivity responses in mice following UV radiation injury (46). This observation suggests that uric acid levels affect cell-mediated immune responses, which supports the idea that inflammasome activation by radiation can suppress immune function by the uric acid–dependent mechanism. Additionally, it has been reported that blocking xanthine oxidase after radiation protected the vascular endothelium integrity in the aorta of rats (47). This provides evidence that blocking uric acid production after radiation injury might have additional beneficial effects that extend beyond the immune system and may also be mediated by inflammasome-independent mechanisms.

In summary, to our knowledge, we report for the first time that whole-body radiation exposure causes sustained inflammasome activation in most major immune cell subsets and that radiation-induced cell death is partly dependent on caspase-1 activation. Furthermore, we provide evidence that inflammasome activation by radiation can be modulated by allopurinol, a drug that is used to reduce uric acid production and reduce uric acid–mediated inflammation. Thus, therapeutic reduction of uric acid synthesis following radiation exposure could help reduce radiation-induced damage to radiation-sensitive tissues and cells and could also help reduce inflammatory complication of radiation
injury and immune system functions. Thus, future studies are needed to address the potential of using drugs to modulate uric acid production or levels as a way to protect individuals from the damaging effects of radiation on the body or a radionuclide accident or event does occur.

Disclosures

The authors have no financial conflicts of interest.

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