NLRP3 inflammasome activation mediates radiation-induced pyroptosis in bone marrow-derived macrophages

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A limit to the clinical benefit of radiotherapy is not an incapacity to eliminate tumor cells but rather a limit on its capacity to do so without destroying normal tissue and inducing inflammation. Recent evidence reveals that the inflammasome is essential for mediating radiation-induced cell and tissue damage. In this study, using primary cultured bone marrow-derived macrophages (BMDM) and a mouse radiation model, we explored the role of NLRP3 inflammasome activation and the secondary pyroptosis underlying radiation-induced immune cell death. We observed an increasing proportion of pyroptosis and elevating Caspase-1 activation in 10 and 20 Gy radiation groups. Nlrp3 knock out significantly diminished the quantity of cleaved-Caspase-1 (p10) and IL-1β as well as the proportion of pyroptosis. Additionally, in vivo research shows that 9.5 Gy of radiation promotes Caspase-1 activation in marginal zone cells and induces death in mice, both of which can be significantly inhibited by knocking out Nlrp3. Thus, based on these findings, we conclude that the NLRP3 inflammasome activation mediates radiation-induced pyroptosis in BMDMs. Targeting NLRP3 inflammasome and pyroptosis may serve as effective strategies to diminish injury caused by radiation.

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Radiotherapy is used extensively with bone marrow transplants and for restricting the growth and spread of a variety of tumors such as prostate cancer, lung cancer, and renal-cell carcinoma, etc.1,3 A limit to the clinical benefit of radiotherapy is not an incapacity to eliminate neoplastic cells but rather a limit on its capacity to do so without destroying normal tissue and inducing inflammation.4 Although the principal pathways of radiation for damaging normal tissue is through the depletion of rapidly dividing cells, such as hematopoietic cells, vascular endothelial cells and somatic stem cells, emerging concepts suggest that exposure to radiation can also result in the activation and damage of peripheral immune cells and pro-inflammatory cytokine release, which in turn may impair the recovery and repopulation of destroyed cells and even result in a systemic response syndrome.4–6

Macrophages are recruited as a first response to radiation-induced damage.7 Alterations in macrophages following radiation have been observed during both early and late phases of tissue injury.8 Radiation polarizes macrophages towards an M1 phenotype, which is known as the pro-inflammatory phenotype, and enhances the secretion of M1 cytokines such as TNF-α, IL-12 and IFN-γ, which participate in pro-inflammatory responses.9,10 Activated M1 macrophages then promote extracellular matrix destruction, cell apoptosis, chronic inflammation (fibrosis) and tissue injury.4 Additionally, Raj et al. found that N-acetyl tryptophan glucoside (NATG) pretreatment overcomes the radiation-induced immune response by affecting macrophages and thus contributes to radioprotection.11 Thus, to explore the mechanism of radiation-induced immune cell, particularly macrophage, activation or damage, it is of paramount importance to find ways to limit the side effects of radiation and to maximize future therapeutic benefit. In the present study, the mechanism of immune system damage under radiation was studied using primary cultured bone marrow-derived macrophages (BMDMs).

Mainly described in macrophages and dendritic cells, pyroptosis is recognized as a model of cell death distinct from apoptosis and is thought of as a regulated form of necrosis.12,13 Unlike apoptosis, pyroptosis is initiated by the recognition of NOD-like receptors (NLRs) to pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs), leading to the assembly of a large multiprotein complex termed inflammasome which recruits and cleaves the precursor of Caspase-1 (pro-Caspase-1, p45) to its active form.14,15 Active Caspase-1 then causes the enzymolysis of the precursors for inflammasome activation and thus contributes to cell and tissue damage.16 However, the role of pyroptosis in radiation-induced macrophage damage remains unclear.

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In recent years, NLRP3 inflammasome upregulation at the expression or activation level has been reported to play an important role in radiation-induced lung inflammation, oral mucositis and skin lesions. Activation of Caspase-1 has also been linked with radiation exposure in the immune cells from spleen or hippocampal neural stem cells. Furthermore, Stoecklein et al. demonstrated that inflammasome activation occurs in many immune cell types following radiation exposure. As Caspase-1 was found to be activated by radiation in many studies, whether the components downstream of active Caspase-1, such as pyroptosis, play a role in radiation-induced immune cell death remains unknown. In this study, using primary cultured BMDMs and a mouse radiation model, we reported here for the first time that NLRP3 inflammasome-mediated pyroptosis is of great significance in radiation-induced BMDM death.

Results

Radiation induced pyroptosis in bone marrow-derived macrophages. Cultured BMDM were exposed to a $^{60}$Co radiation source to attain the desired doses of 5, 10 and 20 Gy. After 24 h, a cell viability assay was performed to evaluate the survival rate of cells. As shown in Figure 1a, groups exposed to radiation (5, 10 and 20 Gy) illustrate a dose-dependent cell loss and reaches low levels by 10 Gy (cell viability 55.27%) and 20 Gy (cell viability 45%, $P<0.001$ versus 0 Gy).

Pyroptotic cell death was assessed by measuring the release of LDH and double-positive staining of activated Caspase-1 and propidium iodide (PI). We detected that radiation (10 and 20 Gy) induced increased activity of LDH dose dependently in BMDM; 10 and 20 Gy radiation induced activity of LDH from 1.318 to 2.442 and 2.782 (OD 490 nm), respectively ($P<0.001$ versus 0 Gy; Figure 1b). To determine whether the death of BMDM induced by radiation is a result of pyroptosis, we exposed BMDM to radiation (5, 10, and 20 Gy) and detected activated Caspase-1 and PI using flow cytometry after 24 h. As shown in Figures 1c and d, the proportion of double-positive (activated Caspase-1 and PI) BMDM (Q3) significantly increased in 10 Gy (29.66%) and 20 Gy (46.27%) radiation ($P<0.001$ versus 0 Gy), while the 5 Gy group (9.67%) showed no significant difference in comparison with the 0 Gy group (5.60%, $P>0.05$ versus 0 Gy).

Radiation promoted pro-inflammatory cytokine production in bone marrow-derived macrophages. ELISA analysis of the pro-inflammatory cytokines in supernatant revealed that both 10 and 20 Gy radiation significantly increased the production of IL-1β, IL-18, TNF-α, IFN-γ, IL-1α, IL-12p40 and MCP-1 (Figure 2). Notably, 5 Gy radiation also significantly induced the release of IL-1β, IL-18 and IFN-γ ($P<0.05$, Figures 2a, b and e), but not TNF-α, IL-1α, IL-12p40 or MCP-1 ($P>0.05$). Nevertheless, radiation stimulation did not have a significant effect on the production of IL-6 ($P>0.05$, Figure 2h). Based on a number of studies of inflammasome activation, we used IL-1β instead of both...
IL-1β and IL-18 to test the activation of Caspase-1 in the latter study, as it was deemed more representative and economical.\textsuperscript{23,24}

Radiation promoted activation of the NLRP3 inflammasome. We performed Western Blot to detect the target protein: NLRP3 (p118), precursors of Caspase-1 (pro-caspase-1, p45), precursors of IL-1β (pro-IL-1β, p32) and cleaved-Caspase-1 (p10). As shown in Figure 3, 10 and 20 Gy radiation induced Caspase-1 cleavage after 3 h ($p < 0.001$ versus 0 Gy) while 5 Gy radiation did not show a significant increase of cleaved-Caspase-1 (p10, $P > 0.05$ versus 0 Gy). Specific comparisons (between treated groups) illustrated no significant difference in cleaved-Caspase-1
between 10 and 20 Gy radiation groups. However, using RT-PCR, we observed that radiation did not significantly affect the mRNA levels of NLRP3 inflammasome-related gene Nlrp3, Caspase-1 or IL-1β (P > 0.05 versus 0 Gy, Supplementary Figure S1).

As the 10 Gy radiation group shows a significant effect in radiation-induced pyroptosis and Caspase-1 activation, we chose 10 Gy as the treatment dosage in our subsequent research.

Nlrp3 knock out suppressed radiation-induced bone marrow-derived macrophages pyroptosis and NLRP3 inflammasome activation. To determine the role of NLRP3 in radiation-induced BMDM pyroptosis, Nlrp3−/− BMDM was isolated from Nlrp3−/− mice, cultured and then exposed to 10 Gy radiation. Using this strategy, we observed that Nlrp3−/− BMDM exhibit resistance to radiation-induced cell death (Figure 4a). Knock out of Nlrp3 rescued 10 Gy induced cell death from 25.98% (wild type (WT)+10 Gy) to 5.45% (Nlrp3−/−+10 Gy, P < 0.001 Nlrp3−/−+10 Gy versus WT+10 Gy). A test of the supernatant LDH showed that knock out of Nlrp3 suppressed 10 Gy radiation induced LDH release from 2.442 (WT+10 Gy) to 1.792 (Nlrp3−/−+10 Gy, P < 0.001 Nlrp3−/−+10 Gy versus WT+10 Gy, Figure 4b).

Furthermore, flow cytometry diagrams indicated that the proportion of pyroptosis induced by radiation was significantly lessened from 31.47% (WT +10 Gy) to 16.83% (Nlrp3−/−+10 Gy, P < 0.001 Nlrp3−/−+10 Gy versus WT+10 Gy, Figures 4c and d).

Immunoblotting analysis (Figures 5a–e) revealed that cleaved-Caspase-1 (p10) was elevated in WT BMDM exposed to 10 Gy radiation, while Nlrp3−/− BMDM showed no elevation of cleaved-Caspase-1 (p10), suggesting that knock out of Nlrp3 can block radiation-induced Caspase-1 activation. Likewise, knock out of Nlrp3 inhibited radiation-induced IL-1β production (P < 0.001 Nlrp3−/−+10 Gy versus WT+10 Gy, Figure 5f).

Nlrp3 knock out protected mice from radiation-induced death and Caspase-1 activation. To address whether Nlrp3 knock out can overcome the lethal effect of radiation, a group of 9.5 Gy radiated Nlrp3−/− mice (Nlrp3−/−+9.5 Gy, n=22) were monitored for 30 days in comparison with a group of 9.5 Gy radiated WT mice (WT+9.5 Gy, n=25). Nlrp3−/− knockout was associated with significantly improved survival at 30 days after 9.5 Gy compared with WT+9.5 Gy group (median survival time: 27 days versus 14 days; Figure 6a, log-rank P = 0.018).

To further investigate the role of the NLRP3 inflammasome in radiation induced damage, cleaved-Caspase-1 (p10) and IL-1β were detected by Western Blot and ELISA, respectively. From Figures 6b–g, we observed that cleaved-Caspase-1 (p10) and IL-1β was induced upon radiation stimulation (both P < 0.001) whereas it was decreased with Nlrp3 knockout (P = 0.0015, P < 0.001, respectively). Similar results were obtained using immunofluorescence analysis (3 h after radiation) for elevated cleaved-Caspase-1 (p10, in green fluorescence) in spleen marginal zone cells (rich in macrophages and
follicular dendritic cells) induced by 9.5 Gy radiation. This effect can also be blocked by knocking out Nlrp3 (Figures 7a and b). Notably, it was difficult to observe staining of cleaved-Caspase-1 in the white pulp cells (rich in lymphocytes) of the spleen.

Discussion

The present study demonstrates that radiation induces NLRP3 inflammasome activation and pyroptosis in BMDMs. The NLRP3 inflammasome activation, IL-1β production, and pyroptosis were downregulated by knockout of Nlrp3. These
although not in Stoecklein et al., we did not detect obvious increases in IL-6 after radiation, Nevertheless, membrane impermeant dyes conjugated annexin V, 7-aminoactinomycin (7-AAD) or PI and pro-inflammatory cytokine release and fluorochrome-pore formation, which permits cytosolic contents such as LDH release (as a marker of pore formation in the plasma membrane). Detection of LDH release (as a marker of pore formation in the plasma membrane) is also used widely to identify pyroptosis. Some labs like Jiahuai et al.’s and Lei et al.’s also have made great efforts to explore the morphologic characteristics of pyroptosis by electron microscopy and have made certain achievements recently that may contribute to the future detection of pyroptosis.

Pyroptosis, defined as Caspase-1-dependent programmed and pro-inflammatory cell death, is distinct from any other programmed cell death and results in cell lysis and pro-inflammatory cytokine release. Pyroptosis is triggered by various pathological stimuli, such as stroke, heart attack, cancer, microbial or liver inflammation and fibrosis. Unlike Caspase-3-dependent apoptosis, pyroptosis is typically mediated by Caspase-1 activation following inflammasome complex formation. Another apparent feature of pyroptosis is pore formation, which permits cytosolic contents such as LDH and pro-inflammatory cytokine release and fluorochrome-conjugated annexin V, 7-aminoactinomycin (7-AAD) or PI enter the cell. Nevertheless, membrane impermeant dyes such as 7-AAD or PI do not stain apoptotic cells. Although Stoecklein et al. discussed the role of radiation-induced immune cell pyroptosis in their good work, there remains some confusion about the methods of pyroptosis detection.

Stoecklein et al. first stained cells with annexin V and cleaved-Caspase-1 (p10) and found annexin V+ cells express higher cleaved-Caspase-1 (p10) than annexin V+ cells after radiation. Then, they stained another group of radiated cells with annexin V and PI and found that radiation increased double-positive cells (annexin V+/PI+). Taken together, they concluded that these annexin V+/PI+ cells may also express more cleaved-Caspase-1 (p10) and be more subject to pyroptotic death. However, in an indirect way, the method cannot exclude annexin V+/PI+/p10− cells from annexin V+/PI− cells, and these type of cells (annexin V+/PI+/p10−) are known as apoptotic cells in the late stage, not pyroptotic cells. Indeed, although controversial, many studies have used relatively direct and exact methods to detect pyroptosis such as double staining with FAM-YVAD-FMK (a marker of activated Caspase-1) and PI or SYTOX Blue DNA intercalation staining (a marker of pore formation in the plasma membrane). Detection of LDH release (as a marker of pore formation in the plasma membrane) is also used widely to identify pyroptosis. Some labs like Jiahuai et al.’s and Lei et al.’s also have made great efforts to explore the morphologic characteristics of pyroptosis by electron microscopy and have made certain achievements recently that may contribute to the future detection of pyroptosis.

In this study, detection of active caspase 1 and PI double stain were used in combination with an LDH release assay to detect and quantify pyroptosis. Our current data confirmed that radiation induces BMDM pyroptosis in a dose-dependent manner. Importantly, the 5 Gy radiation group did not show a significant increase of double-positive Caspase-1 and PI, nor did they show an elevation of LDH activity compared with the control group (0 Gy), which suggests a BMDM tolerance to relatively low doses of radiation (Figure 1). Many studies have shown an active role for macrophages under relatively low doses of radiation (2 Gy or 8 Gy) when many other cell types exhibit a marked loss.

Based on these results and previous research, we favor the hypothesis that low dose radiation activates the
macrophages that then mediate a variety of biological effects while relative high dose radiation kills the macrophages in a pyroptotic manner and mediates various types of severe damage.

As pyroptosis occurs after activation of caspase-1 and many studies that have focused on the inflammatory responses under radiation detected controversial results about the links between radiation exposure and NLRP3 inflammasome activation, we then tested the role of the NLRP3 inflamma-
machine in radiation and in the pyroptosis induced by radiation in cultured BMDM using RT-PCR, immunoblotting and ELISA techniques. Interestingly, the results imply that BMDM exposed to radiation shows evidence of dose-dependent Caspase-1 activation and IL-1β production (Figures 2 and 3) and that the Caspase-1 activation and IL-1β production is dependent on NLRP3, since Nlrp3 knock out diminishes the quantity of cleaved-Caspase-1 (p10) and IL-1β (Figures 4 and 5). This conclusion was confirmed by in vivo research (Figures 6 and 7). More vitally, Nlrp3 knock out can also significantly lessen the proportion of pyroptosis induced by Nlrp3 (Figures 6 and 7). Radiation-induced caspase-1 activation. 21,37,38 Stoecklein AIM2 that can recognize PAMPs may also contribute to infections, other inflammasomes such as NLRP1, NLRC4 or NLRP3 inflammasome-related protein after at least 1 day. 18 The isolation and culture of BMDM was performed as Pineda-Torra et al. described. 43 At first, briefly, animals were killed by cervical dislocation and soaked in 75% ethanol. Then, femurs and tibias were harvested and the bone marrow cells from all bones were flushed out. After centrifuging for 5 min at 310 × g, Erythrocytes were eliminated using Red Blood Cell Lysing Buffer (Sigma-Aldrich, St. Louis, MO, USA). The remaining cells were seeded in plates and incubated in complete medium with 50 mg/ml recombinant mouse M-CSF and incubated for 7 days to form proliferative nonactivated cells (also named M0 macrophages).

Materials and Methods Animals. WT C57BL/6J male mice, 5-7 weeks age, were purchased from Super-B&K Laboratory Animal Corp. Ltd. (Shanghai, China). Male Nlrp3−−/− mice on the C57BL/6J genetic background were from Model Animal Research Centre (AAALAC accredited, Nanjing University, China). Housed in specific-pathogen-free animal facility for at least 5 days under a 12-h light/dark cycle, the animals were given access to water and standard laboratory chow ad libitum. Those knockout mice and cells were identified by PCR (sequences of primers were presented in Supplementary Table S2), DNA sequencing, immunoblotting and immunofluorescence staining (Supplementary Figure S2). All animal procedures used in this study were approved by the Institutional Animal Care and Use Committee of the Second Military Medical University (No. 20120025, Shanghai, China).

In vivo radiation. In vivo, mice were exposed to whole-body radiation by timed exposure to 60Co radiation source (Faculty of Naval Medicine, Second Military Medical University) with a dose rate of 1.63 Gy/min and cumulative radiation dosage of 9.50 Gy. For in vitro assay, BMDM were grown as a monolayer and were exposed to 60Co radiation source to attain the desired dose of 5, 10 and 20 Gy at a dose rate of 1.80 Gy/min.

Cell viability assay. Thirty thousand cells per well were seeded into 96-well plates in 100 μl of RPMI 1640 supplemented with 10% FBS, 0.1 mg/ml Penicillin/ Streptomycin (P/S) and 50 mg/ml recombinant mouse M-CSF and incubated for 7 days as described before. Then, the cells were exposed to 60Co radiation and the number of surviving cells was measured by Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan) after 24 h. Data acquisition was performed on DENLEY DRAGON Wellscan MK 3 (Thermo, Vantaa, Finland). Cell viability was calculated according to the formula: cell viability (%) = ([As− Ab]/[Ac− Ab]) × 100%, where As, Ac and Ab represent the A450 in treated, untreated and blank groups, respectively.

Determination of BMDM pyroptosis. Pyroptotic cell death was assessed by measuring the release of LDH and double-positive staining of activated Caspase-1 and PI in BMDM. Released LDH in the cell culture supernatant was detected using LDH Cytotoxicity Assay Kit (Beyotime, Haimen, Jiangsu, China) and the activated Caspase-1 and PI were detected by FAM-FLICA Caspase-1 Assay Kit (ImmunoChemistry Technologies, LCC, Bloomington, MN, USA) according to the iron radiation. 42 Based on these findings, we assume that radiation does not directly affect the expression of NLRP3 inflammasome-related protein in the early stage.

To the best of our knowledge, we reported here for the first time that NLRP3 inflammasome activation mediates radiation-induced pyroptosis in BMDMs. We further assume that radiation can directly activate the NLRP3 inflammasome without affecting the expression level and indirectly activate other inflammasomes in the later period. Targeting the NLRP3 inflammasome and secondary pyroptosis may represent a novel strategy to limit the radiation-induced loss of immune cells, cascades of pro-inflammatory cytokines and related tissue damage. However, there are still limitations that need further exploration. For example, the mechanism of radiation activation of the NLRP3 inflammasome, the downstream cytokine induction of Caspase-1 activation and the role of other inflammasomes in radiation induced cell or tissue damage are not clear.
manufacturer's instruction. Stained cells were then analyzed by flow cytometry (BD FACSCalibur, Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR). The total RNA was isolated from adhered BMDM (3 h after radiation) using Trizol reagent (Life Technologies, Carlsbad, CA, USA). The reverse transcript (cDNA) was synthesized from 1 μg of total RNA and PrimeScript RT Master Mix (Takara Biotechnology, Dalian, China). qRT-PCR was performed with 2 μl first-strand cDNA solution in combination with a Fast start Universal Probe Master Mix, in a final volume of 20 μl. The primers used in this study are listed in Supplementary Table S1. All samples were run in triplicate and underwent 40 amplification cycles on an Applied Biosystems 7500 Real-Time PCR System (Life Technologies Corporation, USA) following the manufacturer's protocols. Expression values of NLRP3, Caspase-1 and pro-IL-1β were normalized to the value of the endogenous standard GAPDH and calculated by using the comparative cycle threshold (ΔΔCt) method.34

Immunoblot analysis. For Western Blot analysis, adhered BMDM (3 h after radiation) or spleens of mice (3 h after radiation) were lysed and the protein concentrations were measured as we described previously.35 Cleared lysates were separated by 10% SDS-PAGE, transferred onto NC membranes and then blocked for 2 h at room temperature with 5% nonfat dried milk. NLRP3, pro-Caspase-1 (p45) and cleaved-Caspase-1 (p10) detection was accomplished by probing the membranes with anti-NLRP3 Ab (AdipoGen Corp., San Diego, CA, USA), anti-Caspase-1 (p45) Ab (Abcam, Cambridge, UK) and anti-cleaved-Caspase-1 (p10) Ab (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and exposed with an Amersham Imager 600 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The membranes were then stripped, reprobed with anti-β-actin and exposed again to detect the endogenous standard β-actin. Imagel software was then used to scan and quantify the immunobots. The band intensity values of the target proteins were normalized to that of β-actin.

Cytokine measurement. Cell-free culture supernatants were tested for IL-1β, IL-18, TNF-α, IFN-γ, IL-6, IL-12p40 and MCP-1 concentrations using ELISA kits (Dakewe Biotech Company Ltd., Shenzhen, China) following the manufacturer's protocols. Data acquisition was performed on DENLEY DRAGON Wellscan MK 3 (Thermo, Finland).

Immunofluorescence staining. After anesthetizing, the mice were transcardially perfused with normal saline (0.9%) and spleen tissues were isolated after anesthetizing, the mice were transcardially perfused with normal saline (0.9%) and spleen tissues were isolated and fixed in fresh paraformaldehyde solution (4%, PH 7.4). Horizontal slices were prepared and blocked with 1% bovine serum albumin (BSA; Sigma-Aldrich) in PBS containing 0.05% Tween 20 (0.05% PBS-T) for 2 h. Specimens were then incubated with primary described anti-cleaved-Caspase-1 (p10) Ab overnight at 4 °C and containing 0.05% Tween 20 (0.05% PBS-T) for 2 h. Specimens were then incubated with primary described anti-cleaved-Caspase-1 (p10) Ab overnight at 4 °C and Alexa Fluor 488 (green) labeled donkey anti-rabbit IgG were then loaded for 2 h at room temperature in the dark. After washing, DAPI (4′,6-diamidino-2-phenylindole) was used for nuclear staining. Stained specimens were observed with an Olympus Research Inverted System Microscope IX71 (Olympus, Tokyo, Japan). Fluorescence intensity of cleaved-Caspase-1 (p10) was quantified by Image Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA).

Statistical analysis. The data are expressed as the mean ± S.E.M. and analyzed for statistical significance using GraphPad Prism 5.0.1 (GraphPad Software, La Jolla, CA, USA). One-way ANOVA was used to detect statistical significance among group means and Bonferroni post-hoc analysis was used to compare specific groups when ANOVA showed significant differences. P<0.05 was considered to be statistically significant.

Conflict of Interest
The authors declare no conflict of interests.

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