Original Paper

Zymosan-a Protects the Hematopoietic System from Radiation-Induced Damage by Targeting TLR2 Signaling Pathway

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Key Words

Zymosan-A • Radiation • Hematopoietic system • TLR2 • IL-6

Abstract

Background/Aims: The hematopoietic system is vulnerable to ionizing radiation and is often severely damaged by radiation. Molecules affecting radioresistance include Toll-like receptor 2. We investigated whether Zymosan-A, a novel TLR2 agonist, can protect the hematopoietic system from radiation-induced damage after total body irradiation. **Methods:** Mice were exposed to total body radiation after treatment with Zymosan-A or normal saline, and their survival was recorded. Tissue damage was evaluated by hematoxylin–eosin staining. The number of nucleated cells in bone marrow was determined by flow cytometry. Cell viability and apoptosis assay were determined by CCK-8 assay and flow cytometry assay. Enzyme-linked immunosorbent assay was used to detect the level of cytokines. **Results:** Zymosan-A protected mice from radiation-induced death and prevented radiation-induced hematopoietic system damage. Zymosan-A also promoted cell viability and inhibited cell apoptosis caused by radiation, induced radioprotective effects via TLR2, upregulated IL-6, IL-11, IL-12, and TNF- α *in vivo*. **Conclusion:** Zymosan-A can provide protection against radiation-induced hematopoietic system damage by targeting the TLR2 signaling pathway. Thus, Zymosan-A can be potentially effective radioprotectant.

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Introduction

Most patients with tumor receive radiotherapy [1-3]. Inevitable radiotherapy at different doses causes injury to normal tissues and affects tumor cells. The bone marrow hematopoietic system is the most sensitive system in the human body [4, 5]. This sensitivity limits the use of radiotherapy in patients with tumor. Thus, novel and effective radioprotective drugs need to be developed [6-9].

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Toll-like receptors (TLRs) have an essential role in the progress of innate immune responses [10-13]. In 2008, Burdelya et al. reported that the agonist of TLR5 exerted strong radioprotective effects on mice [14]. We then proved that TLR2, TLR4, and TLR9 also play important roles in radioresistance [15-17]. In previous studies, we proved that Pam3CSK4 and HKMT, agonists of TLR2, exhibited significant radioprotective effects on mice [15, 18].

Zymosan-A, which is extracted from the cell wall of yeast (*Saccharomyces cerevisiae*), has been demonstrated as a potent ligand of TLR2 [19, 20]. Zymosan-A can activate mitogen-activated protein kinases and nuclear factor-kappa B (NF- κ B) [19]. However, it was used to induce an inflammation model for multiple organ dysfunction syndrome in many studies [20-23]. In the present study, we demonstrated that Zymosan-A can also provide protection against radiation-induced hematopoietic system damage by targeting the TLR2 signaling pathway after total body irradiation.

Materials and Methods

Chemicals and reagents.

Zymosan-A was purchased from Sigma-Aldrich Corp (St. Louis, MO, USA), and normal saline (NS) was obtained from ChangHai Hospital (Shanghai, China). The apoptosis detection kit was purchased from Invitrogen (Carlsbad, California, USA), and RPMI1640 and fetal bovine serum were supplied by PAA Laboratories. Cell Counting Kit-8 (CCK-8) was purchased from Dojindo.

Animals and treatment.

Male wild-type C57BL/6 mice aged 6–8 weeks old were purchased from China Academy of Science (Shanghai, China). TLR2^{-/-} and TLR4^{-/-} mice were purchased from Model Animal Research Center, Nanjing University. All mice were housed in a laboratory animal room under standard conditions. The experiments were approved by the Laboratory Animal Center of the Second Military Medical University, China in conformance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. The mice were treated with Zymosan-A (50 mg/Kg, dissolved in NS) via peritoneal injection 24 and 2 h before irradiation. The mice were killed by cervical dislocation after radiation.

Cell culture and treatment.

Human intestinal epithelial cell (HIEC) and human B lymphocyte (AHH-1) were obtained from American Type Culture Collection, and cultured in RPMI 1640 with 10% FBS at 37 °C in a 5% CO₂ humidified chamber. Cells were treated with Zymosan-A (40 μ g/mL) 12 and 2 h before irradiation.

Irradiation.

⁶⁰Co source in the radiation center (Faculty of Naval Medicine, Second Military Medical University, China) was used to irradiate mice and cells. Mice were irradiated at the following doses: 7.0, 7.5, 8.5, and 9.0 Gy. Cell lines were irradiated at doses of 4 and 8 Gy.

Cell viability and apoptosis assay.

Cell viability was analyzed by CCK-8. Pretreated cells were seeded into 96-well plates at 5000 cells/well. The cells were counted by absorbance measurements at 450 nm 24 h post-radiation. The cell apoptosis was analyzed using the apoptosis detection kit (Invitrogen, Carlsbad, California, USA). After radiation, the cells were stained using Annexin V-fluorescein isothiocyanate (AV-FITC) and propidium iodide (PI). The cells were then analyzed by flow cytometry (Beckman Cytoflex) in accordance with the manufacturer's instructions.

Number of white blood cells (WBCs) and bone marrow cells (BMCs) and spleen coefficient.

The number of WBCs was obtained by blood cell analysis (Mindray), and the relative number of BMCs was determined by flow cytometry (Beckman Cytoflex). The spleen coefficient was calculated as the ratio of spleen weight (g) to body weight (g) (spleen weight/body weight).

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Bone marrow histological examination.

Mouse femurs were removed and then fixed in 4% paraformaldehyde at 1, 5, 10, 15, and 30 d post-irradiation. The cells were then stained with hematoxylin and eosin (HE).

Enzyme-linked immunosorbent assay (ELISA)

Blood was collected from the mice 24 h post-irradiation and stored at room temperature for 30 min. The blood was then centrifuged at 3000 rpm/min for 10 min. Serum was then collected and stored at -20 °C. The levels of IL-6, IL-11, IL-12, and TNF- α were analyzed by ELISA. All steps were operated as instructed.

Statistical analysis.

Data were expressed as means $\pm$ the standard errors of means. Two-tailed Student's t-test was used to analyze the difference between 2 groups. ANOVA was employed to analyze the difference among 3 groups. Kaplan-Meier analysis was applied to estimate the difference of overall survival between 2 groups. The data were analyzed using SPSS ver. 19 (IBM Corp., Armonk, NY, USA). $P < 0.05$ was considered statistically significant.

Results

Zymosan-A exhibited a significant radioprotective effect in vivo

To prove the radioprotective function of Zymosan-A *in vivo*, C57BL/6 mice were administered with a total of 50 mg/kg Zymosan-A 24 and 2 h before TBI. The results indicated that the survival rates of Zymosan-A group were increased after TBI (Figs. 1A–1D), and the dose reduction factor (DRF) was 1.25 (Zymosan-A vs. NS) (Fig. 1E). These data demonstrated that the TLR2 agonist Zymosan-A exerted a significant radioprotective effect *in vivo*.

Zymosan-A protected the hematopoietic system against radiation-induced damage

HE staining was employed to evaluate the radioprotective effect of Zymosan-A on the hematopoietic system. The hematopoietic system damage in the NS group was found to be more severe than that in the Zymosan-A group. The radiation damage on the structure of the

Fig. 1. Zymosan-A showed a significant radioprotective effect in vivo. (A–D) Mice (weighing 18 g, 10 per group) were treated with Zymosan-A (50 mg/kg, dissolved in NS) via peritoneal injection 24 and 2 h before irradiation and then exposed to 7.0, 7.5, 8.5, or 9.0 Gy TBI. The control mice were treated with NS. The survival was recorded. (E) Linear regression analysis of the survival rate for the mice treated with Zymosan-A or NS post-irradiation. DRF was 1.25.

Fig. 2. Zymosan-A protected the hematopoietic system against radiation-induced toxicity. (A) After TBI at 7.0 Gy, the mice were killed by cervical dislocation, and the femur was collected and stained with HE at Days 1, 5, 10, 15, and 30 post-TBI. All images were 200× magnified. The Fig. presents a typical HE image of 3 independent experiments. (B) The number of PBMCs 1, 5, 10, 15, and 30 d post-TBI was determined. (C) The number of BMCs 1, 5, 10, 15, and 30 d post-TBI was determined. (D) The spleen coefficients 1, 5, 10, 15, and 30 post-TBI were calculated. * $P < 0.05$, ** $P < 0.01$.

Fig. 3. Zymosan-A promoted cell viability and inhibited cell apoptosis caused by radiation. (A) Cells were treated with Zymosan-A (40 $\mu\text{g/mL}$) 12 and 2 h before radiation. Cells stained with AV-FITC and PI were analyzed by flow cytometry 24 h post-radiation (4 and 8 Gy). (B) Data are presented as mean $\pm$ SD of 3 independent experiments. (C) Cells were treated with Zymosan-A (40 $\mu\text{g/g}$) 12 and 2 h before radiation and then counted using CCK-8 assay at 450 nm post-radiation. * $P < 0.05$, ** $P < 0.01$.

bone marrow was alleviated in the Zymosan-A group. The number of nucleated cells was greater in the Zymosan group than in the NS group. Moreover, the bone marrow recovered much faster in the Zymosan-A than in the NS group (Fig. 2A). We also found that treatment with Zymosan-A increased the number of PWBCs, BMCs, and spleen coefficients (Figs. 2B–2D). Combined, these data indicated that Zymosan-A protected the hematopoietic system against radiation-induced damage, potentially inducing death in mice post-radiation.

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Fig. 4. Zymosan-A induced radioprotective effects via TLR2. (A and B) WT, TLR2^{-/-} and TLR4^{-/-} mice were exposed to 6.0 Gy TBI, and their survival rates were recorded. (C and D) WT, TLR2^{-/-}, and TLR4^{-/-} mice (weighing 18 g, 8 per group) were treated with Zymosan-A (50 mg/Kg, dissolved in NS) or NS via peritoneal injection 24 and 2 h before irradiation and then exposed to 6.0 Gy TBI. The survival rates were recorded.

Fig. 5. Zymosan-A upregulated IL-6, IL-11, IL-12, and TNF- α in vivo. IL-6, IL-11, IL-12, and TNF- α were detected by ELISA 24 h post-irradiation. Data were presented as the mean $\pm$ SD of 3 independent experiments. * $P < 0.05$, ** $P < 0.01$.

Zymosan-A promoted cell viability and inhibited cell apoptosis caused by radiation.

To verify the radioprotective function of Zymosan-A *in vitro*, we assayed the cell viability and apoptosis of HIEC and AHH-1 cell lines 24 h after radiation (4 or 8 Gy). We found that Zymosan-A inhibited cell apoptosis in AHH-1 (Figs. 3A–3B) and promoted cell viability in HIEC (8 Gy) (Fig. 3C).

Zymosan-A induced radioprotective effects via TLR2.

In our previous study, we showed that TLR2/4 played critical roles in radioresistance *in vivo* [15, 16]. Compared with the WT mice, the TLR2^{-/-} and TLR4^{-/-} mice were more susceptible to radiation-induced deaths (Figs. 4A–4B). Zymosan-A protected TLR4^{-/-} mice from radiation-induced death, but had no radioprotective effects to the TLR2^{-/-} mice (Figs. 4C–4D). These findings consistently indicated that Zymosan-A induced radioprotective effects via TLR2 but not via TLR4.

Zymosan-A upregulated IL-6, IL-11, IL-12, and TNF- α in vivo.

We verified that Zymosan-A induced radioprotective effects via TLR2. To further determine the possible mechanism involved, we detected the serum IL-6, IL-11, IL-12, and

Fig. 6. Possible mechanism of Zymosan-A radioprotection.

TNF- α levels in the mice pretreated with Zymosan-A; the serum levels of IL-6, IL-11, IL-12 and TNF- α were upregulated *in vivo* 24 h post-radiation (Fig. 5).

Discussion

Many studies indicated that activation of the TLRs signaling pathways can provide protection against radiation-induced damage [14-16, 18, 24, 25]. In our previous studies, we showed that TLR2 and TLR4 played critical roles in radioresistance. TLR2-/- and TLR4-/- mice were more susceptible to irradiation-induced mortality and morbidity [15, 16].

To the best of our knowledge, this study is the first report to prove that Zymosan-A can protect against radiation-induced damage *in vivo* and *in vitro* by targeting TLR2. Zymosan-A, an extract of yeast cell wall, is proven to be a potential agonist of TLR2 [26-28]. After treatment with Zymosan-A, the survival rates of the mice were increased after TBI. HE staining showed that Zymosan-A protected the hematopoietic system against radiation-induced damage, which may lead to death in mice post-radiation. The number of BMC and PWBCs and the spleen coefficients were also increased after treatment with Zymosan-A. By using cultured human cells, we showed that Zymosan-A promoted cell viability and inhibited cell apoptosis caused by radiation. The findings indicated that Zymosan-A protected the hematopoietic system against radiation-induced damage *in vivo* and *in vitro*.

To determine the possible mechanism underlying radioprotection by Zymosan-A, we used TLR2-/- and TLR4-/- mice. The results showed that Zymosan-A protected TLR4-/- mice but not TLR2-/- mice from radiation-induced death. These data consistently indicated that Zymosan-A induced radioprotective effects via TLR2 but not TLR4. Many studies indicated that Zymosan-A can induce NF- κ B phosphorylation and nuclear translocation by targeting TLR2 [21, 22, 29]. Zymosan-A can also upregulate cytokines, including TNF- α ; the IL-1 family; the IL-6, IL-8, and IL-10 family; IL-11; IL-23; the IL-12 family; IL-15; TGF- β ; and G-CSF [30-34]. In addition, IL-6, IL-11, IL-12, G-CSF, and TNF- α are regarded as radiation protection factors [35-40]. In the current study, we proved that Zymosan-A upregulated the serum levels of IL-6, IL-11, IL-12, and TNF- α *in vivo*. Our study proved that Zymosan-A exerts marked radioprotective effects *in vivo* and *in vitro* by targeting the TLR2 signaling pathway (Fig. 6).

In conclusion, our findings showed that Zymosan-A can provide protection against radiation-induced damage by targeting the TLR2 signaling pathway. These data suggested that Zymosan-A can be a potentially effective radioprotectant.

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Disclosure Statement

The authors have no interest of conflicts to disclose.

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