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Naturally-Derived PHA-L Protein Nanoparticle as a Radioprotector through Activation of Toll-Like Receptor 5

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Abstract

High energy ray in medical diagnosis and therapy can benefit patients, but can also cause significant damages to biomolecules such as DNA, as well as free radical generation, inevitably leading to numerous side effects. Small molecular radioprotectors provide an effective route to preserve the healthy tissue and whole body from ionizing radiation, but always have a short circulation time in the body. Inorganic nanoparticles show major protection effect but their heavy metal components considerably jeopardize translational promise due to suboptimal biocompatibility. Herein, we report a novel protein nanoparticle that can overcome limitations of both small molecular and inorganic nanoparticle radioprotectors and can be used as a radioprotector with spontaneous biocompatibility, outstanding pharmacokinetics and improvement on survival rate under exposure to γ -ray irradiation. PHA-L protein nanoparticle serves to clear excessive reactive oxygen species *in vivo*, prevents radiation-induced hematopoietic and gastrointestinal damages and boosts the survival rate of irradiated mice to ~70 %. A detailed study of the mechanism shows PHA-L protein nanoparticle can target and activate the toll-like receptor 5 *in vitro* and *in vivo*, and thus protect irradiated cells by modulating immune responses.

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Importantly, the PHA-L protein nanoparticle can perform highly efficient clearance while eliciting negligible toxicological response.

Graphical Abstract

PHA-L protein can protect cells from irradiation damages. In further mechanism studies, it was observed that PHA-L can bind to TLR5 receptors and make TLR5-NF κ B pathway activated, and thus protect irradiated cells by immune response.

Keywords

Protein nanoparticles; Radiation protection

INTRODUCTION

Pelvic and abdominal cancers are common malignancies that affect the gastrointestinal (GI) functions. Due to their massive size and easy spread to other abdominal organs and lymphatic system, radiotherapy has become a regular strategy to treat pelvic and abdominal cancers.^{1–10} During radiotherapy, many cancer patients are suffered from gastrointestinal injuries,^{11–13} which adversely interrupt the treatment.^{11, 14} Specifically, the radiotherapy can also induce the adverse inflammatory response in the peripheral blood and hematopoietic system, thereby precipitating multi-organ damages.^{15, 16} These acute and chronic complications have become a major challenge for radiation oncologists and medical physicians. Thus, finding a novel therapeutic approach to alleviate radiation-induced injury is urgently needed.^{14, 17–24}

Radiation can cause direct cell death of the rapidly proliferating crypt epithelium, which triggers excessive and potentially injurious inflammatory responses²⁵. Specifically, ionizing radiation emits high energy photons that can donate energy to cellular molecules, kicking out atomic electrons from the inner orbit to produce unstable and highly reactive free radicals.^{26, 27} These radicals quickly react with nearby molecules, resulting in breakage of chemical bonds and oxidation (addition of oxygen atoms) of the affected molecules such as DNA, lipids and proteins, which consequently produces cellular and tissue damages.^{28–30} Thus, the high energy rays can induce marked free radical production and DNA damages.³¹ Although many irradiated cells could repair DNA and protein damage, some cells would die of necrosis or apoptosis, leading to sustained deterioration of the injury of the hematopoietic and gastrointestinal system.³²

Various redox molecules and nanoparticles have shown efficacy in protecting irradiation-induced injury.^{9, 26, 33–35} For instance, small molecule antioxidants such as Amifostine and DIM (3, 3-diindolylmethane)^{36, 37} are protective against γ -ray irradiation-induced injury via reducing free radicals. However, these small molecule antioxidants suffer from short half-life in the blood circulation and adverse neuronal toxicities. Although inorganic nanomaterials such as CeO₂ and sulfide-based nanoparticles can have longer blood circulating time,^{33, 38–42} they have also shown limited efficacy in protecting against radiation-induced injuries of the hematopoietic and gastrointestinal system.

In this work, we report the therapeutic efficacy of a plant-derived PHA-L protein nanoparticle in animal model of radiation injury. Mechanistically, it can activate TLR5 and inhibit apoptosis in gastrointestinal tract, one of the most sensitive organs to radiation. Furthermore, it attenuates radiation-induced free radical production. Multi-organ injury was significantly improved by immune reaction induced ROS levels decrease. The PHA-L protein nanoparticle show low toxicity and high excretion, and promising for treatment of radiation enteropathy and translational medicine.

EXPERIMENTAL DETAILS

Chemicals and reagents

Phytohemagglutinin-L (PHA-L) and 1-(4,5-dimethylthiazol-2-yl)-3,5- diphenylformazan (MTT) were purchased from Sigma (St. Louis, MO, USA). Recombinant human TLR5 protein was obtained from Abcam (Cambridge, MA, USA). The kit for RNA isolation and the first-strand cDNA synthesis were obtained from Invitrogen (Carlsbad, CA, USA). DMEM medium, trypsin-EDTA, and antibiotics were acquired from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) was acquired from Invitrogen. All culture supplies were obtained from the BD-Falcon brand (BD, Franklin Lakes, NJ, USA). The antibodies to TLR5 and beta-actin were obtained from Sigma (St. Louis, MO, USA). Horseradish peroxidase (HRP)-conjugated goat immunoglobulin G (IgG) was purchased from Santa Cruz (Santa Cruz, CA, USA). All other chemicals were of analytical grade.

TEM imaging

To characterize the size of PHA-L nanoparticles, TEM image with negative staining was acquired using a FEI Tecnai Spirit microscope operating at an acceleration voltage of 120 kV. Before the negative staining, an ultrathin carbon-coated copper grid was cleaned by Plasma Cleaner system to enhance the hydrophilia. Then, 0.002 mg/mL protein solution was dropped on the cleaned grid, incubated for 1 minute, and was taken away by filter paper. Lastly, 2 % uranyl acetate was used to stain the protein-coated grid.

Cell viability assay

Chinese hamster ovary (CHO-K1) cells were cultured in 96-well plates, with 3,000 cells per well. Thirty minutes before 4 Gy radiation, PHA-L was dissolved in 1 x PBS and added into culture medium at final doses of 10, 50 and 100 $\mu\text{g/mL}$. Cells were cultured in a CO_2 incubator for 48 hours, before the cell viability was measured using MTT assay.

ROS detection

CHO-K1 cells were seeded onto 6-well plates at the number of 10^5 for each well. Cells were treated with PHA-L (50 $\mu\text{g/mL}$) and exposed to 4 Gy radiation (at a dose-rate of 1Gy/minute). After 24 hours' incubation, cells were incubated with 10 μM DCFH-DA for 20 minutes. Then all wells were washed with PBS 3 times. Cell images were obtained by a fluorescent microscope at 488 nm excitation wavelength (AMG). Cells were also collected for relative ROS level evaluation through a BD flow cytometer.

Clonogenic assay

The clonal assay was carried out on 6-well plates, and five hundred CHO-K1 cells were planted in each well. PHA-L was dissolved in culture medium with a final concentration of 50 µg/mL, and added to CHO cells 30 minutes before exposure to 0, 2, 3, 4, 5, and 6 Gy radiation (at a dose-rate of 1Gy/ minute). All plates were kept in a CO₂ incubator for at least 1 week, replacing the culture medium every 3 days. Colonies were counted, and the data fitted to single-hit multi-target model using the Origin 9.0 software.

Total DNA and BMNC analysis

C57BL/6 male mice were randomly divided into 6 groups, i.e., control, irradiation-only and treatment groups respectively set for 1st day and 7th day observation groups (n=6 per group). All groups received 7.2 Gy (at a dose-rate of 1Gy/ minute) of whole-body irradiation after receiving control vehicle or PHA-L solution (10 mg/kg). At 1 and 7 days after radiation, mice were sacrificed to harvest by flushing bone marrow cells from the femurs into calcium chloride solution (10 mL, 5 mM). The cell suspensions were centrifuged at 2500 rpm for 15 minutes, and the pellets were mixed with perchlorate (5 mL 0.2 M), and incubated for 15 min at 90 °C. After cooling down to room temperature, the reaction mixtures were purified through filter paper (pore size =0.2 µm), and the filtrates were measured for UV-vis absorption at 268 nm (Shimadzu, UV-1750). Similarly, BMNCs were flushed into 1 mL PBS and filtered through nylon meshes to remove fragments of bones and tissues for cytometric analysis (Mindray BC-2800 Vet).

TLR5 Cell reporter experiment

HEK-Blue hTLR5 cell line and its negative control were purchased from InvivoGen (San Diego, CA, USA) and HEK 293 cell line was from the American Type Culture (Manassas, VA, USA). Cells were grown in DMEM supplemented with 10 % FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were incubated at 37 °C in a humidified atmosphere of 95 % air/5 % CO₂. PHA-L was added at three different concentrations, 10, 50 and 100 µg/mL; whereas flagellin (25 ng/mL) was used as a positive control. SEAP cell reporter activity measurement was performed as described by InvivoGen.

Transfections and luciferase assay

The HEK293 (5×10⁵ cells/mL) were seeded to 12-well plates to maintain approximately 70–80 % confluence. Prior to transfection, cells were washed three times with 1 mL of Opti-MEM, and 0.4 µg of plasmids expressing TLR5 and β-galactosidase were transfected using the Lipofectamine 2000 (Invitrogen, CA). After 24 h, the medium was replaced by complete PRMI-1640 and the transfected cells were treated with test samples in the presence or absence of PHA-L. Luciferase and β-galactosidase activities were performed using the luciferase assay system according to the procedure provided by the manufacturer (Promega, Madison, WI). Luciferase activity was normalized by β-galactosidase activity.

Preparation, isolation, and culture of human PBMC cells

Human PBMC were isolated from buffy coats provided by Long Island Blood Bank (Melville, NY). PBMC were prepared from buffy coats by Ficoll density gradient

centrifugation (Ficoll-Paque PLUS, Pharmacia, Piscataway, NJ). Following overnight culture, floating cells were removed, and the adherent cells were used for further experiments.

RNA extraction and RT-PCR

Human PBMC were exposed to different concentrations of PHA-L for 24 h. Total RNA was isolated from human monocytes using the Qiagen RNeasy kit as per the manufacturer's instructions. The total RNA was incubated with oligo-dT15 and dNTPs for 5 min at 65 °C, and then transferred to ice, mixed with a 5 × first-strand buffer, RNase inhibitor, and 0.1 M DTT. The reaction mixture was further incubated for 2 min at 37 °C and for 1 h after the addition of M-MLV reverse transcriptase. The reaction was stopped by heating at 70 °C. The amplified products were separated by 1 % agarose gel electrophoresis. The gels were stained with ethidium bromide and photographed with a Gel Doc XR system (Bio-Rad Laboratories, Hercules, CA).

Cell lysis and immunoblotting

Human PBMC were exposed to different concentrations of PHA-L for 24 h. Equal amounts of proteins were boiled at 95 °C for 5 min, separated on a sodium dodecyl sulfate-polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF, Bio-Rad, USA) membranes. After blocking in 5 % non-fat milk, the membrane was incubated for 60 min with the appropriate primary antibody at room temperature, washed three times with the TBST buffer, and then incubated further for 60 min with HRP-conjugated goat IgG. The immunoreactive bands were visualized by using chemiluminescence technique.

Co-immunoprecipitation assay

Transfected HEK 293 cells were harvested and immunoprecipitated/ immunodetected using anti-HA or anti-cMyc antibodies, as described previously. For co-immunoprecipitation, cell lysates containing equal amounts of proteins were pre-cleared with 100 µL of protein A-coupled Sepharose beads (50 % v/v) (Amersham, UK) for 1 h at 4 °C. Precipitated proteins were then analyzed by Western blot using anti-HA or anti-myc. Band signals were developed using Western Lightning® Plus-ECL, Enhanced Chemiluminescence Substrate (Perkin-Elmer, Boston, MA, USA).

SPR analysis

The Biacore 3000 was used for real-time characterization of TLR5/PHA-L interaction. Briefly, TLR5 protein was immobilized onto a CM5 series chip (GE Healthcare), and the sample flow cell was injected with PHA-L at a flow rate of 30 µl/min for 3 min at 25°C. Increasing concentrations (0.125µM, 0.25µM, 0.5µM, 1.0µM, 2.0µM) of PHA-L were flowed over immobilized MD-2. The dissociation time was set for 15 min. The K_d was estimated using the BIAevaluation software.

Molecular docking and dynamics

ZDOCK program were used to model TLR5-PHA-L interaction complex structure.⁴³ The crystal structures of TLR5 (PDB code: 3V47) and PHA-L (PDB Code: 1FAT) were from the

Protein Data Bank (PDB), and used for the molecular docking. ZDOCK is a rigid-body docking algorithm that performed well in the CAPRI Challenge,⁴⁴ a professional competition for predicting protein–protein complexes. It uses a fast Fourier transform (FFT)-based, initial-stage rigid-body molecular docking algorithm.⁴⁵ The top 2000 predicted ligand orientations from ZDOCK were grouped in clusters to eliminate similar conformations using the ClusPro docking server,⁴⁶ which performs cluster analysis using a pairwise binding site root mean squared deviation (RMSD). The criteria for each group consisted of conformations differing by a maximum backbone RMSD of 6 Å from each other.⁴⁶ The top 20 generated clusters were returned for further evaluation. Molecular dynamics simulation (MDS) is a method depended on Newton law through computer technology for simulating atoms dynamic processes. The software used for MDS here was AMBER 14.⁴⁷ The simulations were run in the NPT ensemble. The ff99 force field was used. The built system contained 1 PHA-L-TLR5 complex, 8 Na⁺ and 36604 water molecules. The residue pairs that generated hydrogen bonds were calculated by the H-bonds plugin of VMD.⁴⁸ The H-bonds plugin counts the number of hydrogen bonds formed throughout a trajectory. The cut-off distance was selected with 3.0 Å. The calculation of binding free energy and alanine scanning were carried out by MM_PBSA progress.⁴⁹ The MM_PBSA method combines the molecular mechanical energies with the continuum solvent approaches. Fifty frames were extracted from the dynamic trajectory for MM_PBSA calculation.

Generation of CRISPR-mediated TLR5 knockout mice

The CRISPR-mediated Tlr5 knockout mice were produced by Beijing View Solid Biotechnology, China. The plasmid pCAG-T7-Cas9 linearized by the NotI restriction enzyme was used as the *in vitro* transcription template. Cas9 mRNA was then transcribed with the purified template by using the mMESSAGEmMACHINE T7 Ultra Kit (Life Technologies). Tlr5-gRNA templates were amplified based on gRNA scaffold using T7 promoter sequence-conjugated primers. After gel purification, Tlr5-gRNA was transcribed with a fast *in vitro* transcription T7 Kit (cat.no.VK010, Beijing View Solid Biotechnology, China) and stored at –80 °C. Zygotes of C57BL/6 mice were microinjected with cas9 mRNA and Tlr5-gRNA using a FemtoJet micromanipulator (Eppendorf, Germany). The injected zygotes were then transferred to pseudopregnant mice. All mice were maintained in a specific pathogen-free facility. The genotype of newborn mouse was identified by sequencing the PCR products amplified with the tail-derived DNA by Tlr5-sens and Tlr5-anti primers. Western blotting analysis was performed to confirm the TLR5 expression. The study was approved by the ethics committee of Institute of Radiation Medicine, Chinese Academy of Medical Sciences.

Mouse survival observation in 30 days after radiation exposure

C57BL/6 mice weighing 20–22 g were obtained from the Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences and housed at a temperature of 20–25 °C under relative humidity (50 ± 10 %) with a 12-h light/dark cycle. All animals were allowed free access to a commercial stock diet and water throughout the experiments. All experiments were conducted according to the guidelines of the Committee on the Care and Use of Laboratory Animals. Male C57BL/6 mice were randomly divided into five groups (n=16 per group), including control, irradiation-only, and three different PHA-L dose groups

(2.5, 5.0, and 10 mg/kg). The control group was given sham irradiation, and the irradiation-only and PHA-L groups received 7.2 Gy radiation (at a dose-rate of 1Gy/ minute). The three PHA-L groups were treated with PHA-L through intraperitoneal injection once per day for 3 days before and 7 days after irradiation. Survival was observed and recorded in each group every day. The survival rate was calculated and graphed. TLR5 knockout mice were obtained as described above and the same experiment protocol was carried out to tell the difference between TLR5 knockout and wild-type mice when they received PHA-L treatment against radiation, but only one dose PHA-L, 10 mg/kg, was used. PHA-L was used or not to treat the radiated wild-type C57BL/6 mice and TLR5 knockout mice with 12 mice in each group.

Intestinal radiation injuries and recovery observation

Male C57BL/6 mice were randomly divided into control, radiation only, and radiation with PHA-L treatment groups, with 6 mice in each group. The control groups were given sham irradiation, and the other two groups received 13 Gy (at a dose-rate of 1Gy/ minute) abdominal irradiation. The PHA-L treatment group was given PHA-L at a dose of 10 mg/kg. The mice were sacrificed 3 days after irradiation. Small intestine tissues were exteriorised and submerged in 10 % neutral formalin for 24 hours before dehydrating and embedding in paraffin. Small intestine radiation injury was then estimated based on morphological changes observed through H&E staining using traditional staining methods.

Apoptosis analysis

Eighteen C57 mice were randomly divided into 3 groups, control, radiation only and PHA-L treated radiation group. The mice were exposed to 13 Gy (at a dose-rate of 1Gy/ minute) abdominal radiation, and sacrificed after 24 hours. The intestine of each mouse was gathered and imbedded into paraffin to make sections. Apoptosis testing was carried according to terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) kit (Beyotime, C1090) as described before.⁵⁰

Hematology, Biochemistry, SOD and MDA analysis

All mice were randomly divided into control, radiation alone and radiation with PHA-L treatment groups (n=6). The mice were intraperitoneally injected with PHA-L (10 mg/kg) 30 minutes before 7 Gy (at a dose-rate of 1Gy/ minute) whole body radiation. After 1 and 7 days radiation, all mice were sacrificed by exsanguination. The blood samples were collected for biochemistry analysis of ALT, AST, TP, ALB, TBIL, BUN, GLOB and CREA, as well as hematology analyses of WBC, RBC, HGB, HCT, MCV, MCH, MCHC and PLT. The liver and lung tissues were harvested, and homogenized using an Ultra Turrax (IKA, T18). The activity of SOD was evaluated by *Total Superoxide Dismutase Assay Kit* with nitroblue tetrazolium (NBT), and the MDA concentration in tissues was tested by Lipid Peroxidation MDA Assay Kit, Beyotime.

Pharmacokinetics and in vivo toxicity study

PHA-L solution (2 mg/mL) was mixed with Cy3 dye, and incubated at 4 °C for 3 days to conjugate Cy3 dye to PHA-L protein nanoparticle. Then the mixed solution was filtrated

through a Millipore ultrafiltration device with 10 kDa MW cutoff to remove the excessive Cy3 dye. After 5 times washing, the (PHA-L)-Cy3 solution was ready for pharmaceutical studies. Three mice were intravenously injected with (PHA-L)-Cy3 at the dose of 20 mg/kg, and blood concentration of Cy3-labeled PHA-L protein was monitored at 5 min, 15 min, 30 min, 1 h, 3 h, 6 h, 12 h, 24 h and 48 h by ultraviolet spectrophotometer at 550 nm. During the period of 48 hours post injection, the urine of every mouse was also collected for absorption measurement. The main organs such as heart, liver, spleen, lung, kidney and testis were homogenized in saline solution. After 3 times centrifugation, the supernatant of each sample was tested through an ultraviolet spectrophotometer. The formulas used for

quantification were as followed: $Excretion = \frac{\sum_1^n V_n \times I_n}{V \times I}$; % ID/g = (absorbance value) / (injected solution absorbance value $\times$ injected volume) $\times$ 100 %. C57 mice were divided into 1 day control, 1 day treated, 30 day control and 30 day treated groups, with 6 mice per group. All mice were intraperitoneally injected with PHA-L and weighted every 2~3 days during 30-day observation. After injection, all mice were sacrificed at 1 and 30 day by exsanguination with anesthesia. Blood sample was used for hematology studies through a blood cell counter (mindray, BC-2800 Vet). Serum was obtained by centrifuge (6000 rpm, 10 min) for biochemistry studies (HT 7150). Main organs including heart, liver, spleen, lung, kidney and testis were collected and fixed in 10 % formalin for 48 hours and embedded into paraffin. Pathology studies were processed according to the routine H&E procedure.

Data presentation and statistical analysis

The 30-day survival rates were analysed with Graph Pad Prism 5. All data are presented as means $\pm$ standard deviations (SDs). Statistical Package for the Social Sciences (SPSS) 19.0 was used for statistical analyses. The significance of differences between groups was determined using the Student–Newman–Keuls test. $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

PHA-L protected against radiation-induced cell death in vitro

Phytohemagglutinin (PHA-L) is the lectin extracted from the red kidney bean, consists of four L-type subunits (“leuko-agglutinin”) held together by noncovalent forces. Under transmission electron microscope (TEM), it exists as PHA-L particles with average diameters about 6.8 nm (Figure 1a). The hydrodynamic size of PHA-L was about 9.6 nm by dynamic light scattering, very close to predicted molecular weight of 126 kDa (Figure 1c). We then examined the cytotoxicity of PHA-L protein, and found that the viability of CHO-K1 cell was not affected by PHA-L even when given at concentration up to 100 μ g/ml. At extremely higher concentrations (e.g., 300 μ g/mL), it slightly reduced cell viability to ~86 % after 48 h incubation, suggesting extremely low cytotoxicity. At relatively lower concentrations (10, 50 and 100 μ g/mL), PHA-L conferred a dose-dependent protection against irradiation-induced cell death (Figure 1d). Furthermore, the dose-dependent protective effect was confirmed by colony forming assay. Both PHA-L treated and untreated cells exhibited a significantly decreased survival upon 2–6 Gy radiation, but PHA-L treated cells still possessed a much higher survival fraction at all radiation doses (Figure 1e).

Consistently, the ROS levels in irradiated cells with or without PHA-L treatment were measured by 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) assay using flow cytometer. As predicted, radiation induced a marked elevation of free radicals levels, but PHA-L treatment significantly attenuated this radiation-induced ROS production (Figure 1f). Fluorescence imaging analysis revealed a marked inhibition of radiation-induced elevation of ROS production (Figure 1g, 1h, 1i). Collectively, these experimental data suggested that PHA-L protein nanoparticles can protect cells against high-energy γ -ray-induced cell death partly through reducing ROS production, similar to other previously reported radioprotectors.³³

To further explore its protective mechanisms, we examined its impact on the activation of radiation-protective cell surface receptors, such as TLR5. HEK-Blue™ hTLR5 cells were generated by co-transfection of the hTLR5 gene and an optimised secreted embryonic alkaline phosphatase (SEAP) reporter gene into HEK293 cells. The SEAP reporter gene was placed under the control of an NF- κ B and AP-1-inducible promoter. Stimulation with a TLR5 ligand activates NF- κ B and AP-1, which then induces the production of SEAP. PHA-L treatment caused a dose-dependent (1.81-, 2.64-, and 2.94-fold) increase in TLR5 activity in HEK-Blue™ hTLR5 cells after stimulation with PHA-L at several doses (10, 50 and 100 μ g/mL, Figure 2a). However, PHA-L treatment did not alter the expression level of TLR5 in negative control cells, suggesting that PHA-L affects the activity of TLR5 receptor. Indeed, in cells transfected with a luciferase reporter construct, PHA-L stimulation markedly elevated the luciferase activity (Figure 2b), suggesting PHA-L as a possible activator of TLR5 signalling in HEK 293 cells. To determine whether PHA-L also affects TLR5 expression, human PBMCs were stimulated with different concentrations of PHA-L, and cellular levels of TLR5 mRNA and protein were assessed by RT-PCR and Western blotting analysis. Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) (Figure 2c, e) and western blot (Figure 2d, f) analyses revealed a dose-dependent induction of TLR5 mRNA (Figure 2c, e) and protein (Figure 2d, f) in human PBMCs by PHA-L. Taken together, these results suggest that PHA-L not only activates TLR5 receptor, but also induces its expression in innate immune cells.

PHA-L protected against radiation-induced injury in vivo

We then explored the therapeutic efficacy of PHA-L protein nanoparticle using a well characterized animal model of radiation enteropathy.³³ PHA-L protein nanoparticles were intraperitoneally injected into C57BL/6 mice at various doses before subject the animals to whole-body γ -ray (¹³⁷Cs, 3600 Ci) irradiation at the dose of 7.2 Gy. PHA-L protein nanoparticle promoted a dose-dependent protection against lethal radiation (Figure 3a), significantly increasing animal survival from 0% in control vehicle group to 38 % and 69 % in PHA-L treatment groups. This protection was more pronounced than previously described DIM³⁷ and WSe₂,⁵⁰ and comparable to that of amifostine and MoS₂.³³

To elucidate its protective mechanisms, we determined the impact of PHA-L administration on the total DNA content of bone marrow cells and number of bone marrow nucleated cells (BMNC), which are two sensitive indicators for ionizing radiation injury. Within 1 day of irradiation, PHA-L administration did not significantly attenuate the radiation-induced

decrease of DNA levels in bone marrow cells (Figure 3b). After 7 days, however, PHA-L significantly attenuated the radiation-induced reduction of total DNA content (Figure 3b), indicative of extensive protection against radiation-induced DNA damage. Similarly, the administration of PHA-L led to a significant attenuation of the radiation-induced reduction of BMNC cell number (Figure 3c) at day 7 after radiation. These protective effects were similar to previously reported sulfide dots.³³

Based on the above results, a dose of 10 mg/kg PHA-L was chosen to verify its protective effects on the hematopoietic system and biochemical responses. PHA-L significantly improved the hematopoietic parameters including the numbers of white blood cell (WBC) and red blood cell (RBC), as well as hemoglobin (HGB) levels (Figure 3d–f). This suggested that PHA-L conveyed its protective effects on the hematopoietic system upon irradiation exposure. Therefore, PHA-L confers protection against radiation damages partly through restoring hematopoietic and liver functions.

The small intestine was particularly sensitive to ionizing radiation, and intestinal injury was an important cause of death for radiation exposure. Histopathological examination of intestines after haematoxylin and eosin (H&E) staining (Figure 3h–j) revealed marked intestinal villi disruption and edema in the radiated animals, but these pathological alterations were clearly ameliorated in the PHA-L treated group (Figure 3h–j).

To further understand the protective mechanisms of PHA-L, we investigated its impact on cellular function and apoptosis in major organs such as the lung and liver. Although PHA-L treatment did not significantly affect the levels of superoxide dismutase (SOD) levels in lung and liver within 1 day of radiation, it exhibited a significant restoration of lung and liver SOD levels at day 7 post PHA-L treatment (Figure 4b, c). Similarly, PHA-L treatment led to a significant reduction of 3,4-methylenedioxymphetamine (MDA) levels in the lung and liver at day 7 post treatment (Figure 4d, 4e). SOD enzymes were responsible for removing superoxide (O_2^-) radicals; whereas MDA was a harmful byproduct causing serotonergic neurotoxic effects. Because intestine is one of the organs most sensitive to radiation, we examined the impact of PHA-L administration on radiation-induced apoptosis in this organ. Indeed, *ex vivo* tissue staining revealed signs of cellular apoptosis in intestine of irradiated animals (Figure 4f–i). No red spots were observed in the tissue slice of healthy mice, demonstrating negligible cell apoptotic signals. Nonetheless, irradiated mice showed lots of red spots associated with significant apoptosis and intestine damages. With PHA-L treatment, no obvious red spots were observed, suggesting a significant protection of PHA-L treatment against radiation-induced apoptosis (Figure 4f).

Molecular modelling of PHA-L/TLR5 interaction

To further elucidate the molecular mechanism underlying PHA-L-mediated protection, we characterized the molecular interaction between PHA-L and TLR5. Human embryonic kidney 293T cells were transfected with pcDNA3/HA + pcDNA3/cMyc-TLR-5, pcDNA3/HA-PHA-L + pcDNA3/cMyc, or pcDNA3/HA-PHA-L + pcDNA3/cMyc-TLR-5. Lysates of transfected cells were subjected to immunoprecipitation using anti-HA or anti-CMYC antibodies. Immunoprecipitation pull-down revealed a possible biochemical interaction between PHA-L and TLR5 (Figure 5a–c). To confirm this possibility, we

performed Biosensor-based Surface Plasmon Resonance (SPR) analysis (Biacore) using purified proteins. With TLR5 protein immobilized on the sensor chip, different PHA-L concentrations (0.125, 0.25, 0.5, 1.0, 2.0 μM) were applied to assess the binding affinity for TLR5 and PHA-L protein nanoparticles (Figure 5d), which estimated a strong binding ($K_d = 0.512 \mu\text{M}$) between TLR5 and PHA-L. To assess the interaction mode between TLR5 and PHA-L, we obtained the 3D structures of the TLR5 (PDB code: 3V47) and PHA-L protein (PDB Code: 1FAT) from the Protein Data Bank and identified the potential interaction complex conformation and their binding sites through molecular dynamics. Starting with the docking complex constructed by ZDOCK, we performed molecular dynamic simulations for 11.5 ns by using AMBER 14. MM-PBSA method was employed to estimate the binding free energy for the PHA-L-TLR5 complex to be around -48.35 kcal/mol , which was a favourably stabilized protein-protein complex. Furthermore, we found 3 key residues, PHE273 (TLR5), GLN98 (PHA-L) and LYS100 (PHA-L), that likely contributed to the low binding energy (Figure 5f). In the light of this result, we concluded the main residue pairs involved in hydrogen bonds in the trajectory were GLN271 (TLR5)-SER98 (PHA-L) and LYS100 (PHA-L)-PHE273 (TLR5) (Figure 5e), which were also confirmed by VMD H-Bond plugin. To make sure these residues are key factors for the PHA-L and TLR5 interaction, we re-calculated their free energy changes using alanine scanning after mutating each of the four residues to alanine (Figure 5g). It corroborated that the four residues are key residues governing the interaction between the two proteins. From the difference in height, we found that SER98 (PHA-L) and LYS100 (PHA-L) are relatively more vital and may play important roles in the binding process of PHA-L and TLR5.

To confirm the critical involvement of TLR5 in PHA-L-mediated protection, $\text{TLR5}^{-/-}$ mice were generated by CRISPR/cas9 technique and employed in parallel experiments. The diagrammatic sketch of how TLR5 gene knockout influence the PHA-L treatment on irradiated mice was shown in Figure 6a. An 8bp deletion was performed as Figure 6b for the TLR5 knockout. The TLR5 protein expression in TLR5 knockout mice was finally confirmed by western blotting analysis (Figure 6c). In contrast to wild-type mice, which were significantly protected by PHA-L treatment (Figure 6d), PHA-L barely exhibited any protective effect on $\text{TLR5}^{-/-}$ mice (Figure 6d). All TLR5 KO mice lost survival after radiation, regardless of PHA-L treatment. Consistently, PHA-L treatment similarly failed to attenuate the radiation-induced reduction of bone marrow DNA content and BMNC cell numbers in the TLR5 KO mice (Figure 6e, 6f). These results proved that TLR5 was a specific receptor for PHA-L to confer protection against radiation injuries.

In vivo Pharmacokinetics and Toxicities of PHA-L

We then evaluated the pharmacokinetics and *in vivo* toxicities of PHA-L protein nanoparticles by labeling it with Cy3 before administering it intraperitoneally to animals. PHA-L protein nanoparticle did not induce any obvious adverse effects on the growth of mice (Figure 7a), and cleared through renal excretion with about 70 % clearance within 48 h of administration (Figure 7b). The blood circulation half-life of PHA-L protein nanoparticle was estimated to be $\sim 4 \text{ h}$ (Figure 7c), which was much longer than that of small molecules such as Amifostine and DIM. *In vivo*, it was predominantly accumulated in the spleen, but also found in the kidney, lung, and other organs rich in innate immune cells (Figure 7d),

supporting its immune-modulating properties.^{51, 52} Unlike most reported metal-based nanoparticles,^{53–59} the PHA-L protein nanoparticle showed ideal pharmacokinetic and excretion profiles.

Notably, PHA-L protein nanoparticle treatment did not affect WBC and RBC counts, or blood levels of ALT, AST, and creatinine (CREA) (Figure 8). Furthermore, PHA-L protein nanoparticles did not cause any pathological changes in any organs, even in the organs (e.g., spleen and kidneys) where PHA-L was abundantly accumulated during a prolonged period of time (up to 30 days, Figure 8). Although inorganic nanoparticles showed promising radiation protection effects, their biocompatibility was still not optimal. In contrast, the PHA-L protein-based nanoparticles might be superior in biocompatibility, because it did not cause any pathological effect in mice with minimal toxicity.

At present, there is an unmet need to develop therapeutic agent against radiation enteropathy with good biocompatibility and efficacy.^{60–63} Amifostine is an FDA-approved radioprotector used in clinical settings during radiotherapy. However, its short half-life in patients limits its efficacy and extensive clinical use. Thus, it is urgent to prolong the blood circulation time of radioprotectors for clinical applications. Our experimental data have suggested PHA-L protein as an ideal candidate with prolonged blood circulation time and pronounced efficacy. In addition, it is important to screen other endogenous ligands of TLR5 or other receptors for efficacy in protecting against radiation enteropathy.^{64,65}

CONCLUSIONS

In summary, we demonstrated that PHA-L protein nanoparticle conferred a dose-dependent and significant protection against radiation-induced cell death *in vitro*, and against radiation-induced enteropathy and animal lethality *in vivo*. Mechanistically, PHA-L exerts its protective effects partly through reducing the production of free radicals and maintaining hematopoietic functions. This effect was partly dependent on the functional activation of TLR5, which supports the potential use of TLR5 ligands as therapeutic agents for the clinical management of radiation injury.

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Figure 1. PHA-L protein nanoparticle protected against radiation-induced cell injury *in vitro*. (a) TEM image and (b) structure of PHA-L protein nanoparticles. (c) Hydrodynamic size of PHA-L protein nanoparticles by dynamic light scattering. (d) Viability of irradiated cells with or without treatment of PHA-L protein. (e) Colony formation of cells treated with PHA-L protein at different radiation doses. (f) Intracellular ROS levels of irradiated cells with or without treatment of PHA-L protein. (g-i) Fluorescence microscopic images of intracellular ROS levels probed by DCFH-DA. ROS dramatically increased with radiation as compared to the control, but these radiation-induced excessive ROS production was effectively scavenged by PHA-L protein nanoparticle treatment.

Figure 2. PHA-L protein nanoparticle activates TLR5.

(a) Secreted Embryonic Alkaline Phosphatase (SEAP) assay of TLR5 activation. PHA-L and flagellin were added to HEK-Blue™ hTLR5 cells and negative control cells at indicated concentrations, and TLR5 activities were tested by the SEAP assay. (b) Luciferase assay of TLR5 transcriptional activities. PHA-L (10 $\mu\text{g/mL}$) and Flagellin (as positive group) was added to HEK 293 cells before Luciferase assay. (c, d) TLR5 mRNA and protein expression levels in human PBMCs after stimulation with PHA-L at indicated concentrations. TLR5 mRNA levels were measured by RT-PCR assay, and its protein levels were determined by Western blot analysis. GAPDH and β -actin were used as loading controls for the quantitative analysis of TLR5 mRNA (e) and protein levels (f).

Figure 3. PHA-L conferred protection against lethal radiation *in vivo*.

(a) Survival rate of irradiated mice without treatment or treated with different concentrations of PHA-L protein nanoparticle and Amifostine (as positive group) ($n=16$ in each group). (b) Total DNA assessed from OD_{268} and (c) number of bone marrow nucleated cells (BMNC) from flow cytometry of irradiated mice 1 and 7 days after treatment of 10 mg/kg PHA-L protein ($n=6$ mice/group). * $P<0.05$ compared with control by paired Student's t-test. (d-f) White blood cell (WBC), red blood cell (RBC) and hemoglobin (HGB) of irradiated mice at 1 and 7 days after treatment. (g-i) Histological analysis of intestinal radiation injury by H&E

staining. Three mice groups were set as control, radiation only and radiation with treatment. 10 mg/kg PHA-L was used to irradiate mice.

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Figure 4. Effects of PHA-L protein on radiation-induced organ dysfunction.

(a) Superoxide dismutase (SOD) levels in (b) lung and (c) liver 1 and 7 days after γ -irradiation with or without treatment of PHA-L protein nanoparticle. 3,4-Methylenedioxyamphetamine (MDA) levels in (d) lung and (e) liver 1 and 7 days after γ -irradiation with or without treatment of PHA-L protein nanoparticle. Both SOD and MDA in the lung and liver was restored to healthy levels 7 days after PHA-L treatment. (g-i) Apoptosis staining of intestinal tissue following 13 Gy abdominal radiation in the absence (h) or presence of PHA-L treatment (i) * $P < 0.05$ as compared with control using paired Student's t-test.

Figure 5. PHA-L protein interacted with TLR5.

(a-c) Co-immunoprecipitation analysis of PHA-L/TLR5 interaction. (a) Western blotting analysis of PHA-L (left panel) and TLR5 (right panel) protein in 293T cells transfected with pcDNA3/HA-PHA-L or pcDNA3/CMYC-TLR5 plasmid. In (b) and (c), six groups of cells transfected with six different plasmids: ① pcDNA3/HA + pcDNA3/cMyc-TLR5; ② pcDNA3/HA-PHA-L + pcDNA3/cMyc; ③ pcDNA3/HA-PHA-L + pcDNA3/cMyc-TLR5; ④ pcDNA3/HA + pcDNA3/cMyc-TLR5; ⑤ pcDNA3/HA-PHA-L + pcDNA3/cMyc; and ⑥ pcDNA3/HA-PHA-L + pcDNA3/cMyc-TLR5. Cell lysates were immunoprecipitated with control IgG (① ② ③ groups, in Panel b and c), c-Myc-specific antibodies (④⑤⑥ groups in Panel b), or HA-specific antibody (④⑤⑥ groups in Panel c). (d) Biacore real-time analysis of TLR5/PHA-L interaction. TLR5 protein was immobilized onto a CM5 series chip, and increasing concentrations of PHA-L were applied. The K_d was estimated using the BIAevaluation software. (e-f) Molecular modelling of the interaction between PHA-L and TLR5. (e) All views and details are shown for key residue pairs that generate hydrogen bonds in the PHA-L-TLR5 complex. (f) Contributions of residues in PHA-L-

TLR5 complex to the total binding energy were graphed by different colour. Blue means less contribution, green for average, and red for most. (g) The binding free energy analysis for the wild-type and mutated complexes. Ball-and-stick models display mutated residues.

Figure 6. Functional TLR5 was required for PHA-L-mediated protection.

(a) Diagrammatic sketch of involvement of functional TLR5 in PHA-L-mediated protection against lethal radiation. (b) Genotyping of $TLR5^{-/-}$ mice generated by CRISPR-mediated deletion of 8bp. (c) Western blotting analysis TLR5 expression in $TLR5^{-/-}$ mice. (d) Survival rate of $TLR5$ KO and WT mice treated with or without PHA-L (10 mg/kg) after irradiation. (e) Average total DNA content and (f) number of bone marrow nucleated cells (BMNC) in WT and $TLR5$ KO mice at 7 days post treatment with PHA-L (10 mg/kg) (n=6 mice/group). * $P < 0.05$ compared with control by paired Student's t-test.

Figure 7. Pharmacokinetics and biodistribution of PHA-L protein nanoparticle.

(a) Changes of animal body weight following treatment with control vehicle or PHA-L protein nanoparticle. (b) Renal excretion of PHA-L was averaged from three representative mice at 48h post treatment. (c) Blood half-life of PHA-L and Amifostine was assessed by monitoring the absorption of serum using ultraviolet spectrophotometer at 550 nm. (d) Biodistribution of PHA-L protein nanoparticle following its administration (10 mg/kg) in indicated organs.

Figure 8. Animal toxicity analysis of PHA-L protein nanoparticle.

Various hematological parameters including White blood cell (WBC), red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) mean corpuscular hemoglobin concentration (MCHC), and platelets (PLT) were analyzed in the 8 top left histograms. Data were collected at 1 and 30 days after 10 mg/kg PHA-L intraperitoneal injection. Blood biochemistry was also analyzed on mice treated with PHA-L protein nanoparticle 1 and 30 days post injection. The results showed mean and standard deviation of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein (TP), albumin (ALB), blood urea nitrogen (BUN), creatinine (CREA), globulin (GLOB) and ALB/GLOB (A/G) in the 8 top right histogram. Representative histological images were presented for the heart, liver, spleen, lung, kidney and testis of mice treated with 10 mg/kg PHA-L protein nanoparticle 1 and 30 days post injection.

Scheme 1.
Illustration of radiation protection with PHA-L through activating TLR5.