Toll-like receptors and radiation protection

Z. LIU1, X. LEI1, X. LI2, J.-M. CAI1, F. GAO1, Y.-Y. YANG1

1Department of Radiation Medicine, Faculty of Naval Medicine, Second Military Medical University, Shanghai, P.R. China
2Department of Radiology, Changhai Hospital, Shanghai, China

Zhe Liu, Xiao Lei and Xiao Li contributed equally to this work

Corresponding Author: Yanyong Yang, MD; e-mail: yyyang2010@163.com
Fu Gao, MD; email: gaofusmmu@163.com

Abstract. – Exposure to ionizing radiation (IR) causes severe injuries to the human body, and normal tissue toxicity also limits the further application of cancer radiotherapy. However, current clinically used radioprotective agents are difficult to produce satisfactory effects. Toll-like receptor (TLR) is a kind of pattern recognition receptor (PRR) that has been extensively studied for radioprotection in recent years. Several TLR family members are closely related to radioprotection. In cultured cells, TLR2, TLR5 or TLR9 agonist was proved to inhibit radiation-induced apoptosis and increase cell survival. TLR5 ligand CBLB502 was reported to alleviate bone marrow and intestinal injuries in mice and rhesus monkeys. Activation of TLR4 by its agonist LPS can protect bone marrow damage and lower mice mortality after irradiation. TLR9 ligand also exhibited protective effects on mid jejunum. Moreover, some kinds of TLR agonists, such as TLR2/6 co-agonist CBLB613, were reported to be more effective in radioprotection than single TLR agonist. In conclusion, TLRs and their ligands provide novel strategies for radiation protection in nuclear accidents as well as protection of normal tissues during cancer radiotherapy.

Key Words: TLRs, Radioprotection, NF-κB, DNA damage.

Introduction

Exposure to a high dose of ionizing radiation (IR) leads to multi-system dysfunction including hematopoietic system, digestive system, reproductive system, etc. Nausea, vomiting, dizziness and other symptoms occur when the body suffers from IR at high doses (> 1 Gy). Currently, anti-oxidants, hormones, cytokines and Chinese herbal medicine are four main kinds of drugs used to produce IR protection. However, the application of these drugs is limited due to obvious side effects, undefined targets or uncertainty of drugs efficacy. Toll-like receptors (TLRs) are important pattern recognition receptors in innate immunity and have been widely studied in recent years. Once activated by specific ligands, TLRs initiate some of downstream signaling pathways, and thus participate in cell proliferation, apoptosis, and immune response regulation. It has been proved that activation of TLRs alleviated radiation damage in vitro and in vivo. Accumulating evidence suggests that TLRs exert lower side effects and higher protective efficiency than traditional radioprotective drugs, which make TLRs as a potential candidate in ionizing radiation protection.

Radiation Injury and Underlying Mechanism

Radiation injury mainly results from direct and indirect effects. For direct effects, ionizing radiation directly acts on macromolecules, such as nucleic acid, protein, and lipid, etc., causing DNA damage, lipid oxidation, enzyme dysfunction, resulting in changes in molecular structure and function. These changes in turn cause series of function impairments and metabolic disorders. Alternatively, ionizing radiation also interacts with water molecules in the body and produces series of active radiolysis products (such as various of free radicals and hydrated electrons), which will influence the biological activity and functions of biological macromolecules. Whole-body exposure to high-dose radiation causes injury involving multiple organs, among which bone marrow and gastrointestinal tract are quite sensitive. The average lethal dose of human CFU-GM is 1.27-1.37 Gy, and cell cycle arrest in intestinal crypt cell can be observed after 1.3 Gy irradiation. Intestinal villi epithelium sheds and loses its barrier function after 10 Gy irradiation. The immune system is also sensitive to ionizing radiation: it can cause damage to both innate and adaptive immune systems, including mucosal barrier damage, cell phagocytosis, T cells, B cells.
dysfunction, etc. In addition, a significant imbalance in immunocytes with a distinct function may occur when the body is subjected to ionizing radiation, which leads to immunosuppression. Toll-like receptors are widely distributed in various cell types and can be activated by a variety of endogenous or exogenous ligands. Up to now, a large number of studies confirmed that activation of TLRs signaling pathways has obvious radioprotective effects, indicating that TLRs have great advantages as novel targets in radiation protection.

**Toll-Like Receptors (TLRs)**

Toll-like receptors (TLRs) were firstly identified by Hoffman in Drosophila in 1996; after that, 13 family members were found in human, named TLR1-13. As a class of pattern recognition receptors (PRRs), TLRs participate in many physiological and pathological processes. TLRs are a kind of type I transmembrane proteins, which consist of three major domains: a leucine-rich extracellular domain, a transmembrane domain and a cytoplasmic domain. The cytoplasmic domain of TLR is a highly conserved structural region similar to an IL-1R intracellular domain, known as Toll-IL-1 receptor domain (TIR). Meanwhile, it was predicted that the extracellular domain of the TLRs forms a horseshoe-like structure. TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11 are largely expressed on the cell surface; however, TLR3, TLR4, TLR7, TLR8, and TLR9 are intracellularly expressed in endosomal or lysosomal compartments and the endoplasmic reticulum. TLRs are widely expressed in a variety of cells and can be divided into three categories according to their distributions: wide type, which widely distribute in mononuclear, macrophages, lymphocyte and so on; limited type, which mainly distribute in myeloid lymphocytes; specific type, which is strictly confined to dendritic cells. TLRs can also be divided into three types according to their ligands: (1) TLRs that recognize lipid species (e.g., TLR1, TLR2, TLR4, TLR6), among which TLR4 recognizes lipopolysaccharide (LPS); (2) TLRs that recognize pathogen proteins, for example TLR5 recognize Flagellin; (3) TLRs that recognize nucleic acid from cells or virus (e.g., TLR3, TLR7, TLR8, TLR9). In addition, TLRs can also produce radioprotective effects by recognizing endogenous damage-associated molecular patterns (DAMPs). TLRs activate its downstream effectors including NF-κB, interferon regulatory factors (IRFs), and stress-activated protein kinase (Jnk) through Myd88 or TRIF-mediated singling pathways, which in turn result in inhibition of apoptosis, promotion of cell proliferation, regulation of cell cycle, secretion of cytokines. Surprisingly, ionizing radiation may affect cell surface expression of certain TLRs in cells. Yoshino and Kashiwakura, and Yoshino et al reported that ionizing radiation increased expression of TLR4 in human monocytic THP1 cells through ROS/JNK/AP-1 axis. Sp1 may be involved in the radiation-induced TLR2 expression. Moreover, ROS, known as product in vivo after exposure to ionizing radiation, was reported to participate in TLR2/4 activation and NF-κB nuclear translocation; NAC, a kind of ROS scavenger, could alleviate this activation effect. These findings revealed a possible relationship between ionizing radiation and TLRs.

**Radioprotective Effects of TLRs Against IR**

TLRs play critical roles in basal resistance to IR in animals and multiple radiosensitive tissues. Several TLR ligands had been proved to exert protective roles against IR both in vitro and in vivo.

**Protective Effects of TLR Ligands on Cultured Cells**

Our group has demonstrated that treatment with Heat-Killed *Mycobacterium tuberculosis*, a TLR2 agonist, at 12 h before irradiation significantly improved viability in both HUVEC and L02 cells, and inhibited cell apoptosis after irradiation. In another study, it was found that colony formation of bone marrow cells isolated from irradiated TLR4 knockout mice was significantly less than that of wild-type mouse, which indicates that TLR4 plays an important role in the protection of bone marrow cells to IR. Tong et al demonstrated that TLR5 agonist CBLB502 increased cell survival rate after irradiation, and inhibited G2M cell cycle arrest as well as cell apoptosis. Previous studies showed that treating CD4+T cells, B cells, and mouse macrophages (RAW246.7) with TLR9 agonist CpG-ODN before irradiation decreased cell apoptosis rate and increased cell viability. Saha et al reported that supernatant from TLR9 ligand treated macrophages increased survival rate of irradiated IEC6 cells after 2-6 Gy irradiation. However, the distinct downstream mechanism of TLRs involving radioprotection still remains to be explored.
Radioprotective Effects of TLRs in vivo

In 2008, Burdelya et al. reported that intraperitoneal delivery of synthetic TLR5 agonist CBLB502 effectively improved survival rate in NIH-Swiss mice. The radioprotective effect of CBLB502 was even better than Amifostine, a classic radioprotective drug approved by Food and Drug Administration (FDA). Pretreatment with CBLB502 before whole body irradiation can reduce the rate of apoptotic cells in intestinal lamina propria and intestine crypt cells after irradiation; the protective effects on hematopoietic stem cells and early progenitor cells were also observed. They also reported that in CBLB502 group, the survival rate increased from 25% to 64% in rhesus monkeys after whole body irradiation. Radiation injuries on hematopoietic system and lymphatic system in rhesus monkeys were also alleviated in CBLB502 pretreatment group. Toshkov et al. also reported a kind of TLR5 agonist, Entolimod, that sufficiently reduced damage in head and neck local fractioned radiation model in mice; treatment with Entolimod 1 h after irradiation was more effective than 30 min model in mice; treatment with Entolimod 1 h after irradiation was more effective than 30 min before irradiation. In another study, Shen et al. reported the protection effects of CBLB502 on bone marrow cells extracted from NIH-Swiss mice. The radioprotective effect of CBLB502 effectively improved survival rate in clinical application. Moreover, compared with CBLB502, Gamma-tocotrienol, Ex-Rad and other radiation protection drugs, CBLB613 exhibits lower toxicity and lower minimal effective concentration. Subhrajit et al. reported that a novel synthetic TLR9 agonist mitigated intestinal radiation injury through myd88-mediated pathway. Pretreatment with TLR9 agonist on C57BL/6 mice at 1 h before whole body irradiation significantly increased the 30-days survival rate. At 1 or 3.5 days after irradiation, large crypt depth and elongated villi in the mid jejunum of mice were observed in TLR9 agonist treated group; the proliferation of crypt cells was also retained. Xylose absorption experiment showed better absorption function in experimental group.

However, Naoki et al. reported that the injection of TLR3 ligand, Poly (I:C) in BALB/c mice, aggravated gastrointestinal symptoms and increased mortality after whole body irradiation. TLR3 knockout mice exhibit resistant to IR; the number of apoptotic intestinal epithelia cells was less and more intestinal crypts were observed compared with wild-type mice. Studies proved that TLR3 mediated P53-dependent apoptosis, but only a small part of intestinal cells underwent p53 dependent apoptosis after irradiation. Further experiments showed that during irradiation, mass RNAs leaked from intracellular to the extracellular, and relatively large RNAs (> 200 nt) can form structures with a bulge and internal loops to combine and activate TLR3, which resulted in extensive apoptosis through the TRIF-RIP1-mediated pathway. They also demonstrated that TLR3 inhibitor significantly increased mice survival after irradiation, sufficiently alleviated diarrhea, vomiting and protected intestinal from tissue damage. These findings suggest that not all TLRs are beneficial for radioprotection, which means that TLRs’ targeting and specificity are highly required (Figure 1).

Possible Mechanism of TLRs in Radioprotection

Myd88-Depended Signaling Pathway

With its C-terminal TIR domain, MyD88 interacts with TLRs intracellular TIR domain, passes activation signal downstream, and finally activates NF-κB. NF-κB is an important nuclear transcription factor, which is involved in
varies gene transcription in cell proliferation and apoptosis inhibition. NF-κB produces radiation protection effects mainly through the following mechanisms: (1) by upregulating the production of cytokines, which suppresses radiation-induced cell apoptosis or participates in tissue restructure, such as IL-1, IL-3, IL-6, GM-CSF, MMP-9, etc.; (2) inducing anti-apoptotic gene expression, such as Bcl-2 gene family, etc.; (3) activating inducing TRAF (TNF-Receptor-Associated-Factor) and IAP (Inhibitor of Apoptosis Proteins) to confront with apoptosis; (4) by upregulating antioxidant gene expression, which, in turn, increases antioxidant factors, such as r-GCS (participate in glutathione synthesis), MnSOD, GP and metallothionein. These factors antagonize oxidative damage caused by radiation-induced oxidative free radicals, thus alleviating oxidative stress injury. TLRs can interact with Myd88 TIR structure domain through TRIAP dependent way (e.g., TLR1, TLR2, TLR4, TLR6) or TRIAP independent way (e.g., TLR5, TLR11) and successively activate its downstream molecules, including IRAK molecular family, TRAF6, TAK1, etc. Eventually, IKK (IκB kinase) is activated. Next, IKK phosphorylates IκB, inducing NF-κB nuclear translocation. In nuclear, NF-κB p65 subunit binds to its target gene promoter and triggers the production of cytokines, such as G-CSF, GM-CSF, which accounts for the radioprotective effects.

It was reported that IFN also produced radiation protection effects as an effector downstream Myd88 pathway. IFN is a kind of protein, which plays important roles in anti-virus and immune regulation; thus, it is conjectured that IFN protects the body from infections post-irradiation. It was also reported that IFN effectively trans-
formed dormant hematopoietic stem cells into activated status through upregulation of Scr-1. In addition, some intracellular TLRs (such as TLR7 and TLR9) activate IRAK-1 and IKKα through Myd88 pathway. These two kinases phosphorylate IRF3/7 and increased the production of IFN (mainly IFNα)55,56.

**TRIF-Depended Signaling Pathway**

Alternatively, most TLRs can interact with TRIF through TRAM (a kind of adaptor connecting TRIF and TLRs) dependent or independent ways. In this pathway, TRIF activates IKK-related kinases (consist of TBK1 and IKKβ) through TRAF359,60. IKK-related kinases induce the phosphorylation of IRF3, which is a main trigger of IFNα59,60. It was also reported that TRIF downstream kinase, such as TRAF6, TAK1 and IKK, can also be successively activated, leading to activation of NF-κB as well as radioprotection. However, most studies indicate that Myd88-dependent pathway is more critical than TRIF-dependent pathway in radioprotection.

**The Effects on TLRs on DNA Damage Repair**

Ionizing radiation causes several types of DNA damage, mainly including single-strand break (SSB) and double strand breaks (DSB). Unpaired or error-repaired DSB will result in gene mutation, chromosomal aberration, genome instability, thereby, causing tumor, variation, and aging61. It was reported that TLRs recognize DAMP (including damaged DNA) released from injured cells62. Using TLRs agonists in vivo and in vitro can promote DNA repairing and increase the expression of genes participating in cell cycle progression63,64. Zheng et al65 reported that TLR9 ligand promoted DNA double-strand breaks (DSBs) repair in CD4 T cells after irradiation, in which they also found that TLR9-engaged cells that showed higher levels of phosphorylated Chk1 and Chk2, indicating activation of cell cycle checkpoint and DNA repair. Another study65 showed that AP-1, a transcription factor induced by TLRs signaling, has many binding sites in the promoters of several DNA repair genes. On the one hand, AP-1 directly promoted nucleotide excision repair (NER) gene expression including ERCC1, XPA, XPB, etc.; on the other hand, AP-1 also increases the expression of cytokines, which helps to promote DNA repair, such as IL-12, IL-18. Moreover, Chen et al66, using microarray assay and RT-PCR, found that more than 10 DNA repairing related genes were upregulated in C57BL/6 mice when treated with CBLB502 (a kind of TLR5 agonist), including Gadd45 gene family, SOD1, and Rad21, all of which participate in DNA repair or anti-oxidization. These studies suggest that TLRs induce a variety of DNA repairing genes and alleviate radiation-induced DNA damage. However, the specific role of TLRs in DNA damage response after irradiation remains to be further studied.

**Potential of TLRs in Cancer Radiotherapy**

Preoperative radiotherapy is now a common treatment strategy in many cancer including rectum cancers and lung cancer53. Radiation-induced normal tissue toxicity is the main side effect in radiotherapy and limits its application when a higher dose is required. Burdelya et al27 reported that pretreatment with CBLB502 before radiotherapy decreased mortality in tumor-bearing mice; however, CBLB502 didn’t show any protective effects on tumor. Subhrajit et al26 reported a significant delay in tumor growth compared with control group when treating tumor-bearing mice with TLR9 agonist before radiotherapy. The underlying mechanism might be that DAMPs released from irradiated tumor cells increase tumor immunogenicity; also, TLR9 agonist also activated anti-tumor immunity74,75. Recently, Jeong et al76 indicated that TLR7 agonist IMQ promoted autophagy in tumor cell lines and increased radiosensitivity in a mouse melanoma model. The authors suggested that IMQ induced the increased frequencies of CD4+ and CD8+ T cells in association with reduced frequencies of Treg and MDSC populations in tumor. These studies indicate that radiotherapy in combination with TLR agonists could produce radioprotective effects on normal tissue and at the same time sensitizing cancer cells to ionizing radiation.

**Perspective**

TLRs and their ligands have been proved to produce radioprotection effects on animals and cultured cells, but there are still some limitations in clinical application. Firstly, besides radioprotective cytokines, TLR ligands also trigger the secretion of TNF-α and IL-1β, which are main factors that produce side effects after TLRs activation77,78. These elevated inflammatory cytokines may induce explosive inflammatory response. Secondly, some TLR agonists consist of relatively high immunogenicity substances or toxic substances, which limit its administration
dosage or radiation protection effects. For example, LPS, a typical TLR4 agonist, might induce severe septicemia in high dose\(^\text{9}\). Moreover, it was reported that TLR5 mainly be activated in the liver and colon, while TLR4 mainly be activated in the spleen and lung\(^\text{7}\). The distinct distribution of TLRs may lead to insufficiency activation in certain organs, which in turn compromises radiation protection effects in whole body.

According to recent reports, there are some researches aiming at developing novel and low toxic co-agonists for TLRs. For example, Xu et al\(^\text{10}\) reported that a TLR2 and TLR4 co-agonist, \textit{E. coli} O111:B4 LPS (sLPS), induced a synergistic effect, compared with both the single TLR ligands. Heat killed \textit{Salmonella typhimurium} (HKST) was reported to be a TLR2, TLR4, TLR5 co-agonist\(^\text{81-83}\). \textit{Salmonella typhimurium} (ST) has been widely used in vaccination for its low toxicity\(^\text{84,85}\). Our group recently reported HKST sufficiently inhibited radiation-induced cell apoptosis, alleviated cell DNA damage, and prolonged animal survival. It is noteworthy that by using gene knockout mice, we demonstrated HKST produces radioprotective effect largely in TLR4 dependent manner\(^\text{86}\). This evidence suggests that TLRs co-agonists may provide stronger and comprehensive radioprotective effects in response to whole body irradiation, but the underlying mechanism of the synergistic effects still needs to be clarified. The crosstalk of TLRs with others signaling pathways, such as DNA repair, anti-oxidant, anti-apoptosis, and autophagy, should also be further studied.

**Acknowledgements**

This study was supported in part by the grants from National Natural Science Foundation of China (No. 31670861, No. 11605289, No. 11635014).

**Conflict of Interest**

The Authors declare that they have no conflict of interests.

**References**

5. Lacave-Lapalun JV, Bendertitter M, Linao C. Flagel -
17. Muzio M, Bossio D, Polentarutti N, D’Amico G, Stop -


41) Zhao Y, Zhan Y, Burke KA, Anderson W/F. Soluble factor(s) from bone marrow cells can rescue lethally irradiated mice by protecting endogenous hematopoietic stem cells. Exp Hematol 2005; 33: 428-434.


Toll-like receptors and radiation protection


