Original Paper

Polymyxin B Attenuates LPS-Induced Death but Aggravates Radiation-Induced Death via TLR4-Myd88-IL-6 Pathway

Ying Cheng^a Jicong Du^a Jiaqi Han^a Weimin Sun^b Fu Gao^a Pei Zhang^a
Hainan Zhao^a Ming Chen^a Jianing Wang^a Mingyu Wang^a Suhe Dong^a
Ding Sun^a Yandong Zhang^c Jianguo Cui^a Jianming Cai^a Cong Liu^a

^aDepartment of Radiation Medicine, Faculty of Naval Medicine, ^bNational Key Laboratory of Medical Immunology & Institute of Immunology, Second Military Medical University, Shanghai, ^cModel Animal Research Center, Nanjing University, Nanjing, China

Key Words

Polymyxin B • LPS • Radiation • TLR4 • Myd88 • IL-6

Abstract

Background/Aims: Polymyxin B (PMB) is a cyclic cationic polypeptide antibiotic widely used to counteract the effects of endotoxin contamination, both *in vitro* and *in vivo*. Lipopolysaccharide (LPS) is an endotoxin that acts as a radiation protection factor. In this study, we focus on the role of PMB in LPS-induced and radiation-induced mortality in mice. **Methods:** Mice received total-body radiation or were pretreated by LPS or PMB, and the survival of mice was recorded. Elisa were used to detect the cytokines levels. **Results:** PMB decreased LPS-induced, but increased radiation-induced mortality in mice. Moreover, PMB could block the LPS-induced radioprotective effect. The ELISA and gene knock-out experiments indicated that PMB reduces TNF- α level to block LPS-induced mortality in mice, and inhibits IL-6, G-CSF and IL-10 to increase radiation-induced mortality via the TLR4-Myd88-IL-6 pathway. **Conclusions:** Our study revealed a role of PMB in LPS-induced endotoxemia and radiation exposure. We infer that the TLR4-Myd88-IL-6 pathway may play a crucial role in the process.

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Introduction

High dose radiation leads injuries of hematopoietic system (HP) and gastrointestinal tract have been well confirmed. However, the involved molecular mechanisms are still unclear.

Toll-like receptors (TLRs) play the key roles in the innate immune response [1-7]. In our previous studies, we have proved that TLR2 and TLR4 play critical roles in radio-resistance.

Y. Cheng, J. Du and J. Han contributed equally to this work.

Jianming Cai and Cong Liu

Department of Radiation Medicine, Second Military Medical University; 800, Xiangyin Road 200433, Shanghai (China)
E-Mail caijianming882003@163.com / victorliu20102020@smmu.edu.cn / victorliu20102020@163.com

Injection of lipopolysaccharide (LPS) or TLR2 agonist Pam3CSK4 into wild type mice will activate TLR4 or TLR2 *in vivo* and induced radio-resistance separately [8, 9].

Polymyxin B (PMB) is a cyclic cationic polypeptide antibiotic produced by soil bacterium *Paenibacillus polymixa* [10-14]. PMB is widely used to eliminate the effects of endotoxin contamination, both *in vitro* and *in vivo*. The involved mechanism is that PMB is the TLR signaling inhibitor and could block the biological effects of LPS through binding to lipid A, the toxic component of LPS, which is negatively charged [15-17]. Moreover, the neutralizing effect of PMB on LPS is dose-related and specific for LPS [18].

We have investigated the importance of TLR signal pathway in radiation resistance for years. In this study, PMB, the inhibitor of TLR signaling, was applied to investigate TLR in radiation resistance.

Materials and Methods

Reagent

Polymyxin B (Catalog #tlrl-pmb) was purchased from InvivoGen (San Diego, CA, USA) and was stored at 4°C. LPS from *Escherichia coli* was purchased from Sigma (Saint Louis, MO, USA).

Total body Radiation

Mice received total-body radiation at the radiation center in the faculty of Naval Medicine, Second Military Medical University, China [19, 20], and the survival of mice was recorded.

Mice

Adult wild type mice and TLR4^{-/-}, Myd88^{-/-} and IL-6^{-/-} mice were purchase from the Model Animal Research Center of Nanjing University (Nanjing, China) as described previously [21].

ELISA

The IL-6, TNF-α, G-CSF and IL-10 level in the serum of mice were analyzed by ELISA kits as previously described [19]. The ELISA kit was purchased from Jianglai-bio com (Jianglai, Shanghai, China).

Statistical analysis

A two-tailed Student's t test was used to analyze the differences between the two groups. ANOVA was used to analyze the difference of three groups. The Kaplan-Meier analysis was used to evaluate the overall survival of treated mice as previously described [19]. All statistical analyses were performed using SPSS Version 19 (IBM Corp., Armonk, NY, USA).

Results

PMB decreased LPS induced mice mortalities but increased radiation induced mice mortalities

Our previous study proved that LPS was radioprotective in mice [9], and PMB is an inhibitor of TLR signaling. Accordingly, we first evaluated the effect of PMB on the survival of LPS-treated and radiation-treated mice. Groups of mice were pretreated with either PMB (50 µg/mouse) for 24 hours before treatment with either LPS (1 and 2 mg/mouse) or total-body radiation (IR) (7 and 9 Gy). Mice were then monitored daily for survival. We found that the mice survival rate went down to zero in three days after LPS treatment without the PMB pretreatment (Fig. 1A/B), while those pretreated with PMB survived longer. Meanwhile, radiation-treated mice were observed for 30 days and we found that mice pretreated with PMB had lower survival rates in both the 7 and 9 Gy groups (Fig. 1C/D). Overall, primarily

Fig. 1. PMB decreased LPS-induced mortality but increased radiation-induced mortality in mice. PMB (100 μ g/mouse) was injected into each mouse via the caudal vein, 30 minutes later, LPS (1 or 2 mg/mouse) was injected via the caudal vein into each mouse. Mice were monitored daily for survival and the survival rates were calculated (A/B). PMB (100 μ g/mouse) was injected into each mouse via the caudal vein, 30 minutes later, each mouse received 7 Gy or 9 Gy total-body radiation. Mice were monitored daily for survival and the survival rates were calculated (C/D). Differences in survival between the two groups of mice were evaluated by Kaplan-Meier analysis. A/B, n=6; C/D n=12.

Fig. 2. PMB decreased the LPS-induced radioprotective effect. Mice were pretreated with PMB (100 μ g/mouse, n=24 mice) for 30 minutes and then treated with LPS (50 μ g/mouse, n=24 mice). Mice that only received LPS were regarded as control. Then, these mice received radiation (7 and 9 Gy, n=12 mice for each dose). The survival status of the mice was recorded daily. The differences in survival between the two groups of mice were evaluated by Kaplan-Meier analysis (A/B).

these data confirmed that PMB is an inhibitor of LPS-induced TLR4 signaling, and additionally PMB exacerbated radiation-induced death.

PMB blocked the LPS induced radioprotection effect.

PMB could exacerbate radiation effects and LPS could induce radioprotection. Accordingly, we next investigated whether PMB could reduce the radioprotective effect of LPS. Mice were pretreated with PMB (1 μ g/mouse) and LPS (50 μ g/mouse) for 24 hours.

Fig. 3. PMB reduced IL-6, TNF- α , G-CSF and IL-10 levels induced by LPS/IR *in vivo*. 30 mice were pretreated with PMB (100 μ g/mouse) for 30 minutes. Afterwards, 10 mice received LPS and 4 hours later, all mice were euthanized for the analysis of IL-6, G-CSF, TNF- α and IL-10 level in serum (ABC). The experiments were performed in triplicate. Data are represented as the mean $\pm$ s.d. * P < 0.05 (Con group: control group).

Fig. 4. The survival rates of WT, Myd88^{-/-}, IL-6^{-/-}, TLR4^{-/-} mice and PMB-treated mice. First, wild type mice were pretreated with or without PMB for 30 minutes. Next, the PMB pretreated mice, Myd88^{-/-}, IL-6^{-/-} and TLR4^{-/-} mice each received either LPS (1 mg/mouse) or radiation (9 Gy), and the survival status of the mice were recorded. The differences in survival between the two groups of mice were evaluated by Kaplan-Meier analysis (A/B).

Subsequently these mice received radiation (7 and 9 Gy) and the survival status of the mice was recorded daily. We found that PMB blocked the LPS induced radioprotective effect (Fig. 2A/B).

*PMB reduced IL-6, TNF- α , G-CSF and IL-10 levels induced by LPS/IR *in vivo*.*

IL-6 and G-CSF were shown to be radiation protection factors [22, 23]. TNF- α and IL-6 were the main cytokines induced by LPS [24, 25]. And IL-10 was typical anti-inflammatory interleukins. Thus, to investigate the involved mechanism of PMB decreasing LPS induced mice mortalities, we tested the IL-6, TNF- α , G-CSF and IL-10 in the serum of mice pretreated with LPS, PMB, LPS combined PMB, with or without PMB before total-body radiation (7Gy). and we found that LPS promoted the IL-6, TNF- α , G-CSF and IL-10 levels in the serum of mice following 24 h stimulation. In mice pretreated with LPS combined PMB, we found that PMB decreased the IL-6, TNF- α , G-CSF and IL-10 levels in the serum induced by LPS. And PMB also reduced the serum levels of interleukins (IL-6, TNF, G-CSF and IL-10) of radiation-treated mice, but the difference is not as sharp as the LPS model. (Fig. 3A/B/C/D).

Fig. 5. Possible mechanism for PMB in different regulation of LPS-induced death and radiation induced death (MOF: multiple organ failure).

TLR4^{-/-}, Myd88^{-/-} and IL-6^{-/-} mice mimic the effect of PMB in different regulation of LPS induced death and radiation induced mortalities

Our previous study showed that IL-6^{-/-} mice were more resistant to LPS-induced death but less resistant to radiation-induced death than wild type mice [19]. Accordingly, in this study, *Myd88^{-/-}*, *IL-6^{-/-}* and *TLR4^{-/-}* mice were used to test the role of PMB in LPS-induced and radiation-induced mortality in mice. Wild type mice were pretreated with PMB for 24 hours. Next, the PMB pretreated mice, *Myd88^{-/-}*, *IL-6^{-/-}* and *TLR4^{-/-}* mice separately received LPS (1mg/mouse) and radiation (9 Gy), and the survival status of the mice was recorded. We found that the *Myd88^{-/-}*, *IL-6^{-/-}*, *TLR4^{-/-}* knockout mice and the PMB pretreated mice had higher survival rates than the wild type mice in the LPS treated groups (Fig. 4A); on the contrary, *Myd88^{-/-}*, *IL-6^{-/-}*, *TLR4^{-/-}* knockout mice and PMB pretreated mice showed lower survival rates than wild type mice in radiation treated groups (Fig. 4B). Additionally, our data revealed that the effect of PMB stimulation is similar to the effect of knocking out *Myd88*, *IL-6* and *TLR4*. Thus, we inferred that PMB may play its role via the TLR4-Myd88-IL-6 pathway.

Discussion

To the best of our knowledge, this is the first report on the role of PMB in different regulation of radiation effects. We found that PMB exacerbated radiation-induced death but aggravates radiation-induced death *in vivo*. In addition, a study of the molecular mechanism in gene knockout mice and data indicated that the TLR4-Myd88-IL-6 pathway may play a key role in mediating the PMB effects.

It is known that the TLR4-Myd88-IL-6 pathway plays very different roles in LPS-induced systemic inflammatory response syndrome (SIRS) and radiation exposure [26-31]. In addition, LPS from Gram-negative bacteria is one of the most potent innate immune-activating stimuli. LPS-inducible cytokines included TNF-α, IL-1 family, IL-6, IL-8, IL-10 family, the IL-12 family, IL-15, TGF-β and G-CSF [17, 30, 32-35]. And those hyperreactive inflammatory responses caused by LPS killed pretreated mice. Our results showed that mice pretreated with PMB had higher survival rates than the wild type mice in the LPS treated groups, and *TLR4^{-/-}*, *Myd88^{-/-}* and *IL-6^{-/-}* mice mimic the effect of PMB in regulation of LPS induced death. So we concluded that Polymyxin B may attenuates LPS-induced death via TLR4-Myd88-IL-6 pathway.

In our previous study [9], exposure to radiation led to significant endotoxemia that also confers endogenous protection against radiation. The circulating endotoxins appear to originate from the gut. Moreover, we also proved that TLR4 play a critical role in basal radio-resistance, and *TLR4^{-/-}* mice were more susceptible to radiation-induced death. In this study, we found that PMB increased the radiation-induced mortality in mice, while gene knock-

out mice mimic the effect of PMB in regulation of radiation induced mortalities. Therefore, we hypothesized that PMB may increase the radiation-induced mortality by disrupting the TLR4 signaling pathway.

Furthermore, we also found that PMB reduced the LPS-induced levels of IL-6, G-CSF, IL-10 and TNF- α *in vivo*. IL-6 and G-CSF are known radiation protection factors. TNF- α is the main factor released in endotoxemia [25, 36]. And IL-10 was typical anti-inflammatory interleukins. Thus, we concluded that mechanism whereby PMB exacerbates radiation-induced death involves the disruption of the production of cytokines (Fig. 5).

In conclusion, our data revealed the role of PMB in LPS treatment and radiation treatment, and the TLR4-Myd88-IL-6 pathway plays important roles by disturbing the production of cytokines.

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Disclosure Statement

The authors declare no conflict of interest.

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