USP22 promotes pro-inflammatory responses in *Pseudomonas aeruginosa*-induced keratitis by targeting TRAF6

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Abstract

Background

*Pseudomonas aeruginosa* (PA)-induced keratitis is characterized by inflammatory epithelial edema, stromal infiltration, corneal ulceration, and can lead to vision loss. In the present study, we aim to study the effect of ubiquitin-specific protease 22 (USP22) on PA-induced keratitis.

Methods

Lentivirus containing control plasmid or shRNA targeting USP22 were used to silence the expression of USP22 in mouse corneas and cultured RAW264.7 cells, the inflammatory processes were detected.

Results

We found the expression level of USP22 was significantly increased in both mouse corneas and cultured RAW264.7 cells after PA stimulation. In addition, we observed that silencing of USP22 attenuate disease progression, downregulate NF-κB pathway and suppressed the expression of pro-inflammatory cytokines after PA stimulation. Most importantly, we found the expression of tumor necrosis factor receptor-associated factor 6 (TRAF6) was decreased by silencing of USP22, and USP22 was found to remove K48-linked poly-ubiquitination chains from TRAF6 to stabilize TRAF6 expression after PA infection.

Conclusion

This data indicated USP22 as a positive regulator of pro-inflammatory responses in PA induced keratitis.

Background

*Pseudomonas aeruginosa* (PA) is the pathogen most commonly associated with the use of contact lenses [1]. PA keratitis is a rapidly developing and destructive ophthalmic disease,
which can lead to ulcer, corneal perforation and even severe vision loss [2]. Despite the rapid progress of modern medicine, there is still a lack of effective treatment for PA keratitis [3]. It is of great significance to identify novel therapeutic targets for PA keratitis treatment. Therefore, keeping the immune homeostasis and regulating the inflammatory immune response of the eyes has become the theme of this paper.

Ubiquitination and de-ubiquitination processes have been found to maintain the stability of a large number of critical proteins and internal environment, and participate in the regulation of important physiological processes [4]. When the ubiquitination chain of lysine 63 (K63) is formed, the activity and other functions of the substrates will be changed [5]. If the substrate is modified by lysine 48(K48) ubiquitin, the target protein will be degraded by proteasome system [6]. Recently, many studies already showed that ubiquitination process is crucial for the regulation of inflammatory responses in PA-induced keratitis [7, 8].

Deubiquitinating enzymes (DUBs) are proteases that reverse protein ubiquitination [9]. Ubiquitin-specific proteases (USPs) are the largest subclass of DUBs with specific targets [10]. Ubiquitin-specific protease 22 (USP22) is a member of USPs in mammals, which contains an N-terminal zinc-finger domain for substrate interaction and a C-terminal ubiquitin-specific peptidase domain for protein deubiquitination [11]. Its expression level is related to tumor metastasis, drug resistance and cell cycle progress, and is crucial in the process of tumor oncogenesis and development, therefore it is considered as a biomarker and treatment target of tumor [12]. While, the role of USP22 in keratitis still remains largely unclear.

In this research, our data showed the expression of USP22 is increased by PA infection. Silencing of USP22 significantly delayed the disease progression of PA-mediated keratitis and attenuated pro-inflammatory cytokines production induced by PA infection. At last, we
found that knockdown the expression of USP22 suppressed NF-κB activation and enhanced K48 linked polyubiquitination level of TRAF6. This data indicated USP22 as a positive regulator of pro-inflammatory responses in PA induced keratitis.

Methods

Cell culture

Mouse macrophage cell line RAW264.7 was obtained from American Type Culture Collection (Manassas, VA) and were grown in a DMEM medium containing 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C under 5% CO₂.

Experimental Infection With Pa

Female C57BL/6J mice (8 week) were purchased from Joint Ventures Sipper BK Experimental Animal (Shanghai, China). The mice were kept at room temperature (controlled at 25°C) with a light-dark cycle of 12 h each day. 6 mice per group for each time point. The experiments were carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals approved by the Animal Ethics Committee of the Scientific Investigation Board of our university and performed as previous reported (7). Briefly, after anesthesia with ether, three 1-mm incisions were made on the left cornea with sterile 25 gauge needle. 5 µL bacterial suspension containing 1 × 10⁶ colony-forming units (CFUs) of PA (ATCC strain 19660) was used locally on the ocular surface. The eyes were examined at indicated time points after the infection for monitoring. After the experiments the mice were sacrificed at indicated time, by an overdose of Isoflurane and euthanized with cervical dislocation under anesthesia to minimize pain and suffering.

Bacterial Plate Counts

5 days after infection, the corneas were collected, bacterial number was detected as
reported [13]. In short, the cornea was homogenized in sterile water containing 0.85% (w / V) NaCl and 0.25% BSA individually. The 10 fold diluent of the sample was coated on the Pseudomonas Isolation Agar (BD Difco laboratories, Sparks, MD) for three times followed by the incubation overnight at 37 °C. The data were reported as 10^5 CFU per cornea ± SD.

**Lentivirus Preparation And Infection**

Lentivirus (Invitrogen, BLOCK-iT™ Lentiviral RNAi Expression System, K4944-00) containing control plasmid or shRNA targeting USP22 were constructed by MDL technology company (MDL biotech, Beijing, China) according to the manufacturer’s instruction. shRNA lentivirus was packaged and titered in HEK293T cells, the enriched lentivirus particle was used for cell infection at 50 MOI with the presence of polybrene. For in vivo infection, the protocol was performed as reported [8]. Lentivirus were subconjunctivally injected into the left eye of C57BL/6J mice (5 µl/mouse at a viral titer of 1 × 10^8) once a week for 3 times before PA infection.

**Quantitative Pcr Analysis**

Total RNA was extracted with TRIzol reagent according to the manufacturer’s instructions (Invitrogen) and SYBR RT-PCR kit (Takara Biotechnology) were used for quantitative PCR analysis. Primer sequences were shown in Table 1.
Table 1
List of primers used in the study.

| Number | Gene  | Sequence (5’-3’) |
|--------|-------|-----------------|
| 1      | USP22 | CCTGCACGTTCGTTGGAAC |
|        |       | TCTCCACGTGTGGTTGGAAC |
| 2      | TNF-α | GCCACCACGCTCTCTGCT |
|        |       | TGAGGGGCTCCGTCATAGAAC |
| 3      | IL-1β | ACGCTACACACCAGCGAG |
|        |       | CATTCCACGATTTTCAGG |
| 4      | IL-6  | ACAACCACGGCTCCCTAC |
|        |       | CATTCCACGATTTTCAGG |
| 6      | GAPDH | AATGACCCCTTCATGAC |
|        |       | TCCACGACGTACTCAGG |

Western Blot Analysis And Ubiquitination Assay

The protocols were performed as described previously (8). The cells or corneas were lysed using RIPA buffer (MDL biotech, Beijing, China), and total protein in the supernatants was quantified using a Bio-Rad quantification assay (Bio-Rad Laboratories, Hercules, CA). Equal number of protein (25 µg) was loaded on sodium dodecyl sulfate polyacrylamide gel electrophoresis to separate them from each other and then transferred to a PVDF membrane (Millipore, Billerica, USA) followed by blockade with 2.5% nonfat dry milk for 1 hour. Antibodies for USP22 (#ab195289), K48-linked ubiquitin (linkage-specific K48, #ab140601), TRAF6 (#ab33915) (Abcam, Cambridge, USA) and the antibodies specific for p65 (#8242), phospho-p65 (#3033), IκBα (##4814), phospho- IκBα(#2859) (Cell Signaling Technology Inc, Beverly, USA) and β-Actin (#sc58673) (Santa Cruz Biotechnology, Santa Cruz, USA) were added and incubated overnight at 4 °C. Subsequently, the membranes were applied with the corresponding horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) and detected with the enhanced chemiluminescence (Thermo Fisher Scientific, Bremen, Germany). The ubiquitination of TRAF6 was detected as described [8].
Statistical analysis

The differences in clinical score between lentivirus treated corneas were tested by the Mann-Whitney U test. The other assays were determined by an unpaired, two-tailed Student’s t test. A p value < 0.05 was considered statistically significant.

Results

USP22 expression was increased after PA infection

In order to illustrate the function of USP22 in PA-induced keratitis, the expression of USP22 after PA infection was examined at first. As shown in Fig. 1A and 1B, we found in mice corneas, both the mRNA and protein levels of USP22 were increased after PA stimulation. After PA infection, innate immune cells such as macrophages and granulocytes were infiltrated in the corneal stroma for bacteria elimination [14]. We next detected the level of USP22 in cultured RAW264.7 cells. Consistently, mRNA and protein levels of USP22 were significantly up-regulated by PA stimulation (Fig. 1C and 1D).

Silencing Of Usp22 Alleviated Pa-induced Keratitis

Lentivirus containing USP22 shRNA plasmid was used to knockdown the expression of USP22. The efficiency of USP22 silence in mice corneas was confirmed by qPCR and western blot assay (Fig. 2A). We next examined the clinical scores of PA-infected corneas, and we found that knockdown the expression of USP22 significantly attenuated PA-induced disease severity (Fig. 2B). Consistently, as shown in Fig. 2C, the bacterial load was greatly decreased in USP22-silenced mice corneas after PA infection.

Silencing Of Usp22 Suppressed Pa-induced Pro-inflammatory Cytokines Production

Inflammatory reaction and pro-inflammatory cytokines production are critical processes in response to PA infection in corneas. Therefore we detected the pro-inflammatory cytokines...
production in mice corneas and RAW264.7 cells after PA stimulation. As shown in Fig. 3A and 3B, both mRNA and protein levels of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) were decreased in USP22-shRNA-treated mice corneas after PA infection. Similar results also observed in RAW264.7 cells.

Silencing Of Usp22 Inhibited Nf-κb Activation

Production of pro-inflammatory cytokines depends mainly on the NF-κB activation in response to PA infection, so we hypothesized if USP22 could affect NF-κB activation in PA-infect macrophages. As shown in Fig. 4A, 4B and 4C, we found that silencing of USP22 greatly suppressed phosphorylation of p65 and IκBα in RAW264.7 cells treated with shRNA-USP22 lentivirus.

Silencing Of Usp22 Aggravated K48-linked Polyubiquitination Of Traf6

TRAF6 was reported as an essential adaptor of NF-κB signaling pathway, we wonder the relationship between USP22 and TRAF6, therefore we examined the protein level of TRAF6 in USP22-silenced RAW264.7 cells after PA infection. Interestingly, we found that knockdown USP22 expression decreased TRAF6 expression both in control or PA-treated macrophages (Fig. 5A). Most importantly, we found that silencing of USP22 greatly enhanced k48-linked polyubiquitination of TRAF6, especially in PA infected RAW264.7 cells (Fig. 5B).

Discussion

In the current study, we demonstrated the expression and function of USP22 in PA-induced keratitis. As far as we know, this manuscript is the first report on the connection between USP22 and PA keratitis.

PA keratitis accounts for approximately three quarters of reported cases of contact lens-
associated use. [13]. The pathogenesis of PA keratitis is very complex and has many factors including bacterial factors and host components. For example, PA can produce a variety of toxic factors, such as exotoxin A, lipopolysaccharide endotoxin (LPS) and exoenzyme ExoU to induce host cell death. [15]. In addition, as the key cells of host immune response, macrophages and monocytes accumulate in the infected area during PA infection. The pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 produced by macrophages and monocytes aim to clean up the infected bacteria, but if not properly controlled, these inflammatory cytokines could aggravate tissue damage even leading to corneal perforation [16]. In the current study, we showed that knock down the expression of USP22 could delay PA keratitis progression and decreased PA bacterial load, indicated USP22 as a positive regulator of PA keratitis. As our data shown, USP22 expression is markedly increased by PA infection both in mice corneas and in vitro cultured RAW264.7 cells, which means that USP22 expression is regulated by PA, and with the accumulation of USP22, PA keratitis tends to be aggravated. Consistently, we found that silencing of USP22 suppressed pro-inflammatory cytokines production in PA-infected RAW264.7 cells, these data suggested USP22 as a pro-inflammatory regulator after PA infection.

NF-κB signaling pathway is well studied as a paradigm for signal transduction and pro-inflammatory cytokines production [17]. Previous studies have found that NF-κB signaling pathway plays a crucial role in the development of bacterial keratitis [18–20]. In the current study, we revealed that silencing of USP22 suppressed phosphorylation level of p65 and IκBα, which indicated USP22 could promote NF-κB activation, therefore increased the pro-inflammatory cytokines expression. Ubiquitination modification has been reported to play great roles in NF-κB activation, and multiple studies showed that ubiquitination regulation is essential for the regulation of progression of PA keratitis [7, 8]. In the present research, we found USP22 could remove K48 linked polyubiquitination chain of
TRAF6, which is the key adaptor of NF-κB signaling pathway, leading to the stability of TRAF6, and therefore promote NF-κB activation and the downstream pro-inflammatory cytokines production.

USP22 is a novel deubiquitinating enzyme, it is considered to be important in many physiological and pathological processes such as cell cycle, cell proliferation, and tumor invasion [21–23]. However, the function of USP22 in PA keratitis is unclear. In the current research, we detected the level of USP22 in PA infected mice corneas and RAW264.7 cells, and we found that USP22 expression is induced by PA infection. Furthermore, we revealed that USP22 promote disease progression of PA keratitis, together with the increased pro-inflammatory cytokines production. At last, we found USP22 enhanced PA-induced NF-κB activation, and USP22 stabilized TRAF6 expression by removing K48-linked polyubiquitination of TRAF6.

Conclusion

For the first time, these findings showed that USP22 positively regulates PA-induced PA keratitis and extends our understanding of the physiological function of USP22. At last, we suggested USP22 as a possible medical target for the treatment of PA keratitis.

Abbreviations

PA: Pseudomonas aeruginosa; USP22: ubiquitin-specific protease 22; TRAF6: tumor necrosis factor receptor-associated factor 6; DUBs: Deubiquitinating enzymes; CFUs: colony-forming units; LPS: lipopolysaccharide endotoxin; TNF-α: tumor necrosis factor-α; IL-1β: interleukin-1β; IL-6: interleukin-6; NF-κB: nuclear factor kappa-B.

Declarations

Acknowledgements

Not Applicable.
Authors’ contributions
DC, DS and YM Performed the experiments. WL and JQ Analyzed the data. YW Wrote the paper. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
The experiments were carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals approved by the Animal Ethics Committee of the Scientific Investigation Board of our university.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Figures
USP22 expression was increased after PA infection. (A) Mouse corneas were infected with PA for indicated time, the protein levels of USP22 were detected. (B) Mouse corneas were infected with PA for indicated time, 6 mice/time point. USP22 mRNA expression were examined. (C) RAW264.7 cells were infected with PA for indicated time, the protein levels of USP22 were detected. (D) RAW264.7 cells were infected with PA for indicated time, USP22 mRNA levels were examined.

Data are representative of three independent experiments (mean± SD).
Silencing of USP22 alleviated PA-induced keratitis. (A) Mouse corneas were infected with shRNA-control-lentivirus or shRNA-USP22-lentivirus followed by PA infection for 5 days, the efficiency of shRNAs were confirmed. (B) Mouse corneas were infected with shRNA-control-lentivirus or shRNA-USP22-lentivirus followed by PA infection for 5 days, clinical scores were recorded. (C) Bacterial load in B. Data are representative of three independent experiments (mean± SD). *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Figure 3

Silencing of USP22 suppressed PA-induced pro-inflammatory cytokines production. (A and B) USP22 expression in mice corneas were knockdown by shRNA-USP22-lentivirus followed by PA stimulation, the mRNA expression levels of TNF-α, IL-1β and IL-6 were examined by qPCR. (C and D) USP22 expression in RAW264.7 cells were knockdown by shRNA-USP22-lentivirus followed by PA stimulation, mRNA expression levels of TNF-α, IL-1β and IL-6 were examined by qPCR. Data are representative of three independent experiments (mean± SD). *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Silencing of USP22 inhibited NF-κB activation. (A) USP22 expression were silenced, followed by PA infection for 24 hours, phosphorylation of p65 and IκBα were examined (B and C) Quantification of protein level of p-p65, IκBα in (A). Data are representative of three independent experiments (mean± SD). *, P < 0.05; ***, P < 0.001.
Silencing of USP22 aggravated k48-linked polyubiquitination of TRAF6. (A) TRAF6 expression were examined in USP22 silenced RAW264.7 cells at 24 hours after PA infection. (B) K48 linked polyubiquitination of TRAF6 in (A) was detected by western blot. Data are representative of three independent experiments (mean±SD).

Supplementary Files

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