Microbial Pathogenesis 90 (2016) 50–54

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Up-regulation of CYLD enhances *Listeria monocytogenes* induced apoptosis in THP-1 cells

Changzhi Xu [a, 1], Ling Yang [a, 1], Yuan Yuan [b, 1], Fei Du [c], Shumin Wang [a], Xiangfang Wang [a], Lin Zhu [a], Buchang Zhang [a, *], David Weaver [a, **]

[a] *Institute of Health Sciences, Anhui University, Hefei, Anhui 230601, PR China*
[b] *The Central Laboratory of Binhu Hospital, The Third Affiliated Hospital of Anhui Medical University, Hefei, Anhui 230601, PR China*
[c] *The Clinical Laboratory of Binhu Hospital, The Third Affiliated Hospital of Anhui Medical University, Hefei, Anhui 230601, PR China*

ARTICLE INFO

Article history:
Received 23 September 2015
Accepted 11 October 2015
Available online 3 November 2015

Keywords:
Listeria monocytogenes
CYLD
Apoptosis

ABSTRACT

Listeria monocytogenes (*Lm*), a facultative anaerobic gram-positive bacterium, causes listeriosis. Immune cell apoptosis is considered to be one pathogenic factor for listeriosis. As a deubiquitinase, CYLD is an important regulator both in innate immune response and apoptosis by negatively modulating NF-κB pathway. However the role of CYLD in *Lm* induced apoptosis remains unclear. Here we found that CYLD is significantly up-regulated in macrophages upon its infection. There is a moderate decrease in *Lm* proliferation and apoptotic cells *in* siRNA-induced CYLD knockdown THP-1 cells. Thereby CYLD may be involved in cell apoptosis mediated by *Lm* infection and its proliferation.

1. Introduction

Listeriosis is caused by *listeria monocytogenes* (*Lm*), a facultative anaerobic gram-positive bacterium. *Lm* is reported to be one of the most virulent food-borne pathogens [1]. This intracellular bacterium causes serious human disease, especially in immunocompromised individuals, pregnant women and the developing fetus [2]. Thus it has been employed as a model to study the interaction and immunity between hosts and intracellular bacterial pathogens [3]. To establish infection, *Lm* must escape and overcome the barriers of the innate immune system first. Macrophages are professional phagocytes to counteract invading pathogens here. To hide from the immune systems and dampen the host inflammatory response, an arsenal of virulence factors have been deployed by *Lm* [4]. The most studied one is the pH-dependent pore forming toxin listeriolysin O (LLO) [5]. *Lm* infection triggers the production of interferon β (IFN-β) [6,7], which can bind to the type I IFN receptor (IFNAR) to stimulate the transcription of IFN-inducible genes. However, LLO could block IFN-β production at the early stages of infection [8].

IFN-β has been demonstrated extensively during viral infection, and is commonly regarded as the first line for viral clearance. However, the role of this cytokine in bacterial infection is more controversial [9]. For example, type I IFN induced by influenza virus infection could enhance susceptibility to a potential secondary *pneumococcal* infection or *S. pneumonia* infection [10,11]. Similarly, type I IFN has been recently reported to negatively regulate the production of CXC chemokines and subsequent neutrophil recruitment [12,13]. The studies of *in vivo* also have shown that mice lacking the type I IFN receptors are resistant to *Lm* infection. Although the mechanisms through which type I IFN enhances *Lm* growth in the murine host remain not fully understood, increased immune cell apoptosis [14,15], inhibition of TNF-α production and enhanced recruitment of a population of $CD11b^+$ cells [16] are three major contribution factors. Here apoptosis plays an important role in bacterial infection. *Lm* could induce lymphocyte apoptosis and treatment with type I IFN could prime resting lymphocytes to undergo apoptosis induced by LLO [14]. Increased apoptosis by type I IFN negatively influences the response to *Lm* infection.

As a deubiquitinase, CYLD is first identified as the gene mutated in family cylindromatosis [17]. Further studies show that CYLD is a negative regulator in innate immunity by negatively regulating NF-κB activity [18,19]. It also could modulate apoptosis by negatively regulating NF-κB signaling pathway [18]. Nishanth G et al. [20] have demonstrated that CYLD could promote severe listeriosis through dampening IL-6/STAT3-dependent fibrin production *in vivo*; Wurm

* Corresponding author.
** Corresponding author.
E-mail addresses: zhbc@ahu.edu.cn (B. Zhang), david.weaver.t@gmail.com (D. Weaver).
[1] These authors have contributed equally to this work.

http://dx.doi.org/10.1016/j.micpath.2015.10.007

R et al. [21] have show that the short form CYLD could induce protective dendritic cell responses against listeriosis and this promotion is inhibited by full-length CYLD. However there are rare reports about CYLD and *Lm* infection in apoptosis regulation to date. In the present study, we found that CYLD is significantly up-regulated in macrophages upon *Lm* infection and knockdown CYLD could reduce *Lm* replication. So we speculated that CYLD is a functional gene anticipating in *Lm* infection. Furthermore we found that knockdown CYLD could reduce THP-1 cell apoptosis through negatively regulating NF-κB signaling pathway. Thus the present study has for the first time demonstrated that CYLD is probably a positive regulator to facilitate *Lm* infection by promoting cell apoptosis in macrophages.

2. Results and discussion

2.1. CYLD is up-regulated in response to Lm infection in THP-1 cells

To investigate the role of CYLD in *Lm* infection, first we wanted to explore whether CYLD expression was regulated by *Lm* challenge in human immune cells. Therefore, THP-1 cells were infected with *Lm* and the expression of CYLD was determined at different times post infection. Fig. 1A and C indicate that CYLD is up-regulated from 3 h to 36 h after *Lm* challenges both in mRNA and protein levels, consistent with the kinetics of *Lm* growth in the infected cells (Fig. 1B). Furthermore, CYLD was also up-regulated in a dose-dependent manner (Fig. 1D and E).

2.2. Knockdown CYLD suppresses Lm proliferation

Since CYLD expression is up-regulated during *Lm* infection, it is indicated that CYLD could functionally participate in the modulation between *Lm* infection and cell survival. Therefore CYLD siRNA was transfected into THP-1 cells to suppress CYLD expression level (Fig. 2A). At 48 h later cells were infected with *Lm* and *Lm* proliferation was measured by counting colony numbers. Colony number of *Lm* in CYLD knockdown cells is less than that in control cells (Fig. 2B and C). These results provide biological evidences that CYLD act as a negative regulator to modulate the anti-bacterial cellular response.

2.3. Knockdown CYLD resists apoptosis induced by Lm

In consideration of the function of CYLD in innate immunity, we tested whether knockdown CYLD could trigger IFN-β production and the activity of NF-κB in THP-1 cells. We found that IFN-β is induced significantly after *Lm* infection for 24 h (Fig. 3A). Due to the delayed response of IFN-β in *Lm* infection, we turn to focus on *Lm* induced cell apoptosis. First, we found that the phosphorylation of p65 is increased in time-dependent manner by immunoblotting, indicating that the activity of NF-κB is consistent with *Lm* infection (Fig. 3B). Then THP-1 cells were transfected with CYLD siRNA followed by *Lm* infection. Results show that cleaved caspase-3 level is reduced, while p65 phosphorylation level is increased in CYLD knockdown cells (Fig. 3C). Then cell apoptosis was tested by flow cytometry and knockdown CYLD also resisted FITC-Annexin V and PI double staining cells ratio (Fig. 3D). These observations suggest that reduced immune cell apoptosis by knockdown CYLD may be a target for listeriosis treatment.

Ubiquitylation and deubiquitylation of target proteins represent an important cellular strategy that enable the cell to quickly response to external environmental changes, including bacterial infection and apoptosis. As a family member of deubiquitinases, CYLD could bind to the NEMO in the IκB kinase complex and remove the K63 ubiqutin chain from TRAF2 to modulate apoptosis [18]. However there is no report about CYLD and *Lm* induced macrophage cell apoptosis. Here we found that: 1, CYLD is significantly up-regulated in response to *Lm* infection; 2, *Lm* growth is reduced in THP-1 cells with lower CYLD expression; 3, we found that knockdown CYLD reduce cell apoptosis in infected cells.

CYLD is an important regulator function both in innate immunity and apoptosis. First, we found that IFN-β is not produced until 24 h later after *Lm* infection. This delayed response of IFN-β is considered to be attributed to the production of LLO [8]. Meanwhile NF-κB is activated early in response to *Lm* infection. So we speculated that up-regulated CYLD is mainly involved in apoptosis regulation by regulating NF-κB pathway. Immunoblotting and flow cytometry analysis indicated that knockdown CYLD could increase the phosphorylation of p65, while decrease the proportion of cleaved ratio of caspase-3 and subsequent apoptosis induced by *Lm*. Additionally, it has been reported that $cyld^{-/-}$ cells could enhance the K63-ubiquitination of STAT3 and activation to promote the production of fibrin, thereby protecting the liver tissue in mice infected with *Lm* [20]. Here we confirmed another mechanism for decreased CYLD to protect cells for counteracting with *Lm* infection.

Taken together, this study shows that CYLD is up-regulated during *Lm* infection in THP-1 cells. CYLD could enhance apoptosis through negatively regulating NF-κB signaling pathway. Thus CYLD is exploited to promote macrophage apoptosis and subsequent bacterial growth. Our results provide a new target and bring a new understanding of the molecular mechanism between CYLD and *Lm* infection.

3. Materials and methods

3.1. Cell culture and transfection

THP-1 cells were cultured in RPMI1640 (Invitrogen), supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen), 100 U/mL penicillin and 100 mg/mL streptomycin. CYLD siRNA (dsRNA oligonucleotides) and negative control were synthesized by GenePharma and transfected with INTERFERIN (Polyplus) according to the manufacturer's instructions. Si-CYLD: 5′-GUAUAGGACAGUAUAUUCATT-3′ (sense); 5′-UGAAUAUACUGUCCU AUACTT-3′ (antisense). Si-ctrl: 5′-AGACCCACUCGGAUGUGAAGA-GAUA-3′ (sense); 5′-UAUCUCUUCACAUCCGAGUGGGUCU-3′ (anti sense).

3.2. Bacteria and bacterial infection

L.monocytogenes strain CMCC (B) 54002 was used in this study. Bacteria were grown in brain heart infusion (BHI) broth, overnight at 37 °C with shaking at 180 rpm. Overnight cultures were diluted into 1:100, grown to mid-exponential phase ($OD_{600nm} = 1.0$) and stored as glycerol stocks at −80 °C in small aliquots until use.

THP-1 cells, differentiated by 12-O-Tetradecanoylphorbol-13-Acetate (TPA) for 24 h, were maintained in 6 well plates. At 1 h before bacterial infection, cells were washed with PBS and cultured with serum-free and antibiotics-free medium. Bacteria at different MOI were added to the monolayer of cells. At 30mins later, cells were washed with PBS three times and supplemented with fresh media containing 100 μg/mL gentamycin. At 1 h later cells were washed with PBS three times and supplemented with fresh media containing 25 μg/mL gentamycin. Finally cells were harvested for indicated detection.

3.3. RNA quantification

Total RNA was extracted from samples and reversed by poly dT. The procedure for detecting relative expression levels of CYLD was