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MINIREVIEW

The Functions of Effector Proteins in *Yersinia* Virulence

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Abstract

Yersinia species are bacterial pathogens that can cause plague and intestinal diseases after invading into human cells through the Three Secretion System (TTSS). The effect of pathogenesis is mediated by *Yersinia* outer proteins (Yop) and manifested as down-regulation of the cytokine genes expression by inhibiting nuclear factor-κ-gene binding (NF-κB) and mitogen-activated protein kinase (MAPK) pathways. In addition, its pathogenesis can also manipulate the disorder of host innate immune system and cell death such as apoptosis, pyroptosis, and autophagy. Among the *Yersinia* effector proteins, YopB and YopD assist the injection of other virulence effectors into the host cytoplasm, while YopE, YopH, YopJ, YopO, and YopT target on disrupting host cell signaling pathways in the host cytosols. Many efforts have been applied to reveal that intracellular proteins such as Rho-GTPase, and transmembrane receptors such as Toll-like receptors (TLRs) both play critical roles in *Yersinia* pathogenesis, establishing a connection between the pathogenic process and the signaling response. This review will mainly focus on how the effector proteins of *Yersinia* modulate the intrinsic signals in host cells and disturb the innate immunity of hosts through TTSS.

Key words: *Yersinia* pathogenesis, Yops, TTSS

Introduction

Yersinia species are Gram-negative bacteria in the family of *Enterobacteriaceae*, in which three *Yersinia* species, *i.e. Yersinia pestis*, *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*, are pathogenic against humans. It was reported that the infection of *Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis* could cause plague, yersiniosis, and scarlatinoid fever, respectively (Viboud and Bliska, 2005). Interestingly, although these three pathogenic *Yersinia* species have different modes of transmission, they all share common virulence factors, termed Yops, and utilize the same protein export pathway, known as the TTSS. TTSS is a general secretion system in the Gram-negative bacteria like pathogenic *Escherichia coli*, *Pseudomonas*, *Salmonella*, *Shigella*, and *Yersinia* spp., which allows bacteria to inject their effector proteins from bacterial membrane into host cells cytoplasm using injectisome, a needle-like complex. Once being injected into the host cytoplasm, Yops target different proteins in the host cytoplasm, stimulating each other or performing antagonistic action in the activation of intracellular enzyme and cell death programs. In this review, we will mainly focus on how *Yersinia* spp. inhibit the host innate immune response through TTSS, in which the recent progress in studies on Yops targeting GTPase, caspase-1, and MAPK/NF-κB pathways will be emphasized.

Pathogenic effect of the effector proteins. Extensive studies have been performed to understand the virulence mediated by the effector proteins of *Yersinia*. It is believed that Yops can efficiently suppress the expression of cytokines in host cells through inhibiting the NF-κB and MAPK pathways by different means. For example, YopJ/P binds to MAPK kinase (MKK) and IκB kinase (IKK), blocking their activation (Mukherjee and Orth, 2008). Other evidence shows that YopM activates the cytoplasmic kinase RSK1, PRK2, thereby inhibiting the caspase-1 pathway to cause the activation of proinflammatory cytokines and cell death (Hentschke *et al.*, 2010). Under other circumstances, the effector proteins can perfectly co-regulate the pathogenesis of *Yersinia*, thereby inhibiting the signaling pathways, inducing the apoptosis of target eukaryotic cells, and contributing to the interference and repression of the host immune system. In the *Yersinia* infecting process, under the assistance of YoB, YopD, and LcrV, six effector proteins, including YopE, YopH, YopJ/P, YopM, YopO/YpkA,

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and YopT, are injected into the infected cells through TTSS (Swietnicki *et al.*, 2005). These Yops interfere the host cell signaling pathways and inhibit the immune responses, facilitating *Yersinia* virulence. Among Yops, YopJ and YopP share more than 95% sequence similarity, and YopO and YpkA share 98% sequence similarity, with YopJ existing in *Y. pestis* and *Y. pseudotuberculosis*, YopO existing in *Y. pestis* and *Y. enterocolitica*, YopP existing in *Y. enterocolitica*, and YpkA existing in *Y. pseudotuberculosis*, respectively (Viboud and Bliska, 2005; Aepfelbacher and Heesemann, 2001). In spite of acting on different aspects, Yops have to function jointly to accomplish pathogenesis. For example, YopE (Fallman *et al.*, 1995) and YopT together facilitate to inhibit phagocytosis and pore formation (Viboud and Bliska, 2001), albeit by distinct mechanisms. In addition, some pathological changes are caused by several effectors, in which the absence or mutation of just one effector can lead to incomplete virulence.

Delivery machinery of the effectors protein into the host cells. The effector proteins consist of over 20 proteins, which are secreted with the same conservative manner known as TTSS. In the pathogenic infection, the injectisome enables the bacteria to directly transport or inject effectors from the bacterial cytoplasm into the host cytosol. Formation of the injectisome includes three steps. Firstly, YscRSTV, YscD, YscJ, YscQ, YscNLK and YscU are assembled to form the basal body of the needle-like complex. Then, YscF joins as a needle over the basal body, and YopN is added into the complex to help the recruitment of LcrV and YopBD. In the last step, YopN is released upon cell contact, and the translocation pore is formed (Viboud and Bliska, 2005; Chen and Anderson, 2011; Jessen *et al.*, 2014; Dewoody *et al.*, 2013).

The expression of the proteins in the *Yersinia* TTSS is sophisticatedly regulated by YopD, LcrH, and LcrQ, at both transcriptional and posttranscriptional levels (Cambronn *et al.*, 2004; Rimpilainen *et al.*, 1992; Wilharm *et al.*, 2003). The increase of the LcrQ level in the bacterial cytoplasm is reported to cause constitutive repression of the TTSS (Rimpilainen *et al.*, 1992). In addition, YopD and LcrH can form a complex of YopD-LcrH, attenuating the translation of mRNA of the TTSS genes through binding to the unstructured 5' end of mRNA to prevent ribosome binding (Chen and Anderson, 2011). More interestingly, the regulation caused by YopD, LcrH, and LcrQ is environmentally responsive to calcium, as high concentration of calcium causes the repression of protein expression (Straley and Bowmer, 1986). The transcription of TTSS genes is also affected by LcrF, as activation of LcrF facilitates the formation of a protein channel through the membranes of *Yersinia*. Afterwards, it gives rise to the formation of a pore on the surface of the plasma membrane of the infected eukaryotic cells to permit the entry of the effector proteins, leading to the inhibition of the host signaling pathways and cell death.

Functional roles of effector protein as virulence factors. When *Yersinia* spp. invades mammalian cells, Yops are injected into the infected cells through the TTSS. Under the assistance of caspase-3 and caspase-7, the effector proteins cause the infected cells to exhibit the features of apoptosis, including membrane bleb, condensation of nucleus, DNA fragmentation, and large cytoplasmic vacuoles (Zheng *et al.*, 1998), finally resulting in cell death. This phenomenon is also regarded as immune silence or immunosuppression. In addition, Yops injection also leads to proinflammatory cell death called pyroptosis with the help of caspase-1 induction. Standing on *Yersinia*'s side, it is complicated and hard to control the balance of survival as the apoptosis induced by *Yersinia* leads to immune response acceleration. The effector proteins contribute to the survival of the host cells, expanding their reproduction scope and consolidating their existence, while on the other hand; they promote the positive immune response to prevent the pathogenic invasion. In the TTSS, YopB and YopD form a pore as an entrance to across the host cell cytomembrane (Montager *et al.*, 2011), from which the other six Yops are injected into the interior of host cell, and admitted to modulate the hosts' immune response. Among these Yops, YopO, YopE and YopT target the GTPase; YopJ/P causes the suppression of cytokines by modulating the NF-κB and MAPK pathway signals; YopH mainly regulates caspase-1 to induce pyroptosis (Bahta and Burke, 2012); and YopM activates the cytoplasmic kinase RSK1 and PRK2 to inhibit the caspase-1 pathway.

The virulence of YopJ/P has been well studied to reveal that barrier dysfunction and systemic disease are mainly caused by the injection of YopJ/P (Philip and Brosdky, 2012). YopJ/P-sufficient *Y. pseudotuberculos* exhibits reduced production of cell death cytokine to cause the bacterial control of the host cell immune response, while YopJ/P-deficiency results in robust cytokines production, controlled bacterial spread, and intact barrier function (Philip and Brosdky, 2012). Additionally, decreased colonization of spleen is observed when YopJ is deficient in the *Y. pseudotuberculosis* infection (Monack *et al.*, 1998), indicating YopJ's ability to manage the dissemination from mucosal tissues (Viboud and Bliska, 2005).

Recent progress on the molecular mechanism of the *Yersinia* effector proteins

YopB and YopD are hydrophobic proteins termed translocators that play important roles in the injection of Yops through the conversed TTSS. It has been

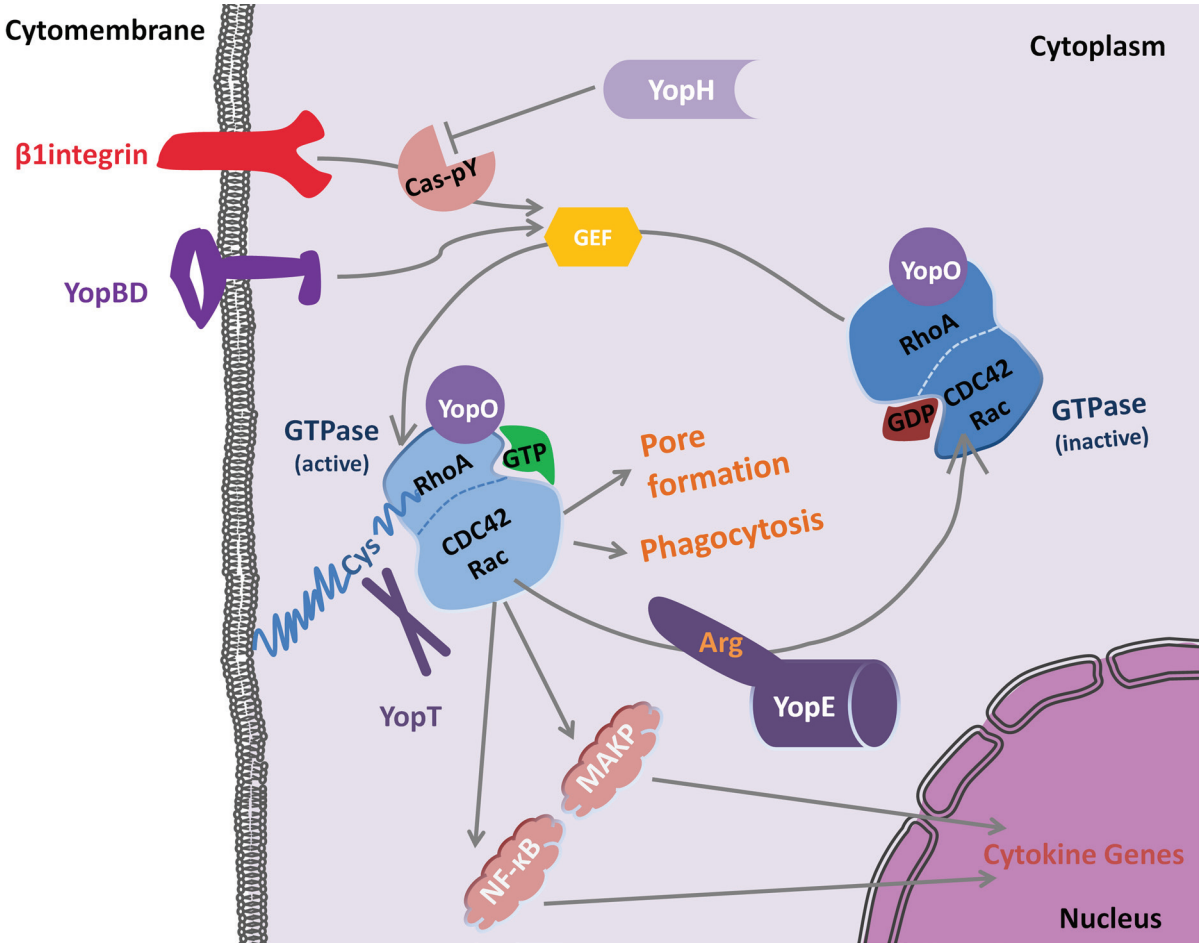

Fig. 1. GTPase-relevant Yops interact with host cell signal pathways.

YopB and YopD form a complex to let other Yops enter into the cells, stimulating the GEF. YopO binds GTPase, interferes with the Rac signals (Groves *et al.*, 2010, Prehna *et al.*, 2006), which damages the actin cytoskeleton. YopH inhibits the GEF activation signal Cas-pY emitted from β1integrin. YopT also targets the GTPase, as it cleaves the junction between cytomembrane and GTPase to repel the latter, resulting in the inactive GTPase. YopE blocks CDC42 and Rac of GTPase (Andor *et al.*, 2001), inducing the transformation of the active GTPase-GTP to inactivate GTPase- GDP to inhibit the pore formation and phagocytosis, which additionally impairs the GTPase activation signal to the MAPK and NF-κB pathways.

identified that YopB and YopD could assist the other effector proteins to enter into cytoplasm through the secretion apparatus called the needle complex (Edqvist *et al.*, 2007). During this process, YopB and YopD form the complex YopBD in the eukaryotic cell membranes with the assistance of the needle tip, constructing a pore on the target cell membrane as an entrance for other virulence effector proteins being injected in.

Interaction between GTPase and effector proteins (Fig. 1). After the virulence factors are transported into the host cell cytosols, the YopE, YopH, YopP and YopT cooperate to damage the actin cytoskeleton (Bliska, 2000) and the capacity of phagocytosis of the dendritic cells (Adkins *et al.*, 2007). YopB and YopD also activate the guanine nucleotide exchange factors (GEF), which can convert the inactive GTPase into its active form, leading to the phagocytosis and pore formation. Under normal circumstance, active GTPase with GTP bound ignites both the MAPK and the NF-κB signaling pathways, causing the genes in nucleus to be transcribed. For example, the expression of proinflammatory cytokines and other factors is enhanced by the active GTPase induced MAPK and NF-κB signaling pathways. Besides YopB and YopD, other Yops can also act on GTPase. YopH, a highly active tyrosine phosphatase (Hamid *et al.*, 1999), inhibits the contact between the β1 integrin, a transmembrane protein of the host cell, and the GEF (Viboud and Bliska, 2005).

YopE reverses the activation of GTPase, and similarly, YopO blocks the conversion between the active and inactive GTPase (Navarro *et al.*, 2007), which together with YopH down-regulate GTPase activation. Different from other Yops, YopT has a unique effect on GTPase. It inhibits phagocytosis and pore formation by scissoring RhoA in GTPase (Shao *et al.*, 2002), expelling the GTPase from the host cell membrane (Schmidt, 2011). Since the cytoskeleton is mainly regulated by GTPases, the destruction of the cytoskeleton

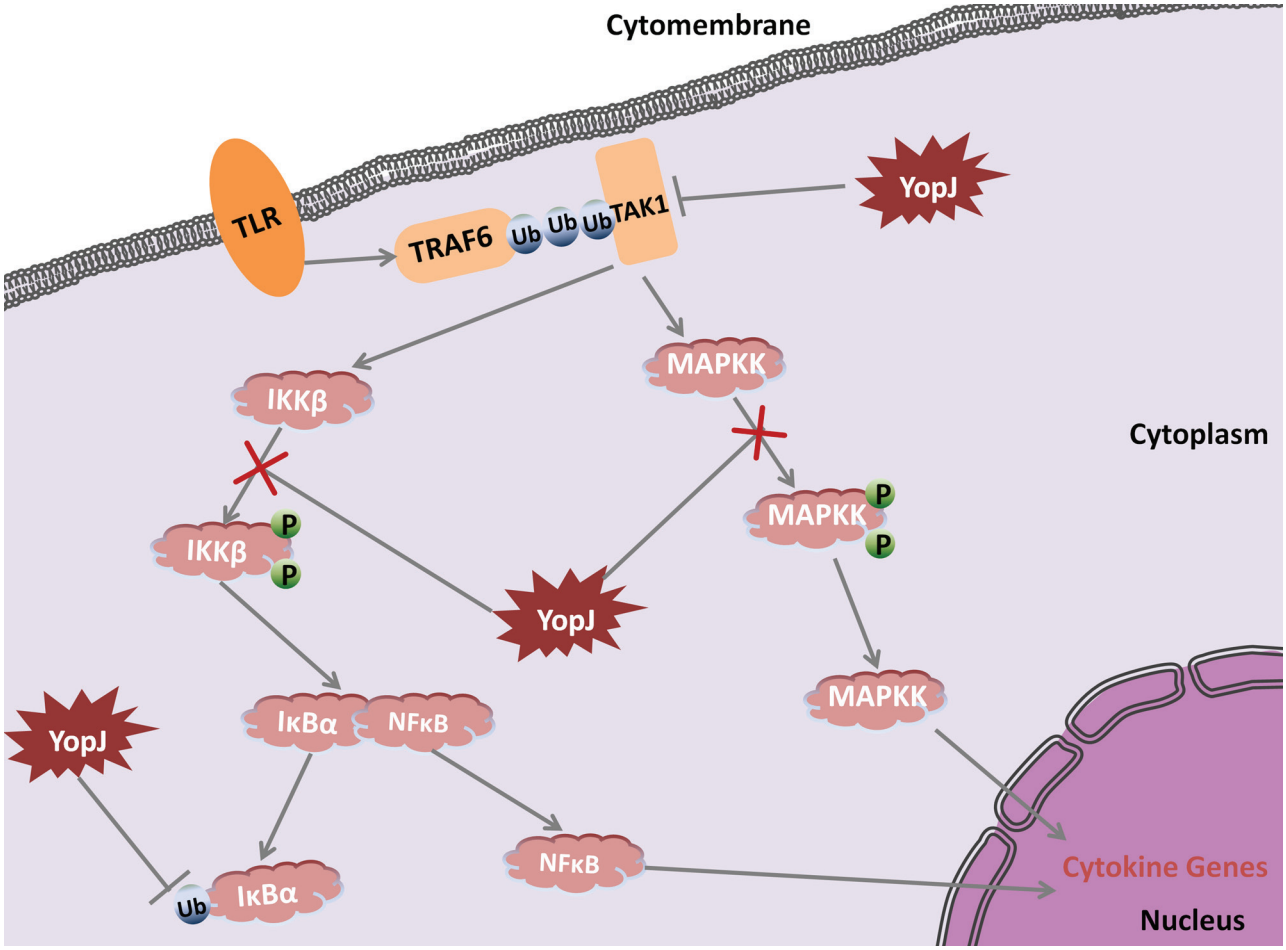

Fig. 2. YopJ inhibits MAPK and NF-κB pathways.

YopJ acetylates MAPKK and IKKβ to block the activation of MAPK and NF-κB, which in turn down-regulates the expression of proinflammatory cytokines and survival factors in cell nucleus. Additionally, YopJ removes Ub from TRAF6 and IκBα, hence leading to the inactivation of TRAF6 and TAK1. The regulation of the TAK1 is the key step to regulate the MAPK and NF-κB pathways.

will be caused by the inactive GTPases, furthermore resulting in cell death.

NF-κB and MAPK – YopJ/P (Fig. 2). Unlike other Yops, YopJ, as an important virulence factor in *Y. pseudotuberculosis*, has unique functions in *Yersinia* virulence. When macrophage or dendritic cells are infected by *Yersinia*, YopJ suppresses the MAPK/NF-κB pathways, hence deterring the formation of cytokines and survival factors. YopJ and its homologous, YopP, share 95–98% sequence similarity, and use the same secretion pathway, TTSS, to enter into the innate immune cells like membranous/microfold cells, dendritic cells, and Peyer's patches in the gut system (Viboud and Bliska, 2005).

After the infection, YopJ/P down-regulates the expression of the proinflammatory cytokine and the prosurvival proteins, and also blocks the formation of inflammasome through inhibiting the NF-κB and MAPK pathways. In addition, YopJ can activate caspase-1, and up-regulate the expression of IL-1β and IL-18 (Vladimer *et al.*, 2012; Luigi and Gabriel, 2012), giving rise to cell death in macrophages. In the interplay with the MAPK pathway, YopJ binds to MKKs (Mukherjee and Orth, 2008) and acts as a serine/threonine acetyltransferase that uses acetyl-coenzyme A (CoA) to modify the critical serine and threonine residues in the activation loop of MAPK kinasese, MKK and MAPK (Mukherjee *et al.*, 2006), thereby blocking the phosphorylation of MAPK to counteract the MAPK signaling pathway (Bliska, 2006; Paquette *et al.*, 2012). During the interaction with NF-κB, YopJ is recognized as a deubiquitinase that binds IKKβ, removing the ubiquitin moieties from critical proteins, such as TRAF6, TRAF2 and IκB (Zhou *et al.*, 2005). It is worth to note that the acetylation of TAK1 (transforming growth factor β-activated kinase 1), a member of the MAPKKK family, is the most important step for the YopJ-induced modulation of the host cell. As a downstream molecule indispensable for the nucleotide-binding and oligomerisation domain 2 (Nod2)-RICK-mediated activation of the NF-κB and MAPK pathways, it is still poorly understood whether YopJ acts directly on TAK1. However, YopJ has been indicated to restrain the NF-κB pathway at the level of TAK1 (Mukherjee and Orth, 2008; Meinzer *et al.*, 2012).

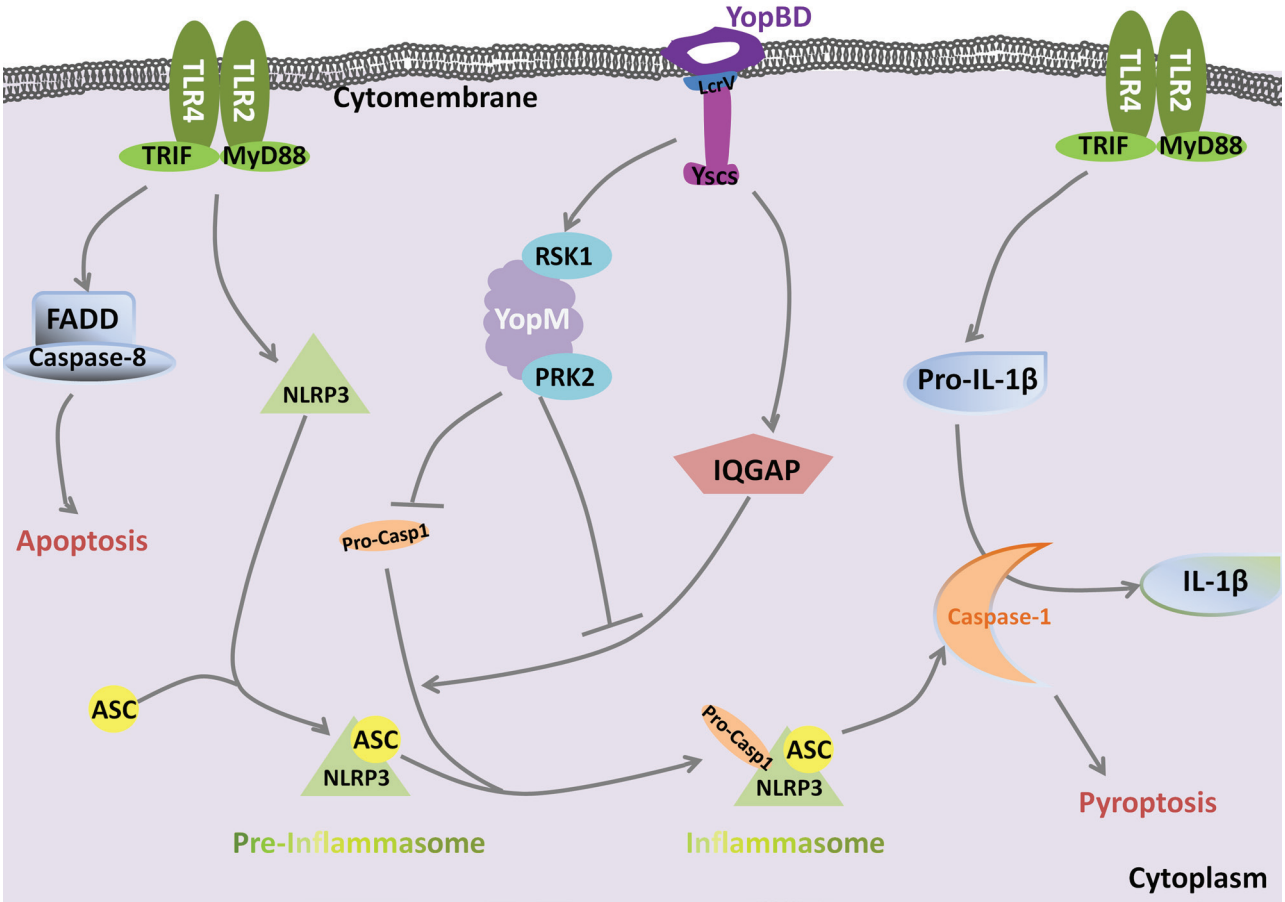

Fig. 3. Caspase-1 and other pathways in *Yersinia*-infected cell.

YopBD, LcrV and Yscs form a Needle-like injectisome across the cytomembrane, translocating the virulent Yops into the cytoplasm. The injectisome activates IQGAP, followed by induction of the caspase-1 medicated cell death, which is inhibited by the YopM-RSK1-PRK2 complex. TLRs, such as TLR2 and TLR4, stimulate FADD/Caspase-8 complex, inducing the apoptosis. In addition, TLRs can also activate the formation of pro-IL-1β and revitalize NLRP3, giving rise to the formation of pre-inflammasome. The assembly of inflammasome requires pro-caspase-1 and the activated IQGAP, which are blocked by the YopM complex. The assembled inflammasome activates caspase-1 and leads to pyroptosis. Besides, caspase-1 is also involved in the transformation from pro- IL-1β to IL-1β (von Moltke *et al.*, 2012).

Autophagy, inflammasome: caspase-1, pyroptosis – YopJ/P, YopM/K (Fig. 3). At the absence of priming, YopJ induces the NLRP3/ASC complex-dependent caspase-1 activation (Zheng *et al.*, 2012) and the NLRP12-activated inflammasome, resulting in the increase of IL-1β secretion (Broz *et al.*, 2010; Hamid *et al.*, 1999). YopJ, as a critical acetylase, acts in the activation loops of the RICK and TAK1 kinases (Meinzer *et al.*, 2012), which are the central mediators of Nod2 signaling. YopJ lowers the affinity of Nod2 for RICK, causing the unbound Nod2 to activate caspase-1, which finally results in the development of IL-1β.

Interestingly, although YopJ activates the caspase-1, the suppression of caspase-1 and inflammasome activation is carried out by YopM at the same time (McDonald *et al.*, 2003). YopM binds caspase-1 to inhibit the activation of caspase-1 (Jorgensen and Miao, 2012) and hamper the formation of the mature inflammasome, thus devastating the protective cell death program pyroptosis against pathogens (LaRock and Cookson, 2012). Additionally, the formation of RSK1 (ribosomal S6 protein kinase) and PRK2 (protein kinase C-related kinase 2) complex is relevant to YopM (Amedei *et al.*, 2011). YopM binds to RSK1 and PRK2, respectively, on its C-terminal tail and the LRR region (Viboud and Bliska, 2005). After binding, YopM blocks dephosphorylation of the activatory phosphorylation sites of RSK1 and activates RSK1-PRK2 complex (Ruckdeschel *et al.*, 2008). The complex formed by YopM and RSK1/Rsk2 blocks the formation of inflammasome through inhibi ting pro-caspase-1 and IQGAP1 signal (Chung *et al.*, 2014). YopM is also recognized as the first bacterial cell-penetrating protein, which can diminish the expression of TNF-α (tumor necrosis factor-α) and some other pro-inflammatory cytokines, such as IL(interleukin)-1, IL-12, IL-15, IL-18 (Kerschen *et al.*, 2004). Evidence also indicates that the release of bioactive IL-1β is also prevented by YopM (Bergsbaken

et al., 2009; LaRock and Cookson, 2012). Besides, the release of lysosomal exocytosis, which is an antimicrobial factor induced by caspase-1 to act on extracellular bacteria, is blocked by YopM (LaRock and Cookson, 2012; Bergsbaken *et al.*, 2011).

Similar to YopM, YopK (known as YopQ in *Y. enterocolitica*) (Holmstrom *et al.*, 1995) is functioned in down-regulating caspase-1 activation and IL-1β secretion. In the absence of all other known Yops, injection of YopK into the cells results in decreased caspase-1 activation (Brodsky *et al.*, 2010). All this evidence together indicates that YopK restricts the injection of effector proteins into the host cytoplasm and also, Caspase-1 activation (Dewoody *et al.*, 2011).

Interplay of the *Yersinia* with gut system and TLRs

Among the three species of pathogenetic bacterium to humans, *Y. enterocolitica* and *Y. pseudotuberculosis* mainly target the human gut system. Unlike *Y. Pestis*, these two enteropathogens are commonly transmitted by food or water (Trcek *et al.*, 2011). Multiple studies have indicated the involvement of Yops in the interaction of *Yersinia* and gut system.

Y. enterocolitica infection typically leads to acute enteritis, enterocolitis, mesenteric lymphadenitis and ulceration, and necrosis of the tissue. Mesenteric lymph nodes become amplified and the focal area of necrosis exhibits leukocytes infiltration (Viboud and Bliska, 2005, Ruckdeschel *et al.*, 1996). Studies of the intestine of *Y. enterocolitica* O9 infected BALB/c mice indicates that the infected mice exhibit splenomegaly, and development of $CD3^+$ total T cells, $CD4^+$ Th cells, $CD8^+$ Tc cells, and $CD11b^+$ phagocytic cells (Ruiz-Bravo *et al.*, 2001). The infection also causes the impaired response and proliferation of normal splenocytes against the mitogens, which in turn promotes the inhibition of lymphocyte responding to mitogens. In addition, the increase of IFN-γ is stimulated by the concanavalin A, but not lipopolysaccharide, which aggrandizes reactive nitrogen intermediates in macrophage. Therefore, the infection of *Y. enterocolitica* may lead to immune attenuation of spleen cells and disrupt the bacterium-pathogens-induced immune responses of host (Dessein *et al.*, 2009).

Y. pseudotuberculosis infection is typically tied to mesenteric adenitis and occasionally with terminal ileum and cecum inflammation (Galindo *et al.*, 2011). The infection normally causes the appearance of micro-abscesses or granuloma-like lesions with central necrosis. Derived from the latter tuberculosis-like lesions, *Y. pseudotuberculosis* disrupts intestinal barrier integrity through intruding the intestinal lymphoid tissue and TLR-2 signaling (Jung *et al.*, 2012). Due to anfractuous cooperation between intestinal epithelial cells and immune cells, the transcellular transport is determined by the pore size of tight junctions, which also limits the access of the luminal commensal pathogens. Studies on *Y. pseudotuberculosis* infected mice indicate that the Peyer's patches barrier dysfunction is largely associated with *Yersinia* virulence and the TLR-2 expressed by the hematopoietic cells. The TLR-2 activation determines the ideal epithelial transcript level of the anti-infective c-type lectin Reg3β, which is regarded as an intestinal resistance to *Yersinia* through controlling the bacterial load in Peyer's patches. After *Y. pseudotuberculosis* infection, TLR-2 plays a crucial role in initiating and regulating the host response due to its secretion in the intestinal epithelial cells, membranous/microfold cells, macrophages and dendritic cells. In humans, it is reported that *Y. pseudotuberculosis* disturbs epithelial gut homeostasis and may induce ileitis. All this evidence together indicates that the infection of *Y. pseudotuberculosis* may disrupt the gut system homeostasis through the interaction between bacterium lipopolysaccharide and TLRs (Dessein *et al.*, 2009; Meinzer *et al.*, 2012).

Future directions

Although the major functions of effector proteins in inhibiting the innate immune system of host cells (Amedei *et al.*, 2011) have been largely clarified, the detailed interplay of the Yops and receptors with the intracellular proteins or genes of the host cells remains unclear.

In the apoptotic pathway, YopM and YopJ both play functional roles in interfering apoptosis. Evidence has been obtained to confirm that the cell death receptor like TNF receptor and Fas could modulate the death-inducing signaling complex consisted of RIPK1, caspase-8, and Fas-associated death domain. However, how these proteins are involved in YopJ-induced cell death (Philip *et al.*, 2014), how YopJ/P inhibits mammalian TGFβ-activated kinase (Paquette *et al.*, 2012), how NOD2 interacts with caspase-1 (Meinzer *et al.*, 2012), and how Yops-induced cell death impacts the local microenvironment and host antibacterial immune responses *in vivo* are still uncertain (Philip and Brodsky, 2012).

Understanding the interactions of needle proteins and TLRs at the amino acids level is another interesting field for future study. The conclusions on how *Yersinia* recognize lipopeptides, impact host defense, and influence the TLR-2 activation on gut permeability are still controversial (Hajjar *et al.*, 2012; Galindo *et al.*, 2011). In addition, identifying the interplay between TLRs and needle proteins will enrich our knowledge of the interaction between host immune responses and TTSS. The investigation of TTSS to understand the formation of

a needle-like receptor, substrate-recognition of TTSS, and the substrate delivery is critical to further interpret the virulence of the *Yersinia*.

Yersinia invasion also significantly impacts the gut system in host cells (Persson *et al.*, 1999). Considering the strong virulence of *Y. enterocolitica* and *Y. pseudotuberculosis*, it can be believed that the understanding of the mechanisms of bacterial invasion interplaying with host gut barrier dysfunction (Zhou *et al.*, 2005) may bring useful clinic applications. All the questions mentioned above are worth being explored, and will produce effective treatment strategies towards these complicated courses of infection.

Conflict of interest

The authors declare that there are no conflicts of interest.

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