NF-κB activation suppresses host cell apoptosis during Rickettsia rickettsii infection via regulatory effects on intracellular localization or levels of apoptogenic and anti-apoptotic proteins

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Abstract

Rickettsia rickettsii, a gram-negative and obligate intracellular bacterium, is the causative agent of Rocky Mountain spotted fever. In human infections, the primary target of R. rickettsii infection is vascular endothelium. Our laboratory has shown that activation of nuclear transcription factor-kappa B (NF-κB) during R. rickettsii infection of cultured human endothelial cells protects against apoptosis by preventing the activation of apical caspases-8 and -9, and the effector caspase-3. To understand upstream signaling mechanisms, we have determined the effect of NF-κB blockade on the status of different Bcl-2 (B-cell lymphoma 2) proteins in this study. Quantitative analysis following TUNEL and Hoechst staining confirmed that infection of endothelial cells with R. rickettsii for 6 h in the presence of a specific NF-κB inhibitor, MG132, resulted in induction of apoptosis. Infection-induced apoptosis of EC was associated with decreased level of Bid and accumulation of Bad, while cytosolic level of Bax remained relatively unchanged. Further, the cellular levels of apoptosis antagonist Bel-2 were found to be down-regulated and apoptogenic mitochondrial proteins Smac and cytochrome c were released into cytoplasm. These results implicate an important regulatory role for NF-κB in controlling the intracellular levels and/or localization of pro- as well as anti-apoptotic proteins of Bel-2 family, the intricate balance of which is a critical determinant of downstream signaling mechanisms governing cell fate during intracellular infection.

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1. Introduction

Rickettsia rickettsii, a gram-negative and obligate intracellular bacterium, is the causative agent of Rocky Mountain spotted fever, the most prevalent rickettsial disease in the United States. The primary target of R. rickettsii in human infections is vascular endothelium, and the process of invasion involves attachment to endothelial membrane via rickettsial outer membrane protein A and induction of phagocytosis. The intracellular organisms then escape from the phagosome and replicate by binary fission predominantly in the cytoplasm of the host cell [1]. Studies using cultured human umbilical vein-derived endothelial cells (EC) indicate that infection with R. rickettsii not only induces characteristic signs of cell damage, but also elicits an active response reflecting altered cellular phenotype. This is evidenced by increased secretion of cytokines and production of several key proteins, which likely contribute to vascular changes during the course of disease [2]. An important cell signaling mechanism participating in the transcriptional regulation during R. rickettsii infection is the activation of nuclear factor-kappa B (NF-κB). The NF-κB family of transcription factors includes a group
of evolutionarily conserved proteins, which play a pivotal role in the regulation of cell adhesion and proliferation, innate immunity and inflammation, stress responses, and host–pathogen interactions [3–7]. Recent studies have defined yet another physiologically important function of NF-κB based on the evidence that it can serve both pro- and anti-apoptotic functions in mediating an array of cellular responses and determination of cell fate [8].

Apoptosis, a physiological process of programmed cell death, occurs in response to a variety of stimuli including infections with viruses and bacteria [9,10]. In disease states, apoptosis represents a pivotal defense mechanism by allowing the host to control the elimination of infected cells. This can inhibit the replication and spread of intracellular bacteria by inducing the inflammatory response and phagocytic mechanisms [9]. Interestingly, a number of pathogenic bacteria display strategies to exploit the intracellular milieu as their permanent or transient habitat. For example, virulent strains of Mycobacterium tuberculosis are capable of evading apoptosis of infected alveolar macrophages [11], and Brucella suis infection prevents the apoptotic death of human mononuclear cells in vitro [12]. It is, therefore, plausible that due to their obligate intracellular lifestyle and dependence on the host cell for energy and nutritional requirements, rickettsiae exert an anti-apoptotic effect to maintain the host cell as a site of infection. Our laboratory has demonstrated that activation of NF-κB is essential to protect the host cells from apoptosis during R. rickettsii infection [13]. Subsequent attempts to identify the mechanisms for this phenomenon revealed that anti-apoptotic actions of NF-κB are mediated by preventing the activation of upstream caspases-8 and -9, and effector caspase-3 [14].

Bcl-2 is a large family of proteins comprised of both proapoptotic (Bax, Bid and Bad) and anti-apoptotic (Bcl-2 and Bcl-X_L) factors. Changes in the intricate balance of intracellular levels and/or subcellular localization of these regulatory proteins likely play a crucial role in mitochondrial dysfunction and induction of apoptosis [15]. In the present study, we have determined the effects of NF-κB inhibition on the regulation of Bid, Bad, Bax, and Bcl-2 proteins during R. rickettsii infection of endothelial cells. To ascertain the involvement of mitochondria in infection-induced apoptosis, the possibility of changes in the release of Second Mitochondria derived Activator of Caspase (Smac) and cytochrome c was also investigated.

2. Materials and methods

2.1. Cell culture

Cultures of human umbilical vein EC were established as described previously [3]. EC at passage two and confluency of about 80-90% were used for all experiments. Wild type and IkBα-M (dominant negative mutant) expressing T24 bladder carcinoma cells were grown in a humidified CO2 (5%) incubator at 37 °C until about 80% confluence. The cells were cultured in RPMI 1640 medium (Life Technologies Inc., USA) supplemented with l-glutamine and geneticin (400 µg/ml; Life Technologies Inc.) and heat-inactivated fetal bovine serum (10% v/v). About 24 h prior to infection and/or treatment, geneticin was withdrawn from the medium.

2.2. Infection and treatment

A plaque-purified seed stock (1 × 10⁷ to 5 × 10⁷ plaque forming units (pfu)/cm²) of R. rickettsii (Sheila Smith strain) was prepared from infected Vero cells as described earlier [14]. T24 or endothelial cells were infected with viable organisms using ~5 × 10⁴ pfu for every cm² of cell culture area. After 2 h incubation to allow for adhesion and invasion, medium containing extracellular infectious organisms was removed by aspiration. The cell monolayer was washed twice and was retained in fresh culture medium for the remainder of infection. Infection of cells on Thermanox coverslips (Nalge–Nunc International, USA) was performed in parallel to monitor the extent and progress of infection by indirect immunofluorescent staining as described [3]. MG132 (Carbobenzoxy-L-Leucyl-L-Leucyl-L-Leucinal), a specific inhibitor of proteasome activity obtained from Peptide International, USA, was used to inhibit infection-induced NF-κB. In all experiments, EC were incubated with MG132 (25 µM) for 30 min prior to and during R. rickettsii infection [13]. Treatment of cells with Staurosporine or Etoposide (Sigma Chemical Co., USA) was used as a positive control for induction of apoptosis.

2.3. Western blot analyses

Total protein extracts from EC or T24 cells were prepared in lysis buffer (50 mM HEPES, pH 7.0, 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 1 mM DTT, and 1 mM phenylmethanesulfonyl fluoride (PMSF)) supplemented with protease and phosphatase inhibitor cocktails (Sigma). The protein content of cell lysates was determined by Bradford assay (Bio-rad, USA). Equal amounts of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% w/v gels, followed by wet tank transfer to nitrocellulose membrane (Bio-rad). The membranes were probed with primary antibodies specific for human Bid, Bad (Cell Signaling Technology, USA); Bcl-2 (Sigma); and α-tubulin (Accurate Chemical, USA). The protein–antibody complexes were visualized using compatible horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotech-
2.4. Immunofluorescent staining

EC plated on gelatin coated glass cover slips were infected with *R. rickettsii* in the presence or absence of MG132 or treated with apoptosis inducing agents. The cells were then washed with cold PBS and fixed using 4% paraformaldehyde or 3.7% formaldehyde for 20 min at 25 °C. Following permeabilization with 0.5% (v/v) Triton-X 100 for 20 min, cells were incubated with primary antibodies against Smac and Bad (1:50 dilution; Cell Signaling Technology), or cytochrome c (1:200 dilution; BD PharMingen, USA), at 37 °C for 30 min in a humidified chamber. The antibodies were diluted in a blocking solution (2% w/v ovalbumin in PBS). For immunofluorescent detection, rhodamine-conjugated goat anti-rabbit IgG (Calbiochem, USA) or Texas red-conjugated goat anti-mouse IgG (Southern Biotechnology Associates Inc., USA) was used at a dilution of 1:100. The cover slips were mounted on a glass slide using Biomedia gel-mount and Fluoromount G (Electron Microscopy Sciences, USA), and were examined under fluorescent microscope (Nikon Eclipse E-800) attached to a Spot Digital Image System (Diagnostic Instruments Inc., USA). The images were captured using Windows-based Spot software, version 3.04 (Diagnostic Instruments Inc.) and saved as tag image format (tif) files in Adobe Photoshop, version 5.0. For each condition, cells were counted in three to five randomly chosen fields and those with moderate or strong immunostaining were considered to be positive. The percentage of positively stained cells among the total number of cells was then calculated to derive the positivity ratio.

2.5. In situ detection of DNA fragmentation

The extent of apoptosis due to infection or treatment with inducers of cell death was determined using terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP nick-end labeling (TUNEL) assay (Intergen, USA) as described earlier [13,14]. The number of positively stained nuclei was determined by analysis of digitized images of TUNEL-stained cell monolayers. A minimum of 500 cells, located in multiple, randomly selected fields were counted for each condition in three different sets of experiments, and data was calculated as percentage of TUNEL-positive cells of arithmetic mean values.

2.6. Hoechst staining

Apoptotic nuclear changes were also investigated by Hoechst staining [17], wherein EC were fixed with 4% paraformaldehyde and permeabilized using 0.5% Triton X 100 as above. The cells were then stained with 10 μM Hoechst 33258 (Sigma). After washing three times with PBS, the stained cells were mounted onto slides using Fluoromount G to evaluate the extent of apoptosis.

2.7. Densitometric analysis

The blots were scanned in the greyscale mode using an HP ScanJet 6300C scanner at a resolution of 600 dpi. Volume analysis was performed using ImageQuant software, version 3.3 (Molecular Dynamics, USA). For comparison, the normalized band intensity for uninfected/untreated control in each experiment was assigned a value of 1. The *t*-test for independent samples was used to determine the differences between study groups. The level of significance was set at *p* < 0.05.

3. Results

3.1. Endothelial cell apoptosis during *R. rickettsii* infection

Infection with concurrent inhibition of NF-κB via proteasomal inactivation by MG132 induced apoptotic nuclear morphology and TUNEL positivity in EC. Fig. 1A shows the results of TUNEL analysis at 6 h post-infection in the presence and absence of MG132. In comparison to those treated with MG132 or infected with *R. rickettsii*, a significantly higher number of infected cells underwent apoptosis in the presence of MG132. Typically, the extent of apoptosis was similar to that triggered by known inducers of apoptosis, staurosporine and etoposide. Staining with Hoechst 33258 revealed that apoptotic cells in all experimental conditions exhibited marked condensation of chromatin, a characteristic feature of apoptosis (Fig. 1B). Such nuclear changes were either completely absent or evident in <5% of cells subjected to treatment with MG132 or infection with *R. rickettsii* alone. As reported previously [13,14], the percentage of apoptotic EC in cultures infected for 6 h with simultaneous inhibition of NF-κB due to MG132 treatment was 38 ± 4%, a statistically significant increase (*p* < 0.05) over infection alone.

3.2. Cleavage of bid

Bid is one of the major proapoptotic proteins, which is cleaved at carboxyl-terminus by caspase-8 to generate its truncated form, tBid. Since activation of upstream caspase-8 plays a significant role in infection-induced
Fig. 1. NF-κB blockade during *R. rickettsii* infection of endothelial cells (EC) induces apoptotic cellular events. EC were either incubated for 6 h with culture medium alone (C) and medium containing MG132 (MG); or were infected in the absence (RR) and presence of MG132 (RR + MG). Treatment with staurosporine (STS) or etoposide (ETP) was used as positive control for induction of apoptosis. Panels A and B show fragmentation of cellular DNA and changes in nuclear morphology as observed under a fluorescent microscope after TUNEL and Hoechst 33258 staining, respectively. Arrows point towards cells with normal nuclear structure while arrowheads indicate TUNEL positive cells in Panel A and condensed nuclei correlating with induction of apoptosis in Panel B. Original magnification ×20. Bar = 10 μm.

Fig. 2. Levels of pro-apoptotic Bcl-2 family proteins during infection-induced apoptosis. (A) Equal amounts of protein from total cell lysates or cytosolic fractions prepared from uninfected and untreated (Control), MG132-treated (MG), *R. rickettsii*-infected (RR) endothelial cells and those infected in the presence of MG132 (RR + MG) were subjected to Western blotting as described in Section 2. The results of a typical experiment for analysis of cellular levels of native Bid (i) and cytosolic levels of Bax (ii) for different conditions are presented. At the end, blots were probed with α-tubulin to control for loading variations. (B) Western analysis of native Bid in wild-type and IκBα super-repressor mutant expressing T24 bladder carcinoma cells. Cultures were left uninfected/untreated (control), infected with *R. rickettsii* (RR), or incubated with staurosporine (STS) and etoposide (ETP). (C) Indirect immunofluorescent staining of Bad in cultured EC. Representative photomicrographs for experimental conditions identical to those described in the legend for Fig. 1 are shown. Cells exhibiting positive staining (indicated by white arrow heads) were clearly evident when infection was carried out in the presence of MG132 or apoptosis was induced by either staurosporine or etoposide. Similar pattern of staining was observed in three independent experiments.
apoptosis [14], we investigated the processing of Bid by analysis of steady-state levels of its native form. Treatment of EC with staurosporine, which was included as a positive control, resulted in \( \geq 75\% \) depletion of native Bid (not shown). The intracellular level of native Bid was decreased by about 50\% in EC subjected to infection with MG132 treatment, whereas \( R. \) rickettsii or MG132 had no independent effect (Fig. 2A (i)). The status of native Bid during infection of wild-type and IxBz-M expressing T24 cells was also evaluated. These cells were stably transfected to express either empty LXSN-vector (wild-type) or a super-repressor IxBz-mutant with altered phosphorylation sites to inhibit NF-kB activation, respectively (13,14). Electrophoretic mobility shift assays using a consensus oligonucleotide probe showed that \( R. \) rickettsii infection of only wild-type, but not IxBz-M expressing, T24 cells was able to induce DNA-binding activity of NF-kB (Sahni et al., unpublished observation). These cells could, therefore, be used without any pharmacologic manipulation to inhibit \( R. \) rickettsia-induced activation of NF-kB. Under culture conditions used in this study, both types of T24 cells expressed nearly identical basal levels of native Bid. Following infection with \( R. \) rickettsii for 6 h, however, Bid levels dropped an average of 33\% in IxBz-mutant, but not in wild-type cells. On the other hand, exposure to etoposide caused depletion of Bid by \( \geq 65\% \) in both mutant as well as wild type cells, while staurosporine had no effect (Fig. 2B).

### 3.3. Alterations in the levels of Bad, Bax, and Bcl-2

Phosphorylation of Bad (Bcl-2-associated death promoter) at serine residues 112 and 136 is necessary for its binding to the chaperone protein 14-3-3. This represents an important regulatory mechanism to prevent interactions of Bid with Bcl-2 and/or Bcl-xL [18]. Dephosphorylated Bad forms heterodimers with Bcl-2 and/or Bcl-xL causing displacement of Bax, which subsequently translocates to the mitochondria and promotes apoptosis [19]. In the present study, we investigated expression of Bad by indirect immunofluorescent staining. During treatment with MG 132 or infection with \( R. \) rickettsii alone, less than 2\% of cells exhibited positive staining, whereas a significantly higher number of cells (21 \( \pm 3\% \), \( n = 3 \)) displayed positivity during infection with simultaneous MG132 treatment, suggesting intracellular accumulation of Bad (Fig. 2C). This was further confirmed by immunoblotting using an antibody capable of detecting total levels of endogenous protein (not shown). Next, we analyzed the levels of Bax in the cytosolic fraction of cells following infection. The amount of cytosolic Bax in \( R. \) rickettsii-infected and MG 132 treated cells was not significantly different in comparison to that seen in untreated and uninfected control (Fig. 2A (ii)). Staurosporine-treated EC, which were included as positive control exhibited \( \geq 50\% \) reduction in the levels of cytosolic Bax.

The Bcl-2 protein is present in a large variety of cells and is able to prevent apoptosis initiated by many stimuli [15,20]. Results shown in Fig. 3 suggest that the Bcl-2 level of endothelial cells remains unaffected by infection with \( R. \) rickettsii. Although exposure to MG132 alone led to an apparent decrease, the steady state level of this protein was found to be significantly reduced in cells undergoing infection in the presence of MG132.

### 3.4. Release of mitochondrial proteins into cytosol

For several apoptotic stimuli, a primary cascade for the activation of caspase-3 through caspase-9 is mediated by release of cytochrome c from the intermembrane space of mitochondria into the cytosol [20]. Smac (also known as Diablo: direct IAP binding protein with low pI) is another protein released into the cytosol, which promotes apoptosis by binding to inhibitor of apoptosis proteins (IAPs) and preventing them from sequestering caspasps [21,22]. Anti-apoptotic members of Bcl-2 family protect against perturbation of mitochondria, whereas proapoptotic proteins (tBid) trigger the release of apoptosis-inducing molecules such as cytochrome c and Smac. By indirect immunofluorescent staining for in situ detection of Smac and cytochrome c, very low cytoplasmic levels of Smac were seen in the control, infected, and MG132-treated EC. In contrast, infection with MG132 treatment resulted in markedly enhanced intensity of staining in a significantly higher number (25 \( \pm 4\% \), \( n = 3 \)) of EC (Fig. 4A). As shown in Fig. 4B, a similar staining pattern was observed for intracellular localization of cytochrome c as well. To consolidate these findings, we carried out immunoblot analysis using cytosolic extracts from EC subjected to infection in the presence and absence of MG132. Again, accumulation
of increased amounts of Smac and cytochrome c in the cytosolic fraction was evident during R. rickettsii infection of NF-κB inhibited EC, as compared to untreated, R. rickettsii-infected, or MG132-treated EC (Fig. 4C).

4. Discussion

Accumulating evidence indicates that a large number of bacterial pathogens including gram-positive, gram-negative, and mycobacteria mediate apoptosis of host cells, projecting this physiologic form of cell death as a critical determinant of pathogenesis. Apoptotic death may serve to facilitate removal of infected host cells, to control inflammatory and homeostatic processes, or to initiate the host defense mechanisms. Not surprisingly, however, strictly intracellular viruses and bacteria use strategies for inhibiting apoptosis to prolong the existence of host cells as sites for replication and proliferation [9,10]. During in vitro infection with R. rickettsii, activation of nuclear transcription factor NF-κB plays a predominantly anti-apoptotic role, since its inhibition via blockade of proteasome activity or genetic manipulation results in rapid apoptosis of host cells [13]. Recently, we have established that infection-induced NF-κB represses the activation of upstream caspases-8 and -9 and the principal downstream death executioner caspase-3 [14]. The present study illustrates that the fate of host EC during Rickettsia infection also depends on the balance between pro-apoptotic and anti-apoptotic members of the Bcl-2 family of apoptosis-associated proteins, the levels of which are known to control the integrity and function of mitochondria.

The death agonist 'BH-3 domain only' Bid is localized in the cytosolic fraction of cells as an inactive precursor. Decreased levels of native Bid in NF-κB-blocked EC and T24 cells expressing super-repressor mutant of IκBa during R. rickettsii infection indicate the cleavage of full-length protein. The cleavage of Bid by activated caspase-8 and subsequent translocation of resulting truncated form of Bid (tBid) to mitochondria may be responsible for the changes in mitochondrial membrane permeability. The likely effect of altered mitochondrial integrity is the release of cytochrome c, the cytoplasmic accumulation of which is a prerequisite for the activation of intrinsic pathway of apoptosis responsible for the processing of procaspase-9 to its enzymatically active form [14]. The results further suggest that up-regulation of steady-state levels of Bad may also contribute as a trigger for the induction of apoptosis signaling mechanisms, since it can modulate the relative permeability of mitochondria. Bad can be dephosphorylated by a vari-
Bax is an integral organelle protein particularly in mitochondria, but most of the Bax proteins are not associated with membrane and appear to be cytosolic [19]. It has been reported that binding of Bid or Bad to anti-apoptotic Bcl-2 displaces Bax from existing dimers to initiate mitochondrial dysfunction. Bax-induced mitochondrial damage has been shown to occur during infection with Chlamydia, another obligate intracellular bacterium, although host cell apoptosis in this case is not dependent on activation of caspases [23]. Apoptotic death of human umbilical venous EC due to lipopolysaccharide (LPS) treatment also correlates with upregulation of Bax protein expression and reduction in intracellular Bcl-2 levels. Also, attenuation of these effects by a combination of vitamin C and E was protective against cell death, suggesting mediation by oxidative mechanisms [24]. Our data suggest that changes in the subcellular localization leading to mitochondrial translocation of Bax are likely not involved in apoptosis due to R. rickettsii infection. Although rickettsiae are known to possess LPS with only weak endotoxic activity [25], induction of oxidative stress during infection of EC with R. rickettsii plays a significant role in the pathogenesis of infection [1,26,27]. It is, thus, possible that preexisting or secreted rickettsial components and infection-induced stress may also determine signaling mechanisms regulating the equilibrium between pro- and anti-apoptotic proteins.

Bcl-2, a death antagonist, has been implicated as an important factor in the control of apoptosis induced by numerous, often unrelated, physiological and pathological stimuli, and has the capacity to suppress both caspase-dependent and caspase-independent pathways of apoptosis [23]. Bartonella, a unique epacellular bacterium, not only infects and survives within EC, but also induces proliferative effects through increased expression of Bcl-2 [28]. Similarly, over-expression of Bcl-2 in epithelial as well as monocyteic cells is able to inhibit apoptosis due to infection with Chlamydia species [22], and induction of endothelial Bcl-2 expression provides protection against human T-cell lymphotropic virus-type I induced apoptosis to extend cell survival [29]. In vivo apoptosis in rectal tissues during acute shigellosis is associated with compromised levels of Bcl-2 [30], and patients with lyme borreliosis have recently been shown to have decreased serum Bcl-2 levels [31]. Studies have also shown that hypoxia-induced apoptosis of aortic EC involves NF-κB-mediated down-regulation of Bcl-2, translocation of Bax, and up-regulation of p53 [32], and decreased Bcl-2 expression is responsible for adriamycin-induced apoptosis of endothelial cells [33]. In contrast, this study shows that abrogation of NF-κB activation via inhibition of proteasome function during infection of EC reduces the expression levels of Bcl-2 resulting in sensitization of infected cells to apoptosis. To the best of our knowledge, this is the first report of Bcl-2 regulation by NF-κB in host cells during infection with R. rickettsii, an obligate intracellular pathogen. Although a number of apoptosis-inducing stimuli also cause increased DNA binding activity of NF-κB, there are reports that NF-κB activation can both promote as well as inhibit apoptotic response depending on the cell type and death stimulator [8]. In this context, it is important to note that proteosome inhibitors lactacystin, MG132, and ALLN prevent TNF-α and staurosporine-induced apoptosis in endothelial and HeLa cells by blocking degradation of Bcl-2 [34]. Furthermore, inhibition of proteosome activity confers protection against apoptosis elicited by TNF-α and IL-1β and attenuates LPS-induced apoptosis by maintaining the levels of anti-apoptotic proteins FLIP and Mcl-1 [35].

It has also been proposed that NF-κB induces multiple anti-apoptotic factors to block apoptosis at a variety of steps along the caspase cascade. The members of IAP family of proteins are endogenous repressors of the terminal caspase cascade and the genes for cIAP-1 and cIAP-2 are controlled by NF-κB function [36]. Recent studies have shown that pathogenic bacteria Chlamydia pneumoniae and Helicobacter pylori prevent host cell apoptosis through NF-κB dependent transactivation of cIAP2 [37,38]. Our results demonstrate the release of mitochondrial apoptogenic factors Smac and cytochrome c into the cytosol. While cytochrome c may participate in the pathway leading to activation of caspase-9 [14], whether or not Smac also promotes apoptosis by reversing IAP-mediated inhibition of apoptotic cell death requires further investigation.

In summary, activation of NF-κB during R. rickettsii infection is crucial for the maintenance of mitochondrial integrity of host cells and determines the cell fate by regulating the intracellular levels of pro- and anti-apoptotic proteins of Bcl-2 family. NF-κB-mediated inhibition of apoptosis and enhanced host cell survival early during the course of infection likely facilitates the growth and multiplication of intracellular rickettsiae and might be critical to the understanding of subsequent signaling mechanisms involved in the determination of host cell responses and pathophysiology of human rickettsial diseases.

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