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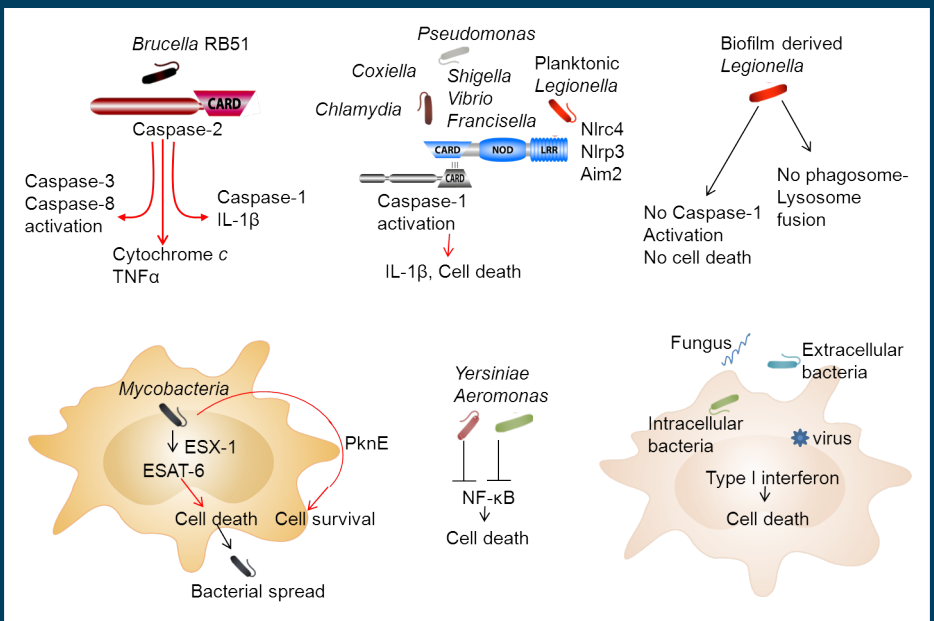
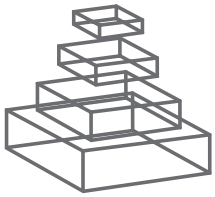


MICROBIAL MODULATION OF HOST APOPTOSIS AND PYROPTOSIS

Topic Editors
Yongqun He and Amal O. Amer

frontiers in
CELLULAR AND INFECTION MICROBIOLOGY



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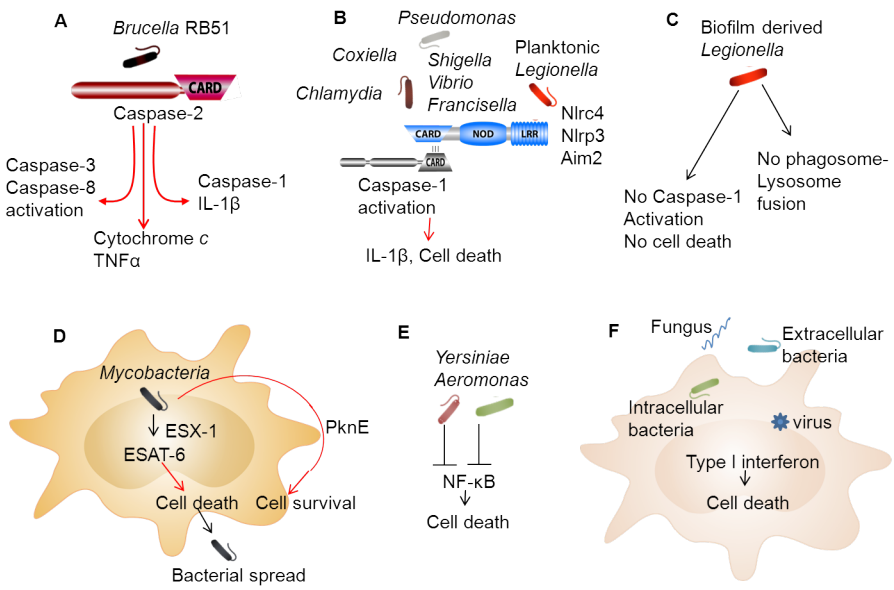
MICROBIAL MODULATION OF HOST APOPTOSIS AND PYROPTOSIS

Topic Editors:
Yongqun He, University of Michigan Medical School, USA
Amal O. Amer, The Ohio State University, USA



Panels in the figure summarize major findings and reviews in the Issue describing "Microbial Modulation of Host Apoptosis and Pyroptosis":

A) Bronner et al.,
B) Cunha and Zamboni; and Casson and Shin,
C) Abu Khweek et al,
D) Aguilo et al; Parandhaman and Narayanan,
E) Rosenzweig and Chopra,
F) Malireddi and Kanneganti.

Due to space limitation, this figure does not include all the details from the above listed papers, and it does not include many findings and reviews reported in other papers authored by Pei et al, Marriott, and Demehri et al.

This image was prepared by the editors Yongqun He and Amal O. Amer

Infectious disease is the result of an interactive relationship between a microbial pathogen and its host. In this interaction both the host and the pathogen attempt to manipulate each other using a complex network to maximize their respective survival probabilities. Programmed host cell death is a direct outcome of host-pathogen interaction and may benefit host or pathogen depending on microbial pathogenesis. Apoptosis and pyroptosis are two common programmed cell death types induced by various microbial infections. Apoptosis is non-inflammatory programmed cell death and can be triggered through intrinsic or extrinsic pathways and with or without the contribution of mitochondria. Pyroptosis is an inflammatory cell death and is typically triggered by caspase-1 after its activation by various inflammasomes. However, some non-canonical caspase-1-independent proinflammatory cell death phenomena have been reported.

Microbial pathogens are able to modulate host apoptosis and pyroptosis through different triggers and pathways. The promotion and inhibition of host apoptosis and pyroptosis vary and depend on the microbe types, virulence, and phenotypes. For example, virulent pathogens and attenuated vaccine strains may use different pathways to modulate host cell death. Specific microbial genes may be responsible for the modulation of host cell death. Different host cells, including macrophages, dendritic cells, and T cells, can undergo apoptosis and pyroptosis after microbial infections. The pathways of host apoptosis and pyroptosis induced by different microbes may also differ. Different methods can be used to study the interaction between microbes and host cell death system.

The articles included in this E-book report the cutting edge findings in the areas of microbial modulation of host apoptosis, pyroptosis and inflammasome.

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EDITORIAL
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Microbial modulation of host apoptosis and pyroptosis

Yongqun He[1]* and Amal O. Amer[2]*

[1] *Unit for Laboratory Animal Medicine, Department of Microbiology and Immunology, Center for Computational Medicine and Bioinformatics, Comprehensive Cancer Center, University of Michigan Medical School, Ann Arbor, MI, USA*

[2] *Department of Microbial Infection and Immunity, Department of Internal Medicine, Center for Microbial Interface Biology, Ohio State University, Columbus, OH, USA*

**Correspondence: yongqunh@med.umich.edu; amal.amer@osumc.edu*

Edited and reviewed by:
Yousef Abu Kwaik, University of Louisville School of Medicine, USA

Keywords: apoptosis, pyroptosis, microbial infection, caspase, inflammasome, *Brucella, Legionella pneumophila, Mycobacterium tuberculosis*

Apoptosis and pyroptosis are two common programmed cell death types induced by various microbial infections. Apoptosis is non-inflammatory programmed cell death and can be triggered through intrinsic or extrinsic pathways and with or without the contribution of mitochondria. Pyroptosis is an inflammatory cell death and is typically triggered by caspase-1 after its activation by various inflammasomes. Non-canonical caspase-11-mediated pyroptosis has been identified. A NLRP3 (cryopyrin)-dependent but casepase-1-independent proinflammatory necrosis called pyronecrosis (Willingham et al., 2007), and a caspase-2-dependent but caspase-1-independent proinflammatory cell death (Chen et al., 2011) have also been reported. Microbial pathogens are able to modulate host apoptosis, pyroptosis, and inflammasomes through different triggers and pathways. The promotion and inhibition of host cell death vary and depend on the microbe types, virulence, and phenotypes.

In this Special Research Topics issue, recent advances in microbial modulation of host programmed cell death, with a special focus on apoptosis and pyroptosis, were captured in a total of 11 research and review articles. The special issue includes three Original Research Articles, five Review Articles, and three Mini Review Articles. Two articles were published for each of the three pathogens: *Brucella* spp. (Bronner et al., 2013; Pei et al., 2014), *Legionella pneumophila* (Abu Khweek et al., 2013; Casson and Shin, 2013), and *Mycobacterium tuberculosis* (Aguilo et al., 2013; Parandhaman and Narayanan, 2014). Modulation of host immune defenses by *Aeromonas* and *Yersinia* species is introduced in Rosenzweig and Chopra (2013). While (Cunha and Zamboni, 2013) summarizes the subversion of inflammasome activation and pyroptosis by eight pathogenic bacteria, (Malireddi and Kanneganti, 2013) introduces the role of type I interferons in inflammasome activation and cell death induced by microbial infections. The apoptosis-associated uncoupling of bone formation and resorption in osteomyelitis is reviewed in Marriott (2013). The intestinal epithelial cell apoptosis in the setting of altered microbiota with enteral nutrient deprivation is reviewed in Demehri et al. (2013).

Brucella causes brucellosis, one of the most common zoonotic diseases in the world in humans and a variety of animal species. The *Brucella*-macrophage interaction is critical to *Brucella* virulence. Virulent smooth *Brucella* strains inhibit macrophage cell death. This is an important strategy employed by several intracellular pathogens to maintain the survival of the eukaryotic cell that represents its niche. Many attenuated rough *Brucella* strains induce macrophage cell death. The Original Research Article (Pei et al., 2014) demonstrates that after smooth *Brucella* invade and replicate inside host macrophages, some smooth bacteria can automatically dissociate into rough mutants that can then cause the macrophage cytotoxicity. The cytotoxicity of infected macrophages is critical for *Brucella* egress and dissemination. The macrophage necrotic cell death also induces inflammatory responses and recruits more macrophages to the infection site.

The rough attenuated *B. abortus* vaccine strain RB51 was found to induce caspase-2-mediated but caspase-1-independent apoptotic and necrotic cell death (Chen and He, 2009). Original Research Article (Bronner et al., 2013) from this special issue further illustrates this mechanism. In RB51-infected macrophages, caspase-2 regulates many genes and several cell death pathways: (i) proapoptotic caspases-3 and -8 activation; (ii) mitochondrial cytochrome *c* release and TNFα production; (iii) caspase-1 and IL-1β production driven by caspase-2-mediated mitochondrial dysfunction. Unlike *S. typhimurium*-induced caspase-1-mediated pyroptosis, RB51-induced pore formation does not contribute to RB51-induced proinflammatory cell death. Therefore, caspase-2 appears to act as a "master regulator" that regulates various genes and pathways and induces a hybrid cell death with features of both apoptosis and pyroptosis. The caspase-2-mediated cell death was also conserved in macrophages treated with cellular stress inducers including etoposide, naphthalene, or anti-Fas (Bronner et al., 2013).

Interesting study by Abu Khweek compared the innate immune response of planktonic and biofilm-derived *L. pneumophila*. *L. pneumophila*, the causative agent of Legionnaire's disease, replicates inside macrophages to establish infection. In the Original Research Article (Abu Khweek et al., 2013), the authors demonstrated that compared to planktonic *L. pneumophila*, biofilm-derived *L. pneumophila* (i) replicate more in murine macrophages, (ii) lacks flagellin expression, (iii) do not activate caspase-1 or -7, (iv) trigger less cell death, and (v) are mostly enclosed in vacuoles that do not fuse with lysosomes. Therefore, biofilm-derived *L. pneumophila* which closely reproduces the natural mode of the bacterial infection in

human is able to evade the innate immune response in murine macrophages.

The canonical pyroptosis is triggered by the inflammasome, a multi-protein complex assembled in the cytosol to activate caspase-1. A non-canonical inflammasome activates caspase-11 and also leads to pro-inflammatory cell death (Kayagaki et al., 2011). Independently of the inflammasome, caspase-11 promotes the fusion of the *L. pneumophila*-containing vacuole with the lysosome (Akhter et al., 2012). The diverse roles of caspase-11 and routes of activation are described in the mini-review (Casson and Shin, 2013) *L. pneumophila* triggers canonical caspase-1-dependent inflammasome activation through one of two pathways: (i) Type 4 secretion system (T4SS)-regulated flagellin, NAIP5, and NLRC4; (ii) host ASC and NLRP3, and a *L. pneumophila*-derived unknown signal. Molecular details on caspase-11 activation in *L. pneumophila*-infected macrophages remain unclear. Interestingly, the inflammasome pathway appears to cross talk with and autophagy, another immune response (Casson and Shin, 2013).

Mycobacterium tuberculosis, another professional intracellular pathogen in this issue, also manipulates cell death. Conflicting results have been reported to support inhibition or induction of apoptosis as a virulence mechanism employed by mycobacteria. This elegant review article (Aguilo et al., 2013), summarizes the evidences showing that ESX-1-induced apoptosis during mycobacterial infection contributes to bacterial virulence. The ESX-1 secretion system regulates the exportation of ESAT-6, a major virulence factor whose secretion is essential for *M. tuberculosis*-induced apoptosis. ESAT-6 appears to trigger the mitochondrial apoptotic pathway through ER-stress activation. ESX-1 dependent apoptosis supports cell-to-cell colonization and bacterial spread. Highly apoptogenic *M. tuberculosis nuoG* mutant showed higher cell-to-cell spread and increased antigen cross-presentation favoring the host. It is evident that apoptosis may benefit the host or mycobacterial pathogen according to different experimental conditions (Aguilo et al., 2013).

The well rounded report (Parandhaman and Narayanan, 2014) summarizes more than 10 different cell death modalities involving *M. tuberculosis*. The paper also reviews how PknE, one of 11 mycobacterial serine/threonine protein kinases, inhibits apoptosis and benefits the bacterial survival.

This issue also comprises a paper (Rosenzweig and Chopra, 2013) that describes toxins secreted by pathogenic *Yersiniae* and most *Aeromonas* species that modulate infected host cell death. The T3SS effector *Yersinia* outer membrane protein J (YopJ) is an acetyltransferase that disrupts MAPK and NF-κB signaling pathways to favor apoptosis and pyroptosis induction. Similarly, *Aeromonas* hydrophila AexU protein induces apoptosis by targeting NF-κB signaling. Additionally *Aeromonas* includes T2- and T6SS effectors that further modulate host immune responses to promote bacterial virulence (Rosenzweig and Chopra, 2013).

The nice paper by Zamboni's group (Cunha and Zamboni, 2013) first reviews different types of inflammasomes that activate caspase-1 (via NLRC4, AIM2, or NLRP3) or caspase-11 and then lead to pyroptosis. These host inflammasomes and pyroptosis pathways can be targeted by microbial factors released via T3SS/T4SS or other mechanisms in different pathogens. This paper reviews the mechanisms employed by eight bacterial species to evade inflammasome activation and pyroptosis induction. These bacteria include *Chlamydia trachomatis*, *Coxiella burnetii*, *Francisella tularensis*, *Legionella pneumophila, Pseudomonas aeruginosa*, *Shigella flexneri*, *Vibrio parahaemolyticus*, and *Yersinia* spp. (Cunha and Zamboni, 2013).

On the host side, the review by Kanneganti's group (Malireddi and Kanneganti, 2013) in this issue introduces the role of type I interferons in inflammasome activation and cell death during infections of five intracellular, four extracellular bacteria, viruses, and fungi.

How cell death can lead to human disease conditions is well described by the review paper (Marriott, 2013) that focuses on osteomyelitis, a severe infection of bone caused by *S. aureus* and *Salmonella* spp. Osteomyelitis is often associated with bone resorption and progressive inflammatory destruction. In the paper Marriott describes the mechanisms underlying the destruction of bone tissue, with a focus on the apoptosis-associated uncoupling of bone formation and resorption in osteomyelitis. Different microbial virulence factors, host response genes and pathways, and their interactions during the formation of osteomyelitis are introduced.

It has been well established that pathogens modulate apoptosis and pyroptosis, but what about microbiota? The paper (Demehri et al., 2013) in this issue introduces a shift in our understanding of intestinal microbiota such as Gram-negative Proteobacteria after enteral nutrient deprivation. The altered microbiota setting leads to increased intestinal proinflammatory cytokines, decreased epithelial cell proliferation, and increased epithelial cell apoptosis. These eventually cause the loss of epithelial barrier function.

The cover image of this E-book summarizes the key findings reported in the original research, review, or mini-review articles included in this e-book.

As briefly introduced above, this special Research Topic issue covers a broad range of cases and reviews demonstrating the modulation of host cell death pathways by different bacterial pathogens and resident microbiota. While huge progress has been made in the past decades, many challenging questions still remain.

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ORIGINAL RESEARCH ARTICLE
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Biofilm-derived *Legionella pneumophila* evades the innate immune response in macrophages

Arwa Abu Khweek[1], Natalia S. Fernández Dávila[1], Kyle Caution[1], Anwari Akhter[1], Basant A. Abdulrahman[1,2], Mia Tazi[1], Hoda Hassan[1], Laura A. Novotny[3], Lauren O. Bakaletz[1,3] and Amal O. Amer[1]*

[1] Department of Microbial Infection and Immunity, Center for Microbial Interface Biology, College of Medicine, The Ohio State University, Columbus, OH, USA
[2] Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, Helwan University, Cairo, Egypt
[3] Center for Microbial Pathogenesis, Nationwide Children's Hospital, Columbus, OH, USA

Edited by:
Yongqun Oliver "He", University of Michigan School of Medicine, USA

Reviewed by:
Sunny Shin, University of Pennsylvania, USA
Cyril Guyard, Public Health Ontario, Canada

***Correspondence:**
Amal O. Amer, Department of Microbial Infection and Immunity, Center for Microbial Interface Biology, Dorothy Heart and Lung Research Institute, Ohio State University, Biological Research Tower, 460 W 12th Ave., Room 706, Columbus, OH 43210, USA.
e-mail: amal.amer@osumc.edu

Legionella pneumophila, the causative agent of Legionnaire's disease, replicates in human alveolar macrophages to establish infection. There is no human-to-human transmission and the main source of infection is *L. pneumophila* biofilms established in air conditioners, water fountains, and hospital equipments. The biofilm structure provides protection to the organism from disinfectants and antibacterial agents. *L. pneumophila* infection in humans is characterized by a subtle initial immune response, giving time for the organism to establish infection before the patient succumbs to pneumonia. Planktonic *L. pneumophila* elicits a strong immune response in murine, but not in human macrophages enabling control of the infection. Interactions between planktonic *L. pneumophila* and murine or human macrophages have been studied for years, yet the interface between biofilm-derived *L. pneumophila* and macrophages has not been explored. Here, we demonstrate that biofilm-derived *L. pneumophila* replicates significantly more in murine macrophages than planktonic bacteria. In contrast to planktonic *L. pneumophila*, biofilm-derived *L. pneumophila* lacks flagellin expression, do not activate caspase-1 or -7 and trigger less cell death. In addition, while planktonic *L. pneumophila* is promptly delivered to lysosomes for degradation, most biofilm-derived bacteria were enclosed in a vacuole that did not fuse with lysosomes in murine macrophages. This study advances our understanding of the innate immune response to biofilm-derived *L. pneumophila* and closely reproduces the natural mode of infection in human.

Keywords: biofilm, inflammasome, flagellin, caspase-1, *Legionella pneumophila*, innate immunity

INTRODUCTION

Legionella pneumophila (*L. pneumophila*) is a Gram negative facultative bacterium with fastidious growth requirements. Although *Legionella* exists as free-living planktonic forms in the environment, they are more commonly found as intracellular parasites of protozoans such as *Acanthamoeba* spp., *Hartmannella* spp., and *Tetrahymena* spp. (Atlas, 1999; Brown and Barker, 1999) and as inhabitants of mixed-community biofilms (Rogers et al., 1994; Lau and Ashbolt, 2009). Replication of *L. pneumophila* within amoeba is utilized as a survival strategy to overcome the low-nutrient environment and increases the resistance to disinfectant (Lau and Ashbolt, 2009). This opportunistic pathogen most often thrives in bacterial communities encased in extracellular polymeric matrix known as biofilm (Costerton et al., 1978; Donlan et al., 2005). Biofilms have been recognized as one of the most important factors of survival and proliferation of *L. pneumophila* in warm, humid environments like showers, air conditioners, and spa baths (Fraser et al., 1979; Fliermans et al., 1981; Sethi and Brandis, 1983; Spitalny et al., 1984; Abu Kwaik et al., 1993; Lettinga et al., 2002). These communities have been identified as a causative source of infection in susceptible hosts who inhale aerosols of contaminated water containing *L. pneumophila*. In the human lung environment, *L. pneumophila* replicates exponentially within alveolar macrophages prior to lysing the host cell and invading other macrophages causing a type of walking pneumonia called Legionnaire's disease or Legionellosis (Horwitz and Silverstein, 1980; Harb and Abu Kwaik, 2000). Legionellosis has two clinically distinct forms: Legionnaires' disease, a severe type of infection, which includes pneumonia and Pontiac fever, a milder self-limiting illness (Lau and Ashbolt, 2009). Approximately 20,000 cases of Legionnaire's disease are reported yearly in the US with no person-to-person transmission (Marston et al., 1997). Thus, using biofilm-derived *L. pneumophila* to study the innate immune response to infection recapitulates natural mode of infection in human.

The murine innate immune response to planktonic *L. pneumophila* has been studied extensively. Nlrc4 and Naip5 detect flagellin monomers in the host cytosol in a process that is dependent upon a functional bacterial type IV secretion system. The sensing of contaminating molecules of flagellin promotes the formation of a multi-protein complex called the inflammasome. Within the inflammasome, caspase-7 is activated downstream

of caspase-1 which results in bacterial restriction via fusion of *L. pneumophila*-containing vacuoles with lysosomes (Coers et al., 2000; Akhter et al., 2009; Amer, 2010). Conversely, human monocytes do not activate this response upon *L. pneumophila* infection and phagosomes containing *L. pneumophila* evade fusion with the lysosome allowing bacterial replication (Roy, 2002; Isberg et al., 2009).

Here we demonstrate that biofilm-derived *L. pneumophila* replicates significantly more than planktonic *L. pneumophila* in murine macrophages due to diminished flagellin expression. Biofilm-derived *L. pneumophila* does not activate caspase-1 or caspase-7, evades fusion with lysosomes, and promotes less cell death. Taken together, our study characterizes the innate immune response to biofilm-derived *L. pneumophila* in murine and human macrophages.

METHODS

BACTERIAL STRAINS

L. pneumophila strain JR32 a wild-type (WT) strain and *flaA* mutant is deficient in flagellin, were kindly provided by Dr. Howard Shuman, University of Chicago. The *dotA* mutant, a JR32-derived strain defective in the Dot/Icm Type IV secretion system was kindly provided by Dr. Craig Roy, Yale School of Medicine. *L. pneumophila* expressing green fluorescent protein (GFP) was used for microscopy.

L. pneumophila GROWTH

L. pneumophila strains were grown on buffered charcoal yeast extract (BCYE) plates at 37°C. Three days later, the bacteria were resuspended in 5 ml of *L. pneumophila* medium (BYE) with additives (ferric nitrate, L-cysteine, thymidine) and vortexed 100× at high speed. For biofilm formation, a bacterial suspension with an optical density (OD) at 600 nm of 3.5 was diluted to 1:2500 in supplemented broth and 200 μl of this suspension was inoculated into each well of an 8-well chamber slide (Thermo Scientific Lab Tek chambered coverglass with cover #155411 and/or #177402). Slides were incubated at 37°C, 5% CO_2 incubator with humidified atmosphere without shaking. Biofilms were fed by delivery of fresh medium to one side of the chamber slide well every 24 h for 6 days using 100 μl of *L. pneumophila* medium. The OD values were 3.4–3.6 and 3.8–4 for the JR32 and *dotA* mutant, respectively. *L. pneumophila* was grown for planktonic culture as previously described (Amer et al., 2006; Akhter et al., 2009, 2012).

MACROPHAGE INFECTION

C57BL/6 mice were purchased from Jackson laboratory. Bone marrow derived macrophages (BMDMs) were prepared from the femurs of 6 to 8-week-old mice as previously described (Akhter et al., 2009, 2012). Isolation and preparation of the human monocyte-derived macrophages (hMDMs) from peripheral blood was carried out as previously described (Santic et al., 2005; Al-Khodor et al., 2008). Planktonic infections were used from post-exponential cultures as previously described (Amer et al., 2006; Akhter et al., 2009). Infection from biofilm derived *L. pneumophila* was carried as follows. Briefly, on day seven, the media was aspirated and transferred to a 50 mL tube. Biofilms were scraped from the chamber slide wells. Chambers were washed 2× with 200 μl of fresh LP medium with the previously mentioned additives and drained into the 50 ml tube. The collected biofilms were vortexed 100× and the OD at 600 nm of the collected suspension was used to calculate the desired multiplicity of infection. Equivalent inocula of planktonic bacteria were used for infection (MOIs).

L. pneumophila is an intracellular pathogen that replicates only within eukaryotic cells since the culture media do not contain required nutrients such as iron and cysteine.

CONFOCAL LASER SCANNING MICROSCOPE VISUALIZATION

On day seven, biofilms were washed gently with 200 μl of sterile saline (0.9% sodium chloride) (Hospira 0409-4888-10), and stained using the Live/Dead BacLight Bacterial Viability Kit (Invitrogen #7007) for 15 min at room temperature protected from the light. Wells were washed 2× with sterile saline and 200 μl of 10% formalin was added for 24 h to fix the biofilm and stored at room temperature protected from the light with before it was visualized using inverted confocal Zeiss LSM 510 META microscope with a 63× water objective. Z-stacks were captured every 1 μm.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

L. pneumophila JR32 and *dotA* strains from post-exponential planktonic and biofilm cultures were used to infect murine macrophages for 24 h. Supernatants were collected and centrifuged at 1200 rpm for 10 min and stored at −80°C as previously described (Abdulrahman et al., 2011). The plates were coated with primary antibody for IL-1β and ELISA were performed according to the manufacture specifications (R&D) (Abdulrahman et al., 2011).

WESTERN BLOT

Macrophage lysates were prepared following infection with either planktonic or biofilm JR32 or *dotA* mutant and immunoblotted with caspase-1, caspase-7, or β-actin antibodies (caspase-1, 1:3000; caspase-7, 1:300). Blots were washed and the corresponding secondary antibody was added. For flagellin detection by western blot, one OD of bacterial culture was pelleted and resuspended in SDS-containing sample buffer from planktonic or biofilm grown bacteria. Eighteen μl were loaded on 12% SDS-PAGE gel. The blot was probed with flagellin antibody (1:100) kindly provided by Dr. Howard Shuman, University of Chicago, followed by the secondary antibody, donkey anti-rabbit (1:5000). Blots were developed after adding ECL Western Blotting Detection Reagent (GE Healthcare Amersham).

MACROPHAGE CYTOTOXICITY ASSAY

Percentage of macrophage necrosis was determined by measuring the release of host cell cytoplasmic lactate dehydrogenase (LDH) using the cytotoxicity detection kit (Roche Applied Science) to the specification of the manufacturer. BMDMs were infected with JR32 or the *dotA* mutant from either planktonic or biofilm culture for 4 or 24 h at an MOI of 0.5. Supernatants were collected and LDH release was calculated as previously described (Abdulrahman et al., 2011).

L. pneumophila COLOCALIZATION WITH LYSOTRACKER

L. pneumophila JR32 from post-exponential planktonic or biofilm cultures were used to infect macrophages plated in 24-well plates containing sterilized coverslips. Lysotracker red (1:500) was added before fixation and 4′, 6-diamidino-2-phenylindole (DAPI) was added after fixation. Coverslips were mounted on slides and viewed using the Olympus Flow View FV10i CLSM. Three hundred bacteria were counted from 2 coverslips for each condition.

CONTACT-DEPENDENT HEMOLYSIS

Sheep RBCs (sRBCs) were diluted in RPMI, and washed 3× by centrifugation for 10 min at 2000× g until the supernatant did not show any signs of hemolysis; the cells were counted using a hemo-cytometer chamber. Reactions were set up in a final volume of 1 ml with a final concentration of 1×10^7 sRBCs/ml. The sRBCs were incubated with the planktonic or biofilm bacteria at an MOI of 20 and RBC lysis was determined as previously described (Kirby et al., 1998; Alli et al., 2000).

SCANNING ELECTRON MICROSCOPY (SEM)

L. pneumophila strains were grown on 12-well plate coverslips for seven days. On the seventh day, the medium was aspirated and the coverslips were washed with 1× DPBS. Coverslips were fixed with 2.5% gluteraldehyde in 0.1 M phosphate buffer pH 7.4, processed and viewed by SEM.

STATISTICAL ANALYSIS

Experiments were performed 2–3 independent times each in triplicate or quadruplicate and yielded similar results. Comparisons of groups for statistical significance were performed using Student's tow tailed *t*-test. *P*-values ≤ was considered significant.

RESULTS

THE Dot/Icm TYPE IV SECRETION SYSTEM PROMOTES ROBUST *L. pneumophila* BIOFILM FORMATION

To reproduce biofilm formation *in vitro*, WT *L. pneumophila* (JR32) and *dotA* mutant were grown for 7 days at 37°C on 5% CO_2 in 8-well chambered coverslips and fed with *L. pneumophila* BYE media every 24 h. On the seventh day, biofilms were stained with Live/Dead stain then observed by confocal microscopy. *L. pneumophila* JR32 produced a thick biofilm with a maximum height of ∼120 μm and exhibited filamentous structures.

The *dotA* mutant, which lacks a functional type IV secretion system, produced a thinner and less filamentous biofilm (**Figure 1A**) that was 60 μm high (**Figure 1B**). The homogenous green color, with lack of red coloring indicates that the constituent bacteria are viable. This result suggests that a functional Dot/Icm type IV secretion system promotes biofilm formation.

To confirm that *L. pneumophila* is able to form biofilm *in vitro* and this process is dependent upon type IV secretion system, we

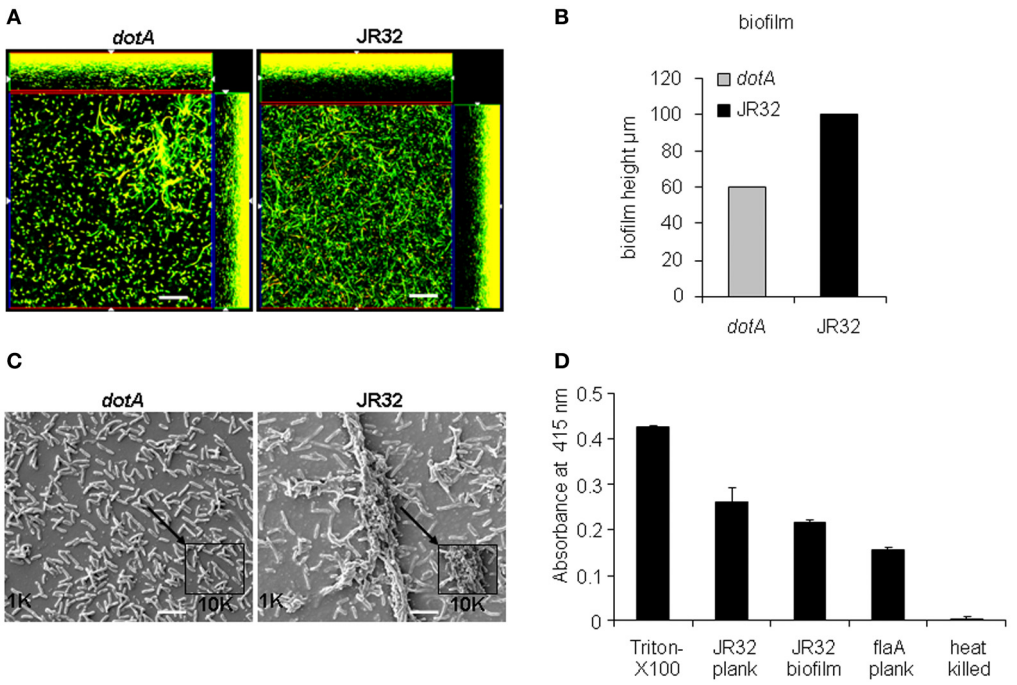

FIGURE 1 | Dot/Icm type IV secretion promotes robust *L. pneumophila* biofilm formation. (A) Representative images showing the Live/Dead staining of WT *L. pneumophila* biofilm (top) or the type IV secretion mutant (*dotA*). Images were captured using inverted confocal Zeiss LSM 510 META microscope with a 63× water objective. Z-stacks were captured every 1 μm. Red stain indicates dead bacteria while green indicates live bacteria, scale = 10 μm. **(B)** Representation of biofilm height in μm. **(C)** Scanning electron microscopy (SEM) of JR32 and *dotA* mutant. Larger images were captured with the 1000× objective lens while smaller images were magnified 10,000×, scale = 10 μm. **(D)** Pore-forming activity of *L. pneumophila* as determined by contact-dependent hemolysis of sheep red blood cells (sRBC) and measured at A_{415} nm. Data are presented as means ± SD of two independent experiments each performed in quadruplicates.

examined biofilm formation using scanning electron microscopy (SEM). Our data showed that JR32 strain formed a robust biofilm with characteristic towers whereas the *dotA* mutant failed to do so (**Figure 1C**). These data confirm that robust biofilm formation requires type IV secretion system.

The pore forming activity of *L. pneumophila* has been shown to contribute to macrophage cytotoxicity and requires a functional type IV secretion system (Kirby et al., 1998; Alli et al., 2000). To examine whether biofilm-derived *L. pneumophila* exhibit pore-forming activity, contact-dependent hemolysis of sheep red blood cells (RBCs) was performed (Kirby et al., 1998). Triton-X100 and heat-killed bacteria were used as positive and negative controls, respectively. The *flaA* mutant lacking flagellin and expresses a functional type IV secretion system was also examined (**Figure 1D**). Biofilm-derived and planktonic *L. pneumophila* and *flaA* mutant were capable of lysing the RBCs, suggesting that biofilm-derived *L. pneumophila* exhibit a functional type IV secretion system.

BIOFILM-DERIVED *L. pneumophila* REPLICATES SIGNIFICANTLY MORE INTRACELLULARLY AND INDUCES LESS MURINE MACROPHAGE DEATH THAN THE PLANKTONIC BACTERIA

WT murine macrophages are restrictive to planktonic *L. pneumophila* replication. However, the murine macrophages response to biofilm-derived *L. pneumophila* is not known. Therefore, we examined the intracellular replication of biofilm-derived *L. pneumophila*. In contrast to planktonic bacteria, the biofilm-derived bacteria replicated significantly as indicated by the colony forming units (CFUs) over time (48–96 h) (**Figure 2A**). Macrophages phagocytozed similar numbers of biofilm-derived and planktonic *L. pneumophila* as demonstrated by the 1 h CFU counts.

Premature cell death restricts *L. pneumophila* replication within murine macrophages (Akhter et al., 2009, 2012). Macrophage death can be detected by measuring the enzymatic activity of lactate dehydrogensae (LDH) released from dead cells using a LDH cytotoxicity assay. Our data demonstrated

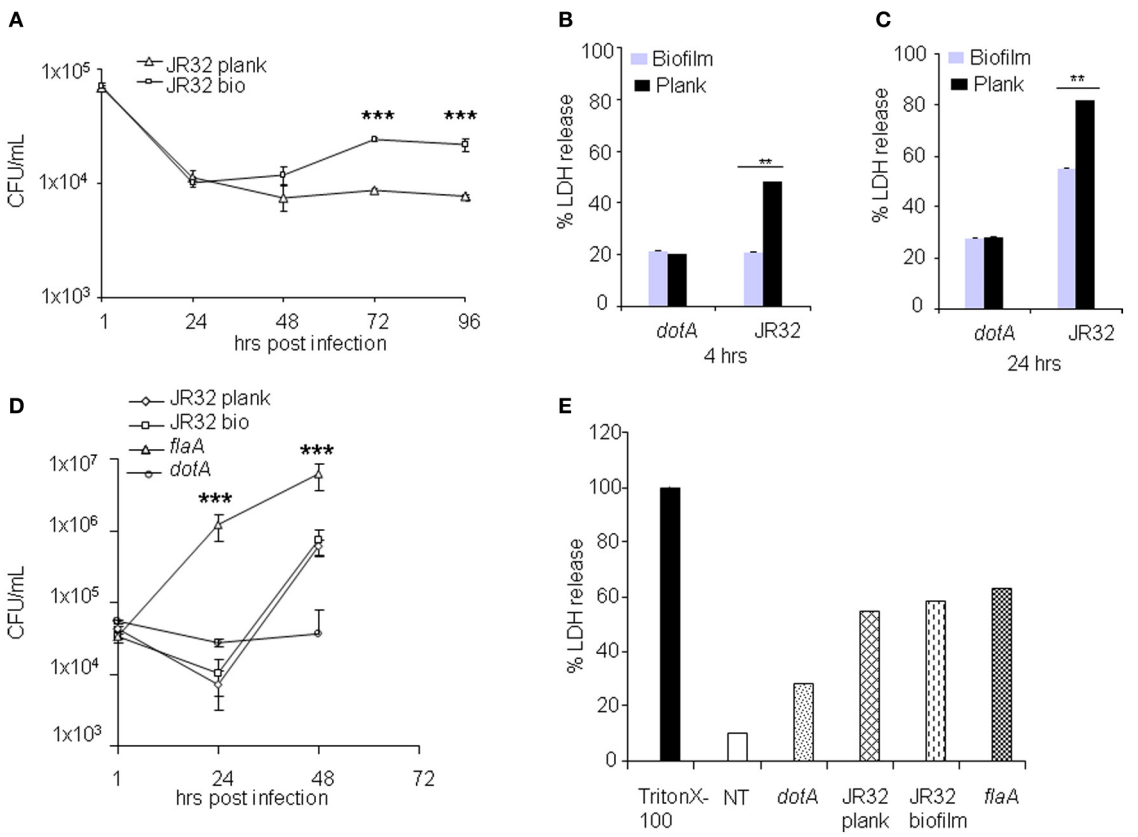

FIGURE 2 | Biofilm-derived *L. pneumophila* replicates significantly more and induces less murine macrophages death than the planktonic bacteria. **(A)** BMDMs were infected with planktonic or biofilm-derived *L. pneumophila* at an MOI of 0.5. CFUs were scored at 1, 24, 48, 72, and 96 h. Data are presented as mean ± SD of two independent experiments each performed in duplicates. Asterisks indicate significant differences (***$P < 0.001$). BMDMs were not infected (NT) or infected with *L. pneumophila* JR32 (planktonic or biofilm) or the *dotA* mutant at an MOI of 0.5 for **(B)** 4 or **(C)** 24 h. The fold change in LDH release was measured from the overall population of macrophages. Data are presented as means ± SD of two independent experiments each performed in quadruplicates. Asterisks indicate significant differences (**$P < 0.01$). **(D)** The hMDMs were infected with *L. pneumophila* strain JR32 (planktonic or biofilm). CFUs were quantified at 1, 24, and 48 h post-infection. Data are representative as means ± SD of quintuplicate samples. **(E)** The hMDMs were not infected (NT) or infected with *L. pneumophila* JR32 (planktonic or biofilm), *dotA* or the *flaA* mutant at an MOI of 0.5 for 4 or 24 h. The fold change in LDH release was measured from the overall population of macrophages. Data are representative of mean ± SD of quadruplicate samples.

that macrophages infected with biofilm-derived *L. pneumophila* produced less LDH at 4 and 24 h post-infection when compared to those infected with planktonic bacteria (**Figures 2B**,**C**). Biofilm-derived and planktonic *dotA* mutants led to the same extent of macrophage death. These data suggest that biofilm-derived *L. pneumophila* induced less cell death in murine macrophages than did planktonic-derived *L. pneumophila*.

HUMAN MACROPHAGES ARE PERMISSIVE TO BIOFILM-DERIVED *L. pneumophila* AS THEY ARE TO PLANKTONIC CULTURES

In contrast to murine macrophages, human monocytes-derived macrophages (hMDMs) are permissive to planktonic *L. pneumophila* at least in part due to diminished caspase-1 and -7 activation (Horwitz, 1983; Abdelaziz et al., 2011a,b). Replication within macrophages is essential for establishing Legionnaire's pneumonia. Thus, we evaluated the intracellular growth of biofilm-derived *L. pneumophila* in hMDMs. The biofilm-derived *L. pneumophila* replicated similar to planktonic *L. pneumophila* in hMDMs (**Figure 2D**). As expected the *dotA* mutant did not replicate whereas *L. pneumophila* mutant lacking flagellin replicated the most (**Figure 2D**). This difference was not due to differential uptake since phagocytosis of all tested strains was similar as shown by the 1 h CFUs (**Figure 2D**).

Furthermore, we tested macrophage death by measuring percentage of LDH released after 24 h of infection. Planktonic and biofilm-derived *L. pneumophila* caused similar amount of LDH release from hMDMs while *dotA* mutant caused less cell death (**Figure 2E**). Human macrophages infected with the *flaA* mutant also released comparable amounts of LDH (**Figure 2E**). These data suggest that biofilm-derived *L. pneumophila* behave similarly to planktonic *L. pneumophila* in hMDMs.

BIOFILM-DERIVED *L. pneumophila* AVOIDS CASPASE-1 AND -7 ACTIVATION IN MURINE MACROPHAGES DUE TO LACK OF FLAGELLIN EXPRESSION

Upon detection of bacterial flagellin by Nlrc4 and Naip5, WT murine macrophages restrict planktonic *L. pneumophila* replication via caspase-1 and -7 activation. Caspase-7 promotes the fusion of the *L. pneumophila*-containing vacuole with the lysosome and bacterial degradation whereas caspase-1 contributes to pyroptosis and IL-1β release (Akhter et al., 2009, 2012). Because biofilm-derived *L. pneumophila* replicate more efficiently in murine macrophages and exhibited less cell death when compared to planktonic, we tested whether mouse macrophages activated caspase-1 in response to biofilm-derived bacteria. The *dotA* and *flaA* mutants were used as negative controls since they both avoid caspase-1 activation. In contrast to planktonic bacteria, biofilm-derived *L. pneumophila* did not promote caspase-1 activation as denoted by the detection of the cleaved active band by western blot (**Figure 3A**). These data indicate that murine macrophages respond to biofilm-derived *L. pneumophila* differentially than to planktonic *L. pneumophila*.

IL-1β maturation is promoted by active caspase-1 in WT macrophages infected with planktonic *L. pneumophila*. Therefore, we tested IL-1β release in culture supernatants from murine macrophages infected with planktonic, biofilm-derived *L. pneumophila* and the *dotA* mutant. Our data demonstrate that murine macrophages infected with biofilm-derived bacteria released

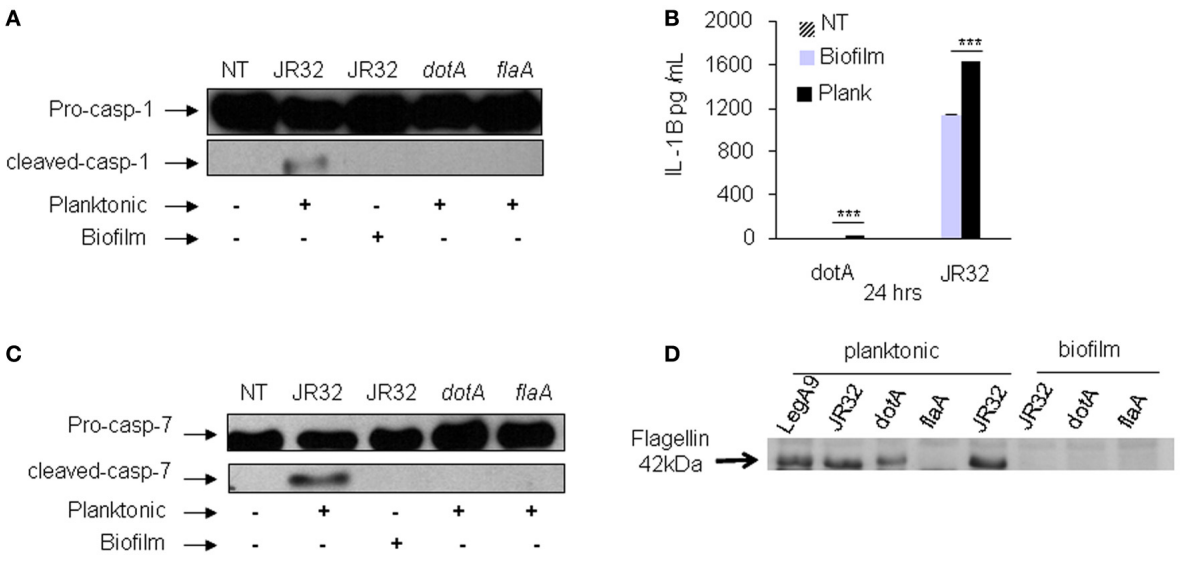

FIGURE 3 | Biofilm-derived *L. pneumophila* did not promote caspase-1 or 7 activation in murine macrophages and showed significantly less IL-1β release due to lack of flagellin expression. (A) Pro and active caspase-1 were detected in cell extracts using caspase-1 antibody. WT BMDMs were either not treated (NT) or infected with *L. pneumophila* JR32 (biofilm or planktonic), the *dotA* or the *flaA* mutant for 2 h. **(B)** The amount of IL-1β was determined in supernatants of WT infected with JR32 (biofilm or planktonic) or the *dotA* mutant after 24 h. Data are presented as means ± SD of one experiment performed in quadruplicate. Asterisks indicate significant differences (*** $P < 0.001$). **(C)** Activation of caspase-7 was detected in cell extracts using caspase-7 antibody. **(D)** Western blot analysis of planktonic and biofilm-derived *L. pneumophila* with flagellin antibody.

30% less IL-1β compared to that released after planktonic *L. pneumophila* infection (**Figure 3B**). This result indicates that the inflammatory response to biofilm-derived *L. pneumophila* in murine macrophages is less than that elicited in response to planktonic bacteria.

During infection of planktonic *L. pneumophila*, murine macrophages activate caspase-7 via the inflammasome complex contributing to bacterial restriction (Akhter et al., 2009, 2012), but this response has not been characterized in biofilm-derived *L. pneumophila*. Therefore, we tested caspase-7 activation of murine macrophages in response to biofilm-derived and planktonic *L. pneumophila*. In contrast to planktonic bacteria, biofilm-derived *L. pneumophila* did not promote caspase-7 cleavage (**Figure 3C**), indicating that biofilm-derived bacteria do not elicit caspase-7 activation, thereby allowing them to evade a restrictive mechanism employed by murine macrophages.

Flagellin mediates restriction of *L. pneumophila* in murine macrophages and the *flaA* mutant has been shown to replicate significantly more than the parent strain (Amer et al., 2006). Since biofilm-derived bacteria replicated significantly in murine macrophages and failed to activate caspase-1 or 7, we hypothesized that biofilm-derived bacteria down regulates flagellin expression. Western blot analysis of bacterial lysates using specific flagellin antibodies demonstrated that biofilm-derived bacteria diminished flagellin expression compared to planktonic bacteria (**Figure 3D**). Collectively, these results indicate that biofilm *L. pneumophila* do not activate the inflammasome because of lack of flagellin expression compared to planktonic *L. pneumophila*.

PHAGOSOMES CONTAINING BIOFILM-DERIVED *L. pneumophila* EVADE FUSION WITH THE LYSOSOMES

In murine macrophages, *L. pneumophila* replication is restricted by caspase-1 and -7 activation that result in phagosome-lysosome fusion, promoting bacterial degradation (Akhter et al., 2009, 2012). We examined the colocalization of planktonic and biofilm-derived bacteria with lysosomes at 1 h post-infection. Approximately 55% of biofilm-derived *L. pneumophila* resided in lysosomes (**Figures 4A**,**B**). Yet, 72% of planktonic bacteria resided in lysosomes. These results suggests that significantly more biofilm-derived *L. pneumophila* evade lysosomal degradation in macrophages allowing the bacteria to survive within the host and replicate as indicated by increased CFUs in (**Figure 4A**).

DISCUSSION

Legionnaire's disease is a severe pneumonia that infects the elderly and the immune compromised. There is no human to human transmission whereas infection occurs by inhalation of contaminated droplets from biofilms lining air conditioners and fresh water fountains. A biofilm is a highly-organized, multicellular community affixed to an inert or biological surface and is the preferred lifestyle of most bacteria. Bacterial

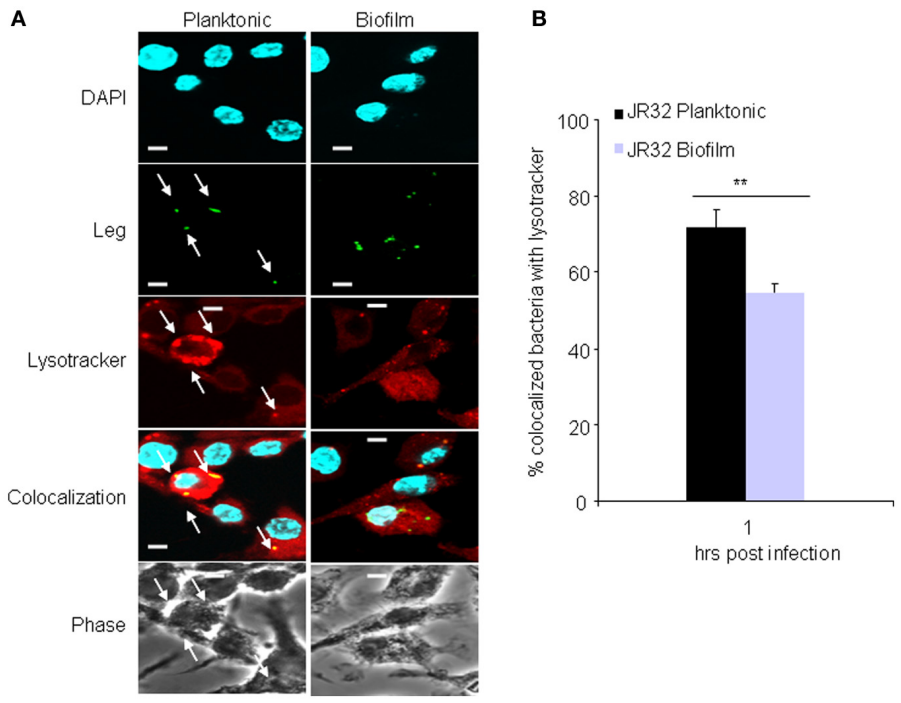

FIGURE 4 | Vacuoles harboring biofilm-derived *L. pneumophila* bacteria significantly evade fusion with lysosomes. (A) Representative images of WT BMDMs infected for 1h with JR32 planktonic or biofilm. Nuclei are stained blue with DAPI and *L. pneumophila* stained green with *L. pneumophila*-specific antibody. Lyso-tracker red was used to stain acidified lysosomes. White arrows indicate *L. pneumophila* colocalization with lysotracker. **(B)** Percent colocalization of *L. pneumophila* with lysotracker. Images were captured with the 60× objective and magnified 3×, scale bar = 10 μm. Data are presented as means ± SD of three independent experiments each performed in triplicates. Asterisks indicate significant difference (** $P < 0.01$).

populations within a biofilm, as opposed to their planktonic counterparts, are highly resistant to eradication (Flemming and Wingender, 2010). While some pathogens form biofilms within infected organs to resist phagocytosis and immune responses, others such as *L. pneumophila* form biofilms in nature and in medical and dental devices to serve as sources of outbreaks of Legionnaire's disease. In this case, the biofilm setting serves as niche where the bacterium is recalcitrant to most antibacterial agents (Rogers et al., 1994; Brown and Barker, 1999; Lau and Ashbolt, 2009).

In this study we reproduced *L. pneumophila* biofilm formation *in vitro*. Since infection occurs through the inhalation of *L. pneumophila*-containing droplets, we also simulated the dispersion of biofilm by vortex. Because once inhaled, the organism adapts an intracellular niche within macrophages, we examined the behavior of biofilm-derived *L. pneumophila* within murine and human macrophages.

We showed that biofilm-derived bacteria are more filamentous than planktonic bacteria (**Figure 1A**). This characteristic may be due to adaptation of the bacteria to a harsh environment providing an energy saving benefit for survival. Alternatively, the filamentous multinucleated characteristic may facilitate rapid division into planktonic form when encountering a parasitic host (Taylor et al., 2009). Notably, macrophages derived from mice and human donors phagocytozed equivalent numbers of biofilm-derived and planktonic organisms as demonstrated at 1 h post infection. Therefore, the filamentous phenotype of *L. pneumophila* within the biofilm did not impact their phagocytosis.

We demonstrated that robust biofilm formation by *L. pneumophila* is promoted by the DotA type IV secretion system. This finding corroborates several studies demonstrating the contribution of secreted bacterial molecules necessary for biofilm formation (Taylor et al., 2009). Secreted bacterial factors are utilized for quorum-sensing, biofilm building, and twitching motility during biofilm formation (Taylor et al., 2009). Therefore, identification and characterization of bacterial factors that are required for *L. pneumophila* biofilm formation can be targeted to prevent or eradicate biofilm formation and transmission of Legionnaires' disease in medical and industrial settings. Furthermore, *L. pneumophila* embedded within multispecies biofilms may respond to signal molecules produced by other bacteria promoting us to rethink current *L. pneumophila* research paradigms (Taylor et al., 2009).

Although the innate immune response of macrophages to planktonic *L. pneumophila* is under investigation by many scientists, the macrophage response to biofilm-derived *L. pneumophila* is yet to be elucidated. In this study, we found that biofilm-derived *L. pneumophila* replicate significantly more in murine macrophages than do planktonic bacteria. This result suggests that studies employing planktonic *L. pneumophila* in murine macrophages may not recapitulate the biofilm-derived mode of infection in humans. Yet, hMDM responded similarly to biofilm-derived and planktonic *L. pneumophila* establishing more validation for human based studies whether using planktonic or biofilm-derived *L. pneumophila*.

For an unidentified reason, planktonic flagellated *L. pneumophila* escapes detection by human NAIP and NLRC4 contributing to the permissiveness of human macrophages to the pathogen (Abdelaziz et al., 2011a,b; Ge et al., 2012). Studies by the Shao group indicated that human NAIP does not appear to respond to *L. pneumophila* flagellin (Ge et al., 2012). We also showed that human NLRC4 does not respond to flagellated *L. pneumophila* (Abdelaziz et al., 2011a,b). Yet, human NLRC4 activates caspase-1 in response to flagellated *Salmonella* (Abdelaziz et al., 2011b). Here we found that biofilm-derived *L. pneumophila* that seems to diminish flagellin expression replicates to the same extent as planktonic cultures in human macrophages. Since *L. pneumophila* flagellin detection is nonetheless avoided in human cells, it is plausible to expect that the *flaA* mutant would replicate to the same extent, however, this was not the case. The planktonic *flaA* mutant replicated more that the parent strain whether planktonic or biofilm-derived. This result suggests that biofilm-derived *L. pneumophila* may still express scarce amounts of flagellin contributing to its modest restriction via a caspase-1 independent mechanism. This remains to be elucidated by further studies.

Unlike planktonic *L. pneumophila*, biofilm-derived *L. pneumophila* do not activate caspase-1 and -7 in murine macrophages. The activation of caspase-1 and -7 promote the fusion of vacuoles containing *L. pneumophila* with lysosomes and bacterial degradation (Amer et al., 2006; Akhter et al., 2009; Franchi et al., 2009). Thus, our results suggest that biofilm derived *L. pneumophila* evades phagosome-lysosome fusion by avoiding caspase-1 and -7 activation. Macrophages infected with biofilm-derived bacteria exhibited significantly less IL-1β release and macrophage death compared to those infected with planktonic bacteria. IL-1β is a pro-inflammatory cytokine activated by caspase-1. Thus, by reducing IL-1β release, biofilm-derived *L. pneumophila* avoids major inflammatory responses and the recruitment of inflammatory cells to the infected lungs.

A better understanding of the innate immune response to biofilm-derived *L. pneumophila* will pave the way for the development of novel diagnosis and treatment strategies.

AUTHOR CONTRIBUTIONS

Arwa Abu Khweek designed and performed the experiments, analyzed the results, and wrote the manuscript. Natalia S. Fernández Dávila, Kyle Caution, Anwari Akhter, Basant A. Abdulrahman, Mia Tazi, Hoda Hassan, Laura A. Novotny, Lauren O. Bakaletz contributed to the performance of the experiments and editing of the manuscript. Amal O. Amer helped in the design of the experiments, interpretation of the results, and editing of the manuscript.

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Modulation of host immune defenses by *Aeromonas* and *Yersinia* species: convergence on toxins secreted by various secretion systems

Jason A. Rosenzweig*[1,2]* and Ashok K. Chopra*[3,4,5,6]*

[1] *Department of Biology, Center for Bionanotechnology and Environmental Research, Texas Southern University, Houston, TX, USA*
[2] *Department of Environmental and Interdisciplinary Sciences, Texas Southern University, Houston, TX, USA*
[3] *Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX, USA*
[4] *Sealy Center for Vaccine Development, University of Texas Medical Branch, Galveston, TX, USA*
[5] *Institute of Human Infections and Immunity, University of Texas Medical Branch, Galveston, TX, USA*
[6] *Galveston National Laboratory, University of Texas Medical Branch, Galveston, TX, USA*

Edited by:
Yongqun He, University of Michigan School of Medicine, USA

Reviewed by:
Richard D. Hayward, University College London and Birkbeck, University of London, UK
Glen C. Ulett, Griffith University, Australia

***Correspondence:**
Jason A. Rosenzweig, Department of Biology, Center for Bionanotechnology and Environmental Research, Texas Southern University, 3100 Cleburne Street, Houston, TX 77004, USA
e-mail: rosenzweigja@tsu.edu;
Ashok K. Chopra, Department of Microbiology and Immunology, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555-1070, USA
e-mail: achopra@utmb.edu

Like other pathogenic bacteria, *Yersinia* and *Aeromonas* species have been continuously co-evolving with their respective hosts. Although the former is a bonafide human pathogen, the latter has gained notarity as an emerging disease-causing agent. In response to immune cell challenges, bacterial pathogens have developed diverse mechanism(s) enabling their survival, and, at times, dominance over various host immune defense systems. The bacterial type three secretion system (T3SS) is evolutionarily derived from flagellar subunits and serves as a vehicle by which microbes can directly inject/translocate anti-host factors/effector proteins into targeted host immune cells. A large number of Gram-negative bacterial pathogens possess a T3SS empowering them to disrupt host cell signaling, actin cytoskeleton re-arrangements, and even to induce host-cell apoptotic and pyroptotic pathways. All pathogenic yersiniae and most *Aeromonas* species possess a T3SS, but they also possess T2- and T6-secreted toxins/effector proteins. This review will focus on the mechanisms by which the T3SS effectors *Yersinia* outer membrane protein J (YopJ) and an *Aeromonas hydrophila* AexU protein, isolated from the diarrheal isolate SSU, mollify host immune system defenses. Additionally, the mechanisms that are associated with host cell apoptosis/pyroptosis by *Aeromonas* T2SS secreted Act, a cytotoxic enterotoxin, and Hemolysin co-regulated protein (Hcp), an *A. hydrophila* T6SS effector, will also be discussed.

Keywords: type 2-, -3, and -6 secretion systems, apoptosis, pyroptosis, actin cytoskeleton, effector proteins

INTRODUCTION

Intricate host-pathogen interactions are constantly evolving as the latter has to combat formidable host immune defenses, resulting in expression/de-repression of genes and molecular mimicry. In some instances, strong immune responses to microbes select for escape mutants with the latter further honing the immune response to that variant pathogen, leading to an ongoing battle, as can be seen typically in human immunodeficiency virus escape mutants (Akahoshi et al., 2012; Yagita et al., 2013) and in *Chlamydia trachomatis* (Nunes et al., 2010). One classic example of this paradigm is programmed host cell death (caused by apoptosis, pyroptosis, and necrosis) which could benefit the host immune system (if clearing an intracellular pathogen) or could be co-opted by the pathogen as a means of eliminating undesirable host cells (e.g., innate immune cells) (Ulett and Adderson, 2006).

Whereas apoptosis is a "self-contained" event that does not stimulate a robust inflammatory response, both pyroptosis and necrosis of host cells release pro-inflammatory cytokines and their cytoplasmic contents into the extra-cellular milieu (Lamkanfi and Dixit, 2010). Apoptosis is a caspase-dependent process that drives embryonic development and is largely characterized by nuclear fragmentation and condensation, blebbing of the plasma membrane, and cell shrinkage. Since all of these physiological consequences occur intracellularly, no host cell cytoplasmic content is released into the extracellular environment, thereby preventing inflammation (Strasser et al., 2000).

Necrosis, by contrast, is a caspase-independent process that results in host cell swelling, disorganized and extensive chromatin hydrolysis, and cytoplasmic leakage (Berghe et al., 2010). Finally, caspase-1-dependent pyroptosis leads to secretion of pro-inflammatory cytokines interleukin-1β (IL-1β) and IL-18. Caspase-1, interestingly, is not involved in apoptosis and is activated by one of four inflamasomes, which contain a member of the nucleotide-binding oligomerization domain-containing protein (Nod)-like receptor family, during pyroptosis (Lamkanfi and Dixit, 2009). Surprisingly, the pathogenic yersiniae can induce apoptosis, necrosis, and pyroptosis depending on the host-cell type infected (Monack et al., 1997; Ruckdeschel et al., 1997, 1998, 2001; Bergsbaken and Cookson, 2007; Zheng et al., 2012). Similarly, *Aeromonas* species are also able to induce apoptosis

(through various caspase activation) (Galindo et al., 2004b, 2006a,b; Martins et al., 2007; Su et al., 2007; Sierra et al., 2007, 2010).

Gram-positive pathogens have also been shown to induce apoptosis in various cell types by disparate mechanisms. Non-secreted lipotechoic acids (LTAs), well-conserved surface antigens on a wide variety of Gram-positive organisms, are such examples that induce apoptosis by distinct mechanisms in various cell types (Ulett and Adderson, 2006). Beyond LTAs that induce host cell apoptosis, *Bacillus anthracis* employs its lethal factor exotoxin (Park et al., 2002; Popov et al., 2002), *Listeria monocytogenes* utilizes listeriolysin, a cytolysin (Carrero et al., 2004), while the streptococci employ hemolysins (Ring et al., 2002; Liu et al., 2004). Ultimately, Gram-positive pathogens' apoptosis-inducing mechanisms are very diverse; they can typically be either intrinsic (e.g., mitochondrial dysfunction) or extrinsic whereby death domains are activated (Ulett and Adderson, 2006). Of the 27-members that belong to the *Aeromonadaceae* family, *A. hydrophila*, *A. veronii,* and *A. caviae* are frequently isolated as human pathogens, with most infections contracted *via* the fecal-oral route or through wounds (Altwegg et al., 1991; Kirov, 1993; Palu et al., 2006; Edberg et al., 2007). *Aeromonas hydrophila* is also a fish pathogen that can negatively impact the fishing industry. The majority of human *Aeromonas* infections result in self-limiting gastroenteritis or superficial skin infections. However, more threatening systemic infections include bacteremia, cellulitis, peritonitis, hemolytic-uremic syndrome, and necrotizing fasciitis (Chopra et al., 1993, 1996; Janda et al., 1994; Merino et al., 1995; Kuhn et al., 1997; Chopra and Houston, 1999; Minnaganti et al., 2000; Brouqui and Raoult, 2001; Galindo et al., 2006a,b; Sha et al., 2013). Alarmingly, it seems as though instances of *Aeromonas*-induced necrotizing fasciitis are on the rise (Huang et al., 2011; Chang et al., 2012; Kao and Kao, 2012; Wu et al., 2012).

Of the 11 known Gram-negative *Yersinia* species, only *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis* are human pathogens. Strikingly, while *Y. pseudotuberculosis* and *Y. enetrocolitica* cause self-limiting gastroenteritis (Galindo et al., 2011), *Y. pestis* (transmitted by the bite of an infected flea) causes radically different diseases (bubonic, septicemic, or pneumonic plague) which have resulted in three major human pandemics as well as the great plagues of London in the mid-late 1600s (Inglesby et al., 2000). Currently, the plague-causing bacterium can be treated with various antibiotics (Rosenzweig et al., 2011a), with levofloxacin recently being approved by the Food and Drug Administration against all forms of plague. However, there is no vaccine against this deadly pathogen (Rosenzweig et al., 2011b; Rosenzweig and Chopra, 2012).

As earlier mentioned, representatives of both the *Yersinia* and *Aeromonas* species are capable of causing gastroenteritis following the fecal-oral route of infection, and they similarly possess a type three secretion system (T3SS). The T3SS multiprotein complex/hyperstructure (Norris et al., 2012) is evolutionarily related to the bacterial flagella (Nguyen et al., 2000; Gophna et al., 2003) and enables rapid translocation of effector proteins directly into the targeted host cell cytoplasm, resulting in a number of anti-host consequences. Interestingly, whereas the yersiniae possess well-studied T3SS weaponry, *Aeromonas* species harbor well-defined T3- and T6-secretion systems (derived from phage injection machinery) along with its two identified effector protein substrates, Hemolysin co-regulated proteins (Hcps) and Valine glycine repeat G proteins (VgrGs) (Sierra et al., 2007, 2010; Vilches et al., 2009; Bergh et al., 2013; Sha et al., 2013). Interestingly, hemolysins in *Streptococcus agalactiae*, a Group B streptococci, have also been shown to induce apoptosis in phagocytic cells (Ulett and Adderson, 2006 and references therein). The yersiniae T6SSs have not been as extensively characterized; however, in *Y. psuedotuberculosis*, it is regulated by the transcriptional factor OmpR and appears to play a role in stress responses, quorum sensing, and maintenance of internal pH homeostasis (Zhang et al., 2011, 2013; Gueguen et al., 2013). In *Y. pestis*, the T6SS was found to secrete an Hcp-like autoagglutination factor (Podladchikova et al., 2011). Finally, *Aeromonas* species also employ the general secretory T2SS pathway to export cytotoxic enetrotoxin Act (with hemolytic, cytotoxic, and enterotoxic activities) into the extracellular milieu (Chopra and Houston, 1999). Within the yersiniae, the T3SS injects into the host 7 *Yersinia* outer membrane protein (Yop) effector proteins that have been identified as YopP/J, -H, -E, YopO/YpkA, YopT, YopK, and YopM; these Yops counteract host immune defenses by various mechanisms (Viboud and Bliska, 2005). Upon first encountering a Gram-negative pathogen, like *Y. pestis*, innate immune cells (e.g., macrophages and/or dendritic cells) recognize non-specific, pathogen-associated molecular patterns/microbe-associated molecular patterns (PAMPs/MAMPs), like lipopolysaccharide (LPS), lipoprotein, or flagellin. When PAMPs/MAMPs associate with their recognition receptors, e.g., Toll-like receptors (TLRs), various mitogen-activated protein (MAP) kinase (MAPK) and nuclear factor Kappa B (NF-κB) signaling pathways are activated resulting in the upregulation of IL-12, -18, and tumor necrosis factor alpha (TNF-α) proinflammatory cytokine production (Matsumoto and Young, 2009).

The yersiniae counteract the aforementioned inflammatory response when YopP/J acetylate I kappa B kinase (IKK) and MAPK kinases (MKKs), preventing their phosphorylation and subsequent activation. The disruption in these signaling events results in innate immune cells undergoing apoptosis (Orth, 2002; Mittal et al., 2006; Mukherjee et al., 2006). A more detailed description of *Yersinia* outer membrane protein J (YopJ) mechanisms of mollifying host defenses is discussed in a later section. YopE, -H, -T, and YopO/YpkA all operate to disrupt actin cytoskeleton re-arrangements and phagocytosis, albeit by attacking unique and distinct targets. YopE is a GTPase-activating protein (GAP), while YopT targets Rac-1, RhoA and Cdc-42, and YopH, which is a tyrosine phosphotase, primarily targets focal adhesion complexes. YopO/YpkA, through its kinase activity, also targets Rac-1 and RhoA as well as actin directly. YopM localizes to the target cells' nuclei and disrupts cytokine IL-15 production by targeting ribosomal S6 protein kinase 1 (RSK1) and possibly protein kinase C-like 2 (PRK2) (Matsumoto and Young, 2009 and references therein). Finally, YopK was found to associate with the translocation pore and is believed to modulate inflammation (Brodsky et al., 2010).

As mentioned earlier, many *Aeromonas* species also possess a T3SS. In fact, within the fish pathogen *A. salmonicida*, four T3SS-associated effectors have been identified: AexT, AopP, AopH, and AopO (Braun et al., 2002; Dacanay et al., 2006; Fehr et al., 2006).

Our laboratory recently identified an AexT-like protein (a novel T3SS effector, AexU) in a diarrheal isolate SSU of *A. hydrophila* (Sha et al., 2007). While *Aeromonas* outer protein P (AopP) disrupts NF-κB signaling downstream of IKKB, unlike YopJ in the yersinaie, it does not disrupt the MAPK signaling pathway (Fehr et al., 2006). On the contrary, AexT and AexU both possess highly cytotoxic ADP-ribosyltranferase activity for host proteins (Braun et al., 2002; Sha et al., 2007). AopO and AopH remain poorly understood and are homologues of yersiniae YopO/YpkA and YopH, respectively (Sha et al., 2007). Interestingly, we also demonstrated that an *A. hydrophila* Δ*aopB* deletion mutant, unable to translocate effector Aops into host cells, exhibited greatly reduced virulence in a murine model of infection (Sha et al., 2005).

T3SS EFFECTOR YopJ'S MECHANISMS OF ANTI-HOST ACTIVITY

The yersiniae T3SS effector YopJ is an acetyltransferase as well as a de-ubiquitinase. Its anti-host activity involves blocking MAPK signaling and NF-κB activation (**Table 1**). This aberrant signaling leads to significantly reduced production of both proinflammatory and anti-apoptotic host cytokines (Monack et al., 1997; Orth et al., 1999, 2000; Mukherjee et al., 2006). Shedding more light on the mechanism of YopJ anti-host activity, a report from Shrestha et al. (2012) identified that YopJ reduced the induction of eukaryotic initiation factor 2 (eIF2) in both yeast and mammalian cells, and that eIF2 signaling was required for YopJ-mediated inhibition of NF-κB activation as well as pro-inflammatory cytokine production.

TLR-2, NF-κB, and Nod2 signaling pathways all targeted by the versatile YopJ

By using recombinant YopJ (rYopJ), it was determined that TLR-2 in murine macrophages was involved in YopJ-mediated apoptotic signaling by increasing production of caspases 3 and 8, IL-1 receptor associated kinase (IRAK)-4, Fas-associated protein with death domain (FADD), and phosphorylation of IκB and MAPK (Pandey and Sodhi, 2011). Together with this TLR-2 apoptotic signaling, the ability of YopJ to target macrophage eIF2 signaling pathway required for inhibition of NF-κB activation as well as pro-inflammatory cytokine production ultimately leads to host cell apoptosis (Shrestha et al., 2012). In a separate study employing a *Drosophila* model system, transforming growth factor (TGF)-β-activated kinase (TAK1), which is part of the immune NF-κB signaling pathway independent of the TLR-2 signaling, was identified as the YopJ serine/threonine acetylation target (Paquette et al., 2012). Following YopJ acetylation of serine/threonine residues in the active site of *Drosophila* TAK1, its phosphorylation was blocked preventing activation of this kinase. Corroborating *Drosophila* studies, YopJ similarly modified and inhibited TAK1 in mammalian cells (Paquette et al., 2012).

Despite an earlier study demonstrating rYopJ activation of TLR-2 signaling in macrophages (Pandey and Sodhi, 2011), *in vivo* studies employing both *Drosophila* and macrophage models of infection clearly demonstrated that native YopJ indeed activated the NF-κB signaling but not the TLR-2 signaling pathway (Paquette et al., 2012). It was proposed that following acetylation of key serine/threonine residues in the active sites of both RIP (receptor interacting protein 1)-like interacting caspase-like apoptosis regulatory protein kinase (RICK) and TAK1, YopJ prevented the interaction of RICK and Nod2, a NACHT-leucine-rich repeats (NLRs) recognition receptors. Further, Nod2 interacted with caspase 1, promoting increased expression/production of IL1-β and dissemination of *Y. psudeotuberculosis* through the gut epithelium (Meinzer et al., 2012). Perhaps this seeming contradiction of YopJ not signaling through TLR-2 during

Table 1 | The mechanisms of action of yersiniae effectors YopJ and YopK.

Effector	Secretion system	Mechanisms of pathogenesis	References
YopJ	T3SS	Acetyltransferase and deubiquitinase that blocks MAPK and NF-κB signaling, causing reduced production of pro-inflammatory and anti-apoptotic cytokines.	Monack et al., 1997; Orth et al., 1999, 2000; Mukherjee et al., 2006
		Reduced induction of eukaryotic initiation factor 4.	Shrestha et al., 2012
		Signals through TLR-2 to increase production of Caspase-3, -8, IRAK-4, FADD.	Pandey and Sodhi, 2011
		Serine threonine acetylation of TAK1 in *Drosophila* preventing its phosphorylation.	Paquette et al., 2012
		Blocks interaction of RICK and Nod2 acetylation of RICK and TAK 1; Nod2 then interacts with caspase-1 to increase expression of IL1-β which promotes bacterial dissemination through the gut.	Meinzer et al., 2012
YopK	T3SS	Regulates pyroptosis (via caspase-1).	Brodsky et al., 2010
		Regulates YopJ-mediated apopotosis in macrophages and facilitates bacterial dissemination.	Peters et al., 2013

MAPK, mitogen-activated protein kinases; NF-κB, nuclear factor Kappa B; TLR-2, toll-like receptor 2; IRAK 4, interleukin 1 receptor associated kinase; FADD, Fas-associated death domain; TAK1, tumor growth factor β-activated kinase; RICK, receptor interacting protein 1-like interacting caspase-like apopotosis regulatory protein kinase; Nod2, nucleotide-binding oligomerization domain-containing protein 2; IL 1-β, interleukin 1 Beta; T3SS, type three secretion system; Yop, Yersinia outer protein.

in vivo studies while rYopJ was shown to signal through TLR-2 *in vitro* underscores one very important point. Different cell types and/or organisms likely possess disparate/specialized receptors used to recognize threatening pathogens. Viewed in this light, one can envision how within one cell type there could exist several unique receptors that could detect the same (or different products) derived from one pathogen. In the very delicate host-pathogen paradigm, every potential detection mechanism must be employed if the host is to successfully subvert the pathogenic threat. Collectively, YopJ has been observed disrupting TLR-2, Nod2, and the NF-κB signaling pathways, making the T3SS-acetyltransferase a potent weapon for the pathogenic yersiniae.

The yopK "switch" for YopJ activity

Importantly, YopJ seems to function in concert with another Yop, YopK, which regulates YopJ activity (**Table 1**). Studies have shown that YopK appeared to regulate pyroptosis (via caspase 1) by *Y. pseudotuberculosis* (Brodsky et al., 2010) and YopJ-dependent apoptosis specifically in RAW 264.7 monocytic cells, thereby facilitating bacterial dissemination in a murine model of pneumonic plague (Peters et al., 2013). Despite YopK appearing dispensable for *Y. pseudotuberculosis* to induce caspase-1 mediated pyroptosis (Brodsky et al., 2010), it was required for optimum virulence of *Y. pestis* in a pneumonic murine model of infection (Peters et al., 2013). Further, cell culture *Y. pestis* infection models have also revealed that caspase 1 activation occurs downstream of cell necrosis, is independent of mitochondrial driven apoptosis, but does require cathepsin B activity (Zheng et al., 2012). Taken together, these data demonstrated that, at least in *Y. pestis*, YopJ's ability to induce apoptosis is regulated by a YopK "switch" downstream of cell necrosis.

T3SS EFFECTOR AexU's MECHANISMS OF ANTI-HOST ACTIVITY

Shortly after a T3SS was identified in both *A. salmonicida* and *A. hydrophila* and implicated in their pathogenesis of both fish and animal/human hosts (Burr et al., 2002; Yu et al., 2004; Sha et al., 2005), we identified a novel T3SS-dependent AexT-like protein, referred to as AexU, in *A. hydrophila* (Sha et al., 2007; Sierra et al., 2007). AexT (**Table 2**), identified in the fish pathogen *A. salmonicida*, is homologous to the *Pseudomonas aeruginosa* ExoT/S and is also a bifunctional effector (Pederson et al., 1999; Sundin et al., 2004). Its amino terminus has YopE-like activity of yersiniae and can depolymerize actin by targeting RhoA, while its carboxy-terminus has highly cytotoxic ADP-ribosyltransferase (ADP-RT) activity for host proteins (Braun et al., 2002).

While the amino terminus of AexU from *A. hydrophila* SSU maintained ∼67% sequence similarity to its AexT counterpart, surprisingly, the AexU carboxy terminus had a unique sequence which did not share similarity with any other known protein in the NCBI database, despite full-length AexU maintaining ADP-RT activity (Sha et al., 2007). Surprisingly, the purified full-length, truncated amino terminus, or truncated carboxy terminus of AexU all exhibited ADP-RT activity; however, the full-length AexU and its amino terminus exhibited higher ADP-RT activity than did the carboxy terminus of AexU alone (Sierra et al., 2007). Since the ADP-RT activity of the *Pseudomonas* homologue (ExoT/S) resides in its carboxy terminus, the aforementioned finding suggested a potentially unique evolution of AexU as an *Aeromonas* T3SS effector.

Table 2 | Aeromonad effector proteins AexT, AexU, Act, and Hcp mechanisms of action.

Effector	Secretion system	Mechanism of pathogenesis	References
AexT	T3SS	Amino terminal activity targets RhoA and promotes actin depolymerization; carboxy terminal ADP-ribosyltransferase activity.	Braun et al., 2002
AexU	T3SS	Bifunctional-like AexT; activation of caspase-3 and -9 and induction of cell rounding, chromatin condensation; also required for virulence in mice.	Sierra et al., 2007
		GAP-activity (amino terminus) promotes apoptosis and disrupts the cell cytoskeleton as well as NF-κB signaling; prevents signaling of c-Jun, JNK, IκBα, and inhibits IL-6 and IL-8 secretion.	Sierra et al., 2010
		GAP-activity disruption of actin cytoskeleton mediated by down-regulating Rac-1; binding to β4-integrin results in cytotoxicity.	Abolghait et al., 2011
Act 2	T2SS	Induced upregulation of apoptosis-related genes.	Galindo et al., 2003
		Activates MEK1, JNK, ERK1/2, and c-Jun of the MAPK pathway; induces membrane blebbing and increased production of mitochondrial cytochrome C, caspase-3, -8, and -9.	Galindo et al., 2004b
Hcp	T6SS	Caspase 3 activation.	Suarez et al., 2008
		Demonstrates anti-phagocytic properties.	Suarez et al., 2010
		Hcp-2 is part of the T6SS apparatus while Hcp-1 negatively regulates motility and protease production.	Sha et al., 2013

RhoA, Rat sarcoma homolog gene family member A; GAP, GTPase activating protein; NF-κB, nuclear factor Kappa B; JNK, c-Jun N-terminal kinase; IL, interleukin; Rac-1, Rac GTPase activating protein 1; MEK1, MAP/ERK kinase 1; ERK1/2, extracellular signal-regulated kinase 1/2; MAPK, mitogen-activated protein kinases.

Supporting this view, findings from a comprehensive genomic study evaluating an *A. veronii* group collection (derived from both clinical and environmental isolates) revealed that all 20 bacterial isolates possessed a functional T3SS as well as both AexU and AexT effectors. However, whereas AexU had a nucleotide substitution rate of ∼17% in its carboxy terminal region, AexT was much better conserved and demonstrated only a ∼4% substitution rate (Silver and Graf, 2009). Perhaps, the AexU carboxy terminus is evolving independently from its amino terminus allowing for not only the possibility of producing varying alleles but also producing a variety of AexU effectors capable of adapting to changing environments within the host (**Figure 1**)?

Immunogenicity of AexU and its contribution to overall virulence

In a cell culture infection model, our laboratory found that *A. hydrophila* AexU (**Table 2**) caused actin reorganization and cell rounding, chromatin condensation, and the activation of caspase 3 and 9, all hallmark features of apoptosis (Sierra et al., 2007). Furthermore, we reported that *A. hydrophila* AexU also possessed GAP activity which strongly promoted apoptosis and disrupted actin cytoskeletal rearrangements of the host cells (Sierra et al., 2010). Additionally, it was noted that *A. hydrophila* AexU prevented phosphorylation of c-Jun [a component of the activator protein 1 (AP-1) transcription factor], c-Jun N-terminal kinase (JNK) and IκBα (thereby disrupting their signaling cascades), and inhibited IL-6 and -8 secretion from HeLa cells. Ultimately, AexU inhibited NF-κB and inactivated Rho GTPases in the host cell (Sierra et al., 2010). For reasons unclear at this time, an AexU variant devoid of both GAP and ADP-RT activities, when produced from an *aexU* null mutant of *A. hydrophila*, induced higher mouse mortality and increased pro-inflammatory cytokine production (Sierra et al., 2010). As was noted with YopJ, perhaps, to increase overall bacterial virulence through inflammation, evolutionary deactivation of AexU's activities provides a valuable "switch" for responding to various hosts?

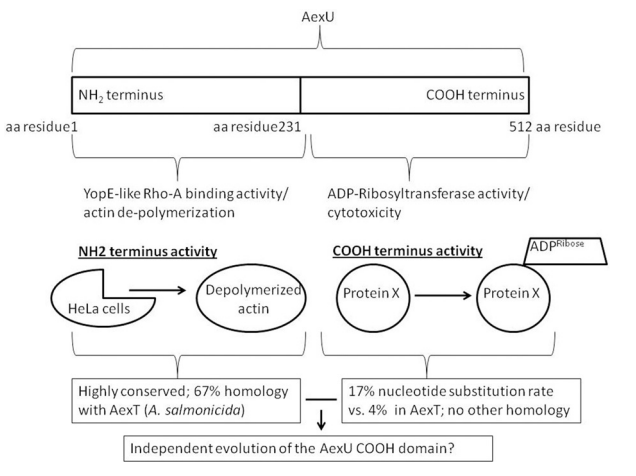

FIGURE 1 | The bifunctional AexU effector protein. The two independent activities of AexU are localized on either the NH2- or the COOH-termini. This allows for the possibility of an independently evolving COOH-terminal activity.

In *P. aeruginosa*, it has been shown that the maturation of IL-1β is negatively regulated by ExoS and is dependent on its ADP-RT activity. In other words, ExoS devoid of this enzymatic activity when produced from the bacteria led to increased IL-1β production and pyroptosis of the host cells. However, AexU seemed to behave differently compared to ExoS as the former without the enzymatic activities was unable to alter IL-1β levels (Sierra et al., 2010). *A. veronii* AexU, in a GAP-dependent manner, was able to similarly disrupt actin cytoskeleton by down-regulating Rac-1 in HeLa cells (Abolghait et al., 2011). Additionally, *A. veronii* AexU was found to co-localize with β4-integrin resulting in cytotxicity for the host cells (Abolghait et al., 2011). Collectively, these data strongly demonstrated AexU's versatility as an effector protein by virtue of its ability to disrupt cell signaling, paralyze the host cell, activate caspases (initiating apoptosis), and interact with β4-integren promoting host cell cytotoxicty. When evaluating AexU's contribution to mouse virulence and immunogenicity, we found that an *A. hydrophila* Δ*aexU* deletion mutant caused significantly less mortality (40% compared to 90–100%) in intraperitoneally-challenged mice than did infection with the isogenic parental strain (Sha et al., 2007). Importantly, rAexU provided protective immunity to mice when subsequently challenged with *A. hydrophila* (Sha et al., 2007). Additionally, the *A. hydrophila* Δ*aexU* deletion mutant was unable to disseminate within infected mice leaving their lungs, liver, and spleens relatively sterile (Sierra et al., 2010). The aforementioned findings raise the possibility of developing potential subunit and/or live-attenuated vaccine candidates for *A. hydrophila*, which is an emerging human pathogen.

Aeromonas T2SS CYTOTOXIC ENTEROTOXIN (ACT) AND T6SS HCP EFFECTOR

In addition to a functional T3SS, *Aeromonas* species also possess a T6SS (Suarez et al., 2008) as well as secrete a potent enterotoxin Act (**Table 2**) via the T2SS (Chopra and Houston, 1999). In efforts to better understand the host cell response to Act, our laboratory obtained transcriptome profiles of Act-exposed murine RAW 264.7 cells. Not surprisingly, of the 76 differentially expressed genes identified in Act-treated macrophages, many were involved in immune responses, including inflammation (Galindo et al., 2003). Additionally, several apoptosis-related genes were also found to be up-regulated including (but not limited to) Bcl-10 (promotes activation of NF-κB and maturation of pro-caspase 9), BimEL (involved in p38 and JNK-associated apoptosis), and TNF receptor associated factor 1 (TRAF1, which regulates activation of NF-κB and JNK). These transcriptome results with respect to apoptosis-related genes were confirmed by performing real-time PCR as well as functional assays for apoptosis (Galindo et al., 2003).

Since primary host cells may vary in their responses to a stimulant compared to the transformed cell lines, our laboratory evaluated transcriptome profiles of primary murine peritoneal macrophages after treatment with Act. We observed 66% differential gene expression, mirroring our results seen with Act-exposed RAW 264.7 cells (Galindo et al., 2004a,b). However, differential expression of 28 genes unique to primary macrophages was also observed. The pro-apoptotic B-cell leukemia/lymphoma

2 (Bcl-2) and Myeloid differentiation primary response 116 (MyD116) genes were upregulated, while interferon consensus binding proteins 8 (IRF-8—involved in immune responses) was downregulated in Act-treated primary cells (Galindo et al., 2004a). When the effect of Act on human HT-29 colonic epithelial cells' transcriptome profiles was evaluated, we noted upregulation of genes involved in immune responses (e.g., IL-8) and apoptosis (e.g., Bcl-2-like genes) as well as phosphorylation of MAPKs (e.g., p38 kinase, extracellular signal-regulated kinase 1/2 [ERK1/2], and JNK) (Galindo et al., 2005), mirroring what was observed earlier in our mouse macrophage studies (Galindo et al., 2003, 2004a,b). Further, through proteomic analysis, we determined that Act increased phosphorylation/activation of cyclic AMP-response element binding protein (CREB), c-Jun, protein kinase C, and signal transducer and activator of transcription 3 (STAT3) (Galindo et al., 2005).

Realizing that Act induces apoptosis in both cultured and primary macrophages, we elucidated the molecular mechanisms and specifically interrogated the MAPK signaling pathway. We found that, in various cell types, Act exposure resulted in activation of JNK and ERK1/2. Furthermore, Act induced activation/phosphorylation of MAPK upstream factors MKK3/6, MKK4 and MAP/ERK kinase 1 (MEK1) as well as downstream transcription factor c-Jun (Galindo et al., 2004b). With regards to apoptosis, Act induced classical membrane blebbing, increased production of mitochondrial cytochrome *c* and apoptosis-inducing factor, in addition to caspase-3, -8, and -9 activation (Galindo et al., 2004b). When we screened for interactions between Act and both human and yeast proteins (using proArrays), Act was found to bind 9 human proteins (out of ∼1800 proteins screened). Of the 9, synaptosomal-associated protein 23 (SNAP23), galectin-3, and guanylate kinase 1 (GUK-1) were knocked down in murine macrophages and HT-29 epithelial cells (using small inhibitory RNA), with the former two resulting in reduced induction of apoptosis following Act exposure (Galindo et al., 2006a,b). Interestingly, we also observed that DNA adenine methyltransferase (Dam) and Glucose inhibited division protein (GidA) both work to positively influence *act* gene expression and its associated hemolytic activity (Erova et al., 2012). Interestingly, the Gram-positive pathogen, *Staphylococcus aureus*, also secretes a potent, pro-apoptotic enterotoxin, the superantigen enterotoxin B. The aforementioned enterotoxin specifically targets T-cells and activates FAS receptor signaling (Ulett and Adderson, 2006 and references therein).

The *Aeromonas* T6SS has two identified effector protein substrates, Hcps and VgrGs (Sha et al., 2013). *A. hydrophila*'s Hcp (**Table 2**) is a powerful effector substrate and once translocated into the targeted host cell cytoplasm, apoptosis ensues following caspase 3 activation (Suarez et al., 2008). We also demonstrated that Hcp paralyzes macrophages thereby preventing phagocytosis (Suarez et al., 2010). Curiously, multiple copies of Hcp are present in T6SS-possessing bacteria suggesting either redundancy of function and/or dosage-related functional influences (Mougous et al., 2006). The *Aeromonas* gene duplications and various alleles are likely a byproduct of co-evolution occurring in both bacterial pathogens and their respective hosts.

In *A. hydrophila* SSU, the 2 Hcp paralogs cluster to two regions of the chromosome and influence virulence-associated properties differently, demonstrating little functional redundancy (Seshadri et al., 2006; Suarez et al., 2008; Sha et al., 2013). Hcp-2, located inside the T6SS cluster appeared to function structurally in forming the T6SS apparatus while Hcp-1, located at a distal chromosomal site functioned more as an effector (Sha et al., 2013). More specifically, only Hcp-1 worked to negatively regulate bacterial motility and protease production (both required for optimal virulence) whereas both paralogs were required for optimal virulence and dissemination to peripheral organs in a murine model of infection (Sha et al., 2013). When considering the impressive arsenal available to *A. hydrophila* that includes a T3SS, a T6SS as well as a potent T2SS secreted Act, it becomes less surprising that human infections caused by this emerging pathogen are on the rise.

CONCLUSION

In context of an intricate host-bacterial pathogen co-evolutionary paradigm, at times it become difficult to determine whether the resulting outcomes better benefit the host or the pathogen. For example, following inhibition of NF-κB and MAPK signaling pathways, *Yersinia* species through their generic PAMP/danger signals (e.g., LPS or flagellin), can induce pyroptosis a specialized inflammation-associated apoptosis that involves the activation of caspase 1 (Philip and Brodsky, 2012 and references therein). Inflammation is, in reality, a double-edged sword. If of short duration and localized, it can serve to reduce extent of bacterial infection preventing systemic spread of the pathogen. However, if persistent and/or systemic, inflammation can damage host tissue and potentially promote bacterial spread contributing to bacterial pathogenesis. These two scenarios underscore the intricacies of the host-pathogen interaction as well as reveal how co-evolution can be shaped. The yersiniae T3SS effector, YopJ, is a perfect example of such an ambiguity. Despite its potent immunomodulatory capabilities, YopJ was largely dispensable for virulence in a rat model of bubonic plague (Lemaitre et al., 2006). Further, there has been evolutionary selective pressure against excessive YopJ secretion in order to achieve maximal virulence during plague infections (Zauberman et al., 2006; Brodsky and Medzhitov, 2008). What does this all mean? Does YopJ's powerful ability to induce inflammation benefit the pathogen or the host? Perhaps viewed in this light, the cost to benefit ratio nears "1" making YopJ a "circumstantial virulence factor" depending on the *Yersinia* pathogen in question, the route of infection, the immunodisposition of the host, etc.

The T3SS is a powerful vehicle of effector protein delivery shared by many Gram-negative pathogens. The pathogenic *Aeromonas* species also possess a functional T3SS that delivers 4 effector proteins into targeted host cells. The YopJ homolog, AopP disrupts NF-κB signaling downstream of IKKB but, unlike YopJ in the yersinaie, does not disrupt the MAPK signaling pathway (Fehr et al., 2006). AexU, is an extremely versatile *Aeromonas* T3SS bifunctional effector that possesses both GAP activity (like the yersiniae YopE) as well as ADP-RT activity. Like YopJ, AexU induces apoptosis and targets NF-κB signaling. However, unlike in the yersinaie which lack clearly defined T6SS-virulence factors,

Aeromonas species possess well-defined T6SS-associated virulence factors and even a T2SS-secreted toxin (Act) creating a much wider arsenal. The T6SS Hcp paralogs sharing limited functional redundancy suggest that co-evolution might have shaped the gene duplication event as well as provide the necessary selection pressure that maintains the multiple copies in the chromosome. Taken together, T3SSs effectors in both the yersiniae and *Aeromonas* species as well as T2- and T6SS effectors in *Aeromonas* species converge on modulating the host immune response to promote bacterial virulence.

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Role of type I interferons in inflammasome activation, cell death, and disease during microbial infection

R. K. Subbarao Malireddi and Thirumala-Devi Kanneganti*

Department of Immunology, St. Jude Children's Research Hospital, Memphis, TN, USA

Edited by:
Amal O. Amer, The Ohio State University, USA

Reviewed by:
Mikhail A. Gavrilin, The Ohio State University, USA
Dario S. Zamboni, Universidade de São Paulo, Brazil

***Correspondence:**
Thirumala-Devi Kanneganti, Department of Immunology, St. Jude Children's Research Hospital, MS #351, 262 Danny Thomas Place, Suite E7004, Memphis, TN 38105-2794, USA
e-mail: thirumala-devi.kanneganti@StJude.org

Interferons (IFNs) were discovered over a half-century ago as antiviral factors. The role of type I IFNs has been studied in the pathogenesis of both acute and chronic microbial infections. Deregulated type I IFN production results in a damaging cascade of cell death, inflammation, and immunological host responses that can lead to tissue injury and disease progression. Here, we summarize the role of type I IFNs in the regulation of cell death and disease during different microbial infections, ranging from viruses and bacteria to fungal pathogens. Understanding the specific mechanisms driving type I IFN-mediated cell death and disease could aid in the development of targeted therapies.

Keywords: type I IFN, IFNβ, NLR, caspase-1, inflammasome, cell death, Nlrp3

INTRODUCTION

Interferons (IFNs) are broadly classified into three groups, which are denoted as type I, II, and III based on the specific receptor utilization for their signal transduction. The type I IFN family comprises subtypes of IFNα (13 subtypes), IFNβ, IFNω, and IFNε (Pestka et al., 2004; Hertzog and Williams, 2013). All of the type I IFNs bind to a common heterodimeric receptor, called the IFNα/β receptor (IFNAR), composed of two chains, IFNAR1 and IFNAR2, that are associated with the tyrosine kinases Tyk2 and Jak1. Activated Tyk2 and Jak1 recruit and phosphorylate several signal transducer and activator of transcription (STAT) family members (**Figure 1**) (Pestka et al., 2004; Platanias, 2005). Activated STAT1 forms a dimer with STAT2, leading to the recruitment of IRF9 and subsequent formation of a heterotrimeric complex called IFN-stimulated gene factor 3 (ISGF3) (**Figure 1**). This complex translocates to the nucleus, where it binds upstream IFN-stimulated response elements (ISRE) and activates the transcription of type I IFN-inducible genes (Pestka et al., 2004; Platanias, 2005). Type I IFNs are classically known for their antiviral immune responses; however, several studies have demonstrated that a wide range of non-viral pathogens can also induce their expression. However, the specific mechanisms and physiological consequences of IFN responses to such pathogens are poorly understood. Various studies have attributed contrasting roles and differential outcomes to type I IFNs in immune responses to diverse microbial pathogens. The ability of IFNs to regulate cell death has been known for a long time and recent studies have started to reveal the specific mechanisms involved.

During the course of evolution, the arms race between bacterial pathogens and host organisms has resulted in the development of virulence mechanisms by microbes and the reciprocal development of host counter strategies to efficiently defend against them. Cell death has emerged as one of the important aspects of such a race between microbes and the host, exploited by both, while the final outcome is dependent on the specific pathogen and cell types involved. The in depth molecular mechanisms of how type I IFN signaling causes differential outcomes during different microbial infections remains to be established. Here we discuss the role of type I IFNs in regulating cell death and disease in various infection models and highlight its emerging role in inflammasome activation.

Abbreviations: IFN-I, type I interferon; IFNAR, Interferon-α/β receptor; ISRE, Interferon stimulated response element; ISGs, Interferon stimulated genes; TLR, TOLL-like receptor; NLR, NOD-like receptor.

INTRACELLULAR BACTERIA

LISTERIA

Listeria monocytogenes (LM), a gram-positive intracellular bacterium that grows rapidly inside host cells, is known to potently induce type I IFN production in mouse (Havell, 1986; O'Riordan et al., 2002) and human (Reimer et al., 2007) macrophages. LM causes life-threatening infections in immunocompromised individuals and may lead to septic abortion in pregnant women (Pamer, 2004). Upon internalization by phagocytes, LM escapes the early phagosome by secreting a hemolytic toxin, Listeriolysin O (LLO) (Portnoy et al., 1988). *Listeria* mutants that do not express the LLO toxin fail to escape the phagosome or to induce IFN-β production. LM-induced type I IFN production is largely independent of TLR signaling and is instead mediated by cytoplasmic RLR- and STING-dependent pathways through the TBK1-IRF3 signaling axis (Ishikawa et al., 2009; Woodward et al., 2010). In addition, *in vivo* studies demonstrated that *Irf3*$^{-/-}$ and *Ifnar*$^{-/-}$ mice, which neither make nor respond to type I IFNs, respectively, are highly resistant to *L. monocytogenes* infection (Auerbuch et al., 2004; Carrero et al., 2004; O'Connell et al., 2004). Furthermore, priming with poly(I:C), a

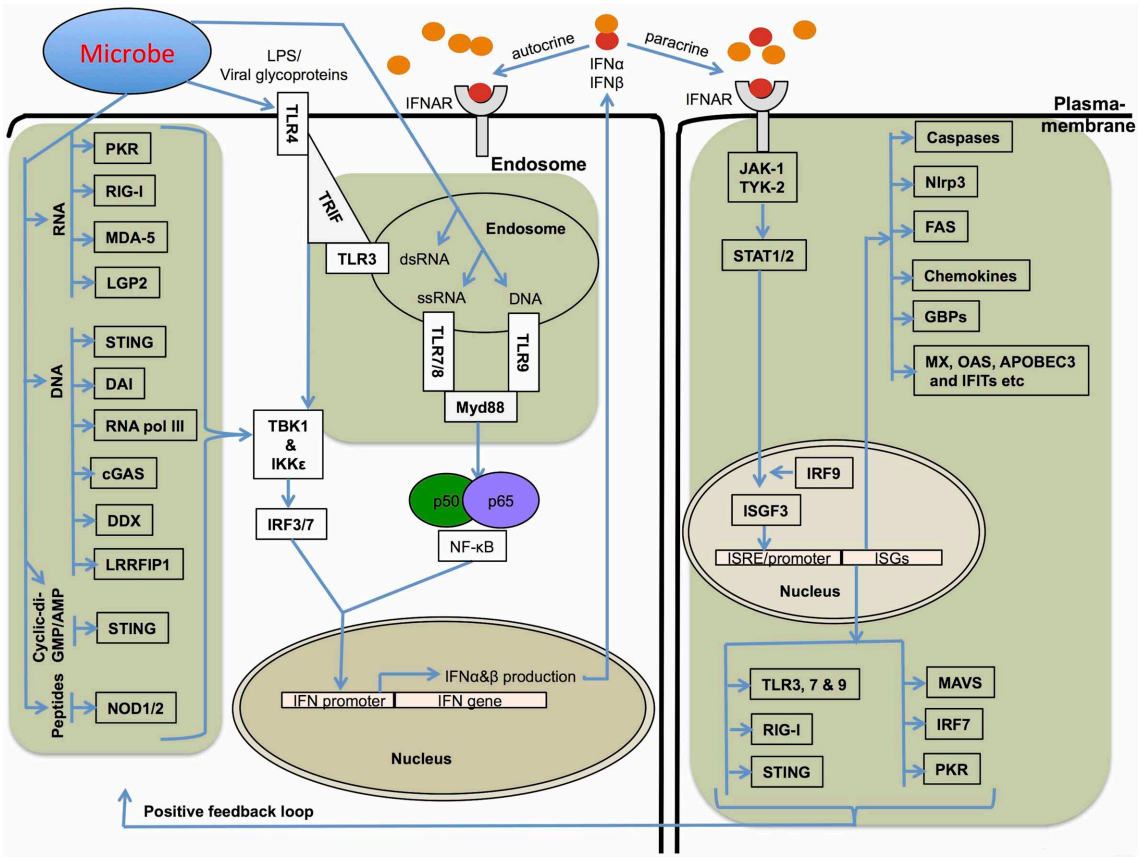

FIGURE 1 | Mechanisms involved in type I IFN production and its receptor signaling. Several different stimuli including pathogen derived LPS, glycoproteins, RNA and DNA can induce type I IFNs (IFNα and IFNβ) via upstream pattern recognition receptors. TLRs sense the endosomally located PAMPs (pathogen associated molecular patterns) and DAMPs (damage associated molecular patterns) and recruit TRIF (TRL3 and TLR4) or Myd88 (TLR4, 7, 8, and 9), which further transfer the signals downstream to IRF3 and IRF7. RLRs, NLRs, DAI, STING, and related receptors sense nucleic acids in the cytoplasm. The RNA sensors, RIG-I, MDA-5, and LGP2, and DNA sensor STING use the adapter protein MAVS (mitochondrial antiviral signaling protein) to transfer signals to IRFs for type I IFN transcription. RIG-I and MDA-5 also sense ligands generated by RNA polymerase III from cytoplasmic DNA PAMPs. All these pathways utilize the common downstream kinases, TBK1/IKKε for activating Interferon transcription factors. Type I IFNs bind to IFNAR receptor and activate a robust transcriptional pathway through a JAK-STAT signaling pathway. The transcriptional complexes activated by type I IFN signaling bind to specific ISRE/GAS sequences and lead to the expression of several genes important for cell death, cell proliferation and immune responses.

well known type I-inducing agent, results in enhanced death rate in LM-infected WT mice but not in *Ifnar*$^{-/-}$ mice (O'Connell et al., 2004), indicating a detrimental role for type I IFNs during *Listeria* infection. Moreover, LM-infected *Ifnar*$^{-/-}$ mice were shown to have reduced lymphocyte and splenocyte apoptosis and antibody-dependent neutralization of LLO controlled infection that resulted in reduced pathology (Auerbuch et al., 2004; Carrero et al., 2004; O'Connell et al., 2004). However, Rayamajhi et al. have proposed an alternative mechanism, where type I IFNs-dependent down-regulation of IFNγ receptor results in heightened susceptibility of wild type mice to *Listeria* infection (Rayamajhi et al., 2010).

Listeria is known to trigger assembly of multiple types of inflammasomes that include Aim2, Nlrc4, and Nlrp3 for caspase-1 activation in mouse macrophages. During *Listeria* infection, type I IFNs have significant roles in regulation of inflammasome activation and pyroptosis (Henry et al., 2007; Kim et al., 2010; Rathinam et al., 2010; Wu et al., 2010). A few studies have attempted to dissect the relative contributions of each of these inflammasomes and demonstrated that the detection of DNA by AIM2 receptor was indispensable for inflammasome activation and pyroptosis during *Listeria* infection in human PBMCs and mouse macrophages (Kim et al., 2010; Rathinam et al., 2010; Sauer et al., 2010). In addition, Sauer et al. demonstrated that pyroptosis was totally dependent on Aim2, while the Nlrp3 and Nlrc4 inflammasomes were dispensable for this process (Sauer et al., 2010). These reports implicate that type I IFNs play a role in the efficient induction of inflammasome activation and pyroptosis.

LEGIONELLA

Legionella pneumophila is an intracellular, gram-negative bacterial pathogen that replicates in host macrophages and causes a severe pneumonia called Legionnaires' disease. Lipmann et al. reported that *L. pneumophila*-infected mouse macrophages produce IFNβ in a STING- and IRF3-dependent manner (Stetson

and Medzhitov, 2006a; Lippmann et al., 2011). By contrast, Monroe et al. have demonstrated that *L. pneumophila* RNA also stimulated a Rig-I-dependent IFN response and proposed that *L. pneumophila* RNA, or host RNA, rather than *L. pneumophila* DNA, as the primary ligand that stimulates the host IFN response (Monroe et al., 2009). IFN-αβ inhibits *L. pneumophila* replication in the permissive A/J or CD1 mouse macrophages (Schiavoni et al., 2004). Furthermore, Bastian et al. have reported that *Legionella* is controlled by IFNβ induced in human lung epithelial cells via MAVS and IRF3 (Opitz et al., 2006). Dendritic cells (DCs) and macrophages are capable of restricting *L. pneumophila* growth through NAIP5-dependent caspase-1 activation and cell death. However, DCs were shown to undergo a more rapid apoptosis than macrophages, leading to enhanced restriction of *Legionella* growth (Nogueira et al., 2009). Indeed, eliminating the pro-apoptotic proteins BAX and BAK or over-expressing the anti-apoptotic protein BCL-2 were both found to restore *L. pneumophila* replication in DCs (Nogueira et al., 2009). Furthermore, a sub-population of DCs, plasmacytoid DCs (pDCs) is known to express higher levels of IFNs (Liu, 2005), which can potentially contribute to the higher cell death responses to *Legionella*. How *Legionella*-induced type I IFN promotes cell death is not well understood currently, however, one possible mechanism might be that Type I IFN-dependent up regulation of pro-cell death molecules like BAK and TRAIL can potentiate apoptosis (Fuertes Marraco et al., 2011; Cohen and Prince, 2013).

MYCOBACTERIUM

Mycobacterium spp. are pathogenic intracellular bacteria that cause tuberculosis (TB) and leprosy. Human and mouse myeloid cells secrete type I IFNs in response to mycobacterial infections (Pandey et al., 2009; Berry et al., 2010; Novikov et al., 2011). Blood based profiling has identified type I IFN-induced genes as the most striking characteristic signature of active TB (Berry et al., 2010). In addition, Wu et al. have reported that several TB-induced genes have key transcription factor binding sites for STATs, IRF-1, IRF-7, and OCT-1 (Wu et al., 2012). IFN-β and its downstream genes, including interleukin-10 (IL-10), were induced in monocytes by *M. leprae in vitro* and were preferentially expressed in progressive lepromatous lesions (Teles et al., 2013). Manca et al. have reported that type I IFNs enhance the virulence of *M. tuberculosis* by suppression of Th1 type immune responses. They have also shown that treatment with purified IFN-α/β increases lung bacterial loads, resulting in reduced survival in mice (Manca et al., 2001). In addition, another study reported that treatment with exogenous type I IFN results in a striking loss of mycobacteriostatic activity in monocytes and macrophages (Bouchonnet et al., 2002). Furthermore, Mayer-Barber et al. have shown that both IL-1α and IL-1β are critical for host resistance to TB and provided evidence that type I IFNs suppressed IL-1 production (Mayer-Barber et al., 2011). However, early clinical studies suggested that type I IFNs have beneficial effects against pulmonary TB (Giosue et al., 1998; Palmero et al., 1999). Together, these studies indicate that the role of type I IFNs in mycobacterial infections is debatable and requires further research to establish a clear consensus.

FRANCISELLA

Francisella tularensis is a gram-negative bacterium and causes tularemia. *F. tularensis* is classified as a Class A agent (having a high potential for use as a bioweapon) by United States regulators, due to its high virulence and ability to spread rapidly. Cytosolic recognition of *Francisella* induces type I IFN production in an IRF3-dependent manner (Henry et al., 2007). *Francisella* induces Aim2 inflammasome-dependent pyroptosis, which further depends on the ability of the bacterium to induce type I IFN production (Henry et al., 2007; Fernandes-Alnemri et al., 2010). Consistent with this, *Francisella*-induced Aim2 inflammasome activation and IL-1β secretion are abrogated in macrophages derived from bone marrow of *Irf3*- and *Ifnar*-deficient mice (Fernandes-Alnemri et al., 2010; Jones et al., 2010; Rathinam et al., 2010). Wild-type *Francisella* that can escape into the cytosol induces type I IFN and Aim2 inflammasome activation and host cell death (Mariathasan et al., 2005; Henry et al., 2007; Jones et al., 2010). This observation underscores the importance of cytosolic recognition of bacteria or their components (DNA in the case of *Francisella*) as an important innate immune mechanism to trigger inflammasome activation. Although studies in mice certainly support an important role for Aim2 in immune responses to *Francisella*, its role in human monocytes seems to be less prominent due to its lack of expression and/or induction in response to type I IFNs (Gavrilin and Wewers, 2011).

SALMONELLA

Salmonella enterica serovar Typhimurium (*S. Typhimurium*) is a gram-negative, intracellular pathogen that is quickly cleared by macrophages. This pathogen is a leading cause of acute gastroenteritis worldwide, which is transmitted primarily via the consumption of contaminated food or water. Induction of rapid cell death is a virulence strategy for this pathogen and contributes to dampening host innate immune responses (Lindgren et al., 1996). Robinson et al. have reported that *Salmonella* exploits type I IFN signaling for eliminating macrophages to establish infection (Robinson et al., 2012). Their studies also revealed that type I IFN-induced cell death of the macrophages is mediated by the classical RIP1-RIP3 dependent necroptosis pathway (Robinson et al., 2012). *Salmonella* is detected by NLRP3 and NLRC4 inflammasomes resulting in casapse-1 activation and pyroptosis (Franchi et al., 2006; Miao et al., 2006; Broz et al., 2010). Broz et al. have suggested the existence of an IFN-inducible regulator of caspase-11 that is crucial for activation of non-canonical Nlrp3 inflammasome activation in mutant *Salmonella*- [type 3 secretion system mutant, i.e., *Salmonella* pathogenicity island 1(SPI-1)] infected mouse macrophages (Broz et al., 2012). Their study revealed that *Salmonella* induces expression and activation of caspase-11 through a Toll-like receptor 4 (TLR4)-dependent and TIR-domain containing adaptor-inducing IFN-β (TRIF)-mediated IFNβ signaling pathway. Consistent with this, *Ifnar1*$^{-/-}$ or *Irf3*$^{-/-}$, or *Stat-1*$^{-/-}$ macrophages infected with mutant *Salmonella* did not process the casapase-11 or activate the non-canonical cell death pathway. Furthermore, *in vivo Casp1*$^{-/-}$ mice are more susceptible to Salmonella infection than the *Casp1*$^{-/-}$ *Casp11*$^{-/-}$ mice (Broz et al., 2012). Thus, these results indicate that caspase-11

mediated cell death results in detrimental effects to the host. Together, it is evident that *Salmonella* exploits type I IFN signaling to rapidly kill the immune cells to cause disease in the host.

EXTRACELLULAR BACTERIA

STAPHYLOCOCCUS

Staphylococcal infections have recently emerged as a significant problem to human health, due to the emergence of antibiotic resistant strains that cause life-threatening infections, especially in post-influenza exposures (Klevens et al., 2007; Martin et al., 2009; David and Daum, 2010). *S. aureus* infected mouse and human epithelial cells produce type I IFN in a STAT3-dependent manner in response to its virulent protein A (Martin et al., 2009). In mouse myeloid DCs and macrophages, TLR9 and IRF1 have important roles (Schmitz et al., 2007), while the cell wall component lipoteichoic acid (LTA) utilizes the IRF1-STAT1 axis in mouse macrophages to induce type I IFNs (Liljeroos et al., 2008). Absence of IFNAR signaling results in protection against lethal *S. aureus* pneumonia infection compared to wild-type control mice (Martin et al., 2009). Following recognition of *S. aureus* α-hemolysin, mouse macrophages undergo pyroptosis in an Nlrp3 inflammasome-dependent manner (Mariathasan et al., 2006; Craven et al., 2009). In contrast to Martin et al., a recent report demonstrated that IFNα induces phospholipid scramblase 1 (PLSCR1) in human lung epithelial cells as part of an innate protective mechanism to a bacterial pore-forming toxin (Lizak and Yarovinsky, 2012) and another study demonstrated a protective role of CpG DNA (a potent inducer of type I IFN production) in a mouse model of *S. aureus* pneumonia (Roquilly et al., 2010). Furthermore, Kaplan et al. have shown that phagosomal degradation and cytosolic release of intracellular ligands are essential for the induction of IFN-β in mouse and human DCs, which is required for the host defense against *S. aureus* during cutaneous infection in mice (Kaplan et al., 2012). Taken together, these reports suggest that type I IFNs can have both protective and detrimental roles during *S. aureus* infection. The disease outcome is variable and may depend on the immune status of the host, the site of infection and the specific strains causing the infection.

STREPTOCOCCUS

S. pneumonia causes acute lung infections and activates type I IFN expression (Joyce et al., 2009; Parker et al., 2011). DAI (DNA-dependent activator of IFN-regulatory factors) dependent recognition of bacterial DNA is proposed to be responsible for inducing type I IFN expression through cytoplasmic DNA sensing pathway involving STING, TBK1 and IRF3-dependent signaling pathways (Parker et al., 2011). Type I IFN treatment enhances protection of mice against *S. pneumoniae* (Weigent et al., 1986). However, prior exposure to influenza A virus leads to increased susceptibility to bacterial infections in a type I IFN-dependent manner (Morens et al., 2008; Shahangian et al., 2009). As opposed to bacterial infection alone, type I IFNs produced during secondary infection with *S. pneumonia* inhibits production of chemokines like CXCL1 and CXCL2 and sensitizes hosts to secondary bacterial pneumonia (Shahangian et al., 2009).

PSEUDOMONAS

Pseudomonas aeruginosa is a causative agent of pneumonia and infection in cystic fibrosis (CF) patients is associated with significant mortality (Zhuo et al., 2008). *P. aeruginosa* induces type I IFN expression predominantly through the TLR4-TRIF-IRF3 axis (Parker et al., 2012). TLR4 signaling was shown to be important for clearance of *P. aeruginosa* from the lungs and preventing sepsis in infected hosts (Faure et al., 2004; Ramphal et al., 2005; Skerrett et al., 2007; Cohen and Prince, 2013). Similarly, *Trif* and *Irf*3 deficiencies in mice resulted in reduced expression of type I IFN-induced chemokines including CXCL10 (IP-10) and CCL5 (RANTES) and abrogated neutrophil recruitment to the lungs leading to impaired bacterial clearance (Power et al., 2007; Carrigan et al., 2010). These results indicate a protective role for type I IFNs during *P. aeruginosa* infection (Roy et al., 2013). *P. aeruginosa* infection of mouse macrophages activates the Nlrc4 inflammasome and induces pyroptosis in a flagellin independent manner (Sutterwala et al., 2007). However, later studies demonstrated a requirement of cytosolic flagellin for Nlrc4 inflammasome activation (Miao et al., 2008; Arlehamn and Evans, 2011). Bacterial expression of specific adhesins, lipopolysaccharide, and a functional type III secretion system were all shown to be necessary to evoke apoptosis (Sutterwala et al., 2007) and the cytotoxin, ExoU-expressing *P. aeruginosa* strain has been shown to inhibit caspase-1 dependent pyroptosis (Sutterwala et al., 2007).

ANTHRAX

Bacillus anthracis is a gram-positive, aerobic bacterium that causes severe pulmonary, gastrointestinal, and cutaneous infections (Dixon et al., 1999). Production of the lethal toxin (LeTx) by this bacterium causes extensive cell death, tissue damage and systemic disease. LeTx is composed of a protective antigen (PA) and lethal factor (LF). Gold et al. found that endogenous IFNs (type I and II) inhibit the germination of *B. anthracis* spores, but exogenous application enhanced inflammation thereby increasing mortality (Gold et al., 2007). In addition, Walberg et al. showed that recombinant murine IFNβ or type I IFN inducers like poly(I:C) provide marked protection against "Inhalation Anthrax" (Walberg et al., 2008). *B. anthracis* LeTxs activate the Nlrp1b inflammasome and pyroptosis in mice (Boyden and Dietrich, 2006; Bergsbaken et al., 2009). It is recognized that macrophages from inbred mice may or may not be sensitive to *B. anthracis*-induced pyroptosis based on the presence of the Nlrp1b inflammasome responsive $Nlrp1b^{S/S}$ or non-responsive $Nlrp1b^{R/R}$ alleles (Moayeri et al., 2010; Terra et al., 2010). In this case, pyroptosis of macrophages is believed to counter anthrax infection since IL-1β released during this process helps to generate protective neutrophil responses. It will be essential to study the contribution of type I IFN in pyroptosis induction during *B. anthracis* infection and investigate whether type I IFN can promote apoptosis of neutrophils and initiate other possible detrimental effects.

VIRUSES

IFNs were originally discovered as antiviral molecules. Viruses are considerably smaller than other microbial pathogens but represent a major threat to human and other animal health. Extensive

progress has been made in understanding the mechanisms of type I IFN production in response to viruses (Stetson and Medzhitov, 2006b; Gonzalez-Navajas et al., 2012; MacMicking, 2012). The diverse mechanisms of cellular entry and tropism of viruses are detected by different TLRs that are strategically located in different cellular compartments (**Figure 1**). Endosomally-located PRRs including TLR3, 7, 8, and 9 are known to trigger type I IFNs (**Figure 1**). TLR7 and TLR9 have particularly important roles in plasmacytoid DCs. Once the virus enters the host cytoplasm, several cytoplasmic receptors such as RIG-I-like receptors (RLRs RIG-I, MDA5 and LGP2) and NOD-like receptors (NOD1 and NOD2) monitor the cytoplasm for microbial PAMPs and initiate type I IFN production and associated immune responses (**Figure 1**).

Programmed cell death is a critical host defense against viruses and type I IFNs are known to be involved to this process. Multiple viruses have been discovered to encode proteins that function to subvert host-induced cell death during infection (Bowie and Unterholzner, 2008; Galluzzi et al., 2010; Kaminskyy and Zhivotovsky, 2010; Gregory et al., 2011). Death of the infected cells is detrimental to viral replication and amplification of viral progeny. However, death of the infected cells can also facilitate viral egress and enhance pathogenesis. Therefore, different viruses have evolved complex mechanisms to enhance or inhibit different forms of cell death (Kaminskyy and Zhivotovsky, 2010). Microarray based studies revealed that a large number of genes are regulated by type I IFNs and several of them are involved in cell death (Der et al., 1998; de Veer et al., 2001; Hertzog and Williams, 2013; Rusinova et al., 2013). However, the mechanisms driving cell death that involve proteins encoded by many of these genes are still awaited.

Uncontrolled chronic viral infections can result in sustained expression of type I IFNs with detrimental pathophysiological outcomes. Two recent studies reported the role of type I IFNs in viral persistence during lymphocytic choriomeningitis virus (LCMV) infection (Teijaro et al., 2013; Wilson et al., 2013). Results from these reports show that robust and acute type I IFNs secreted during early in the infection serve to control viral replication and spread by promoting apoptosis of infected cells and enhancing T cell activation. During chronic infection, prolonged expression and exposure to type I IFNs leads to lymphocyte exhaustion, and reduced viral clearance due to the presence of increased immunosuppressive molecules like IL-10 and PD-L1. Loss of circulating pDCs has been documented in chronic viral infections in mice and humans, which correlates with uncontrolled viral loads, reduced T cell counts and onset of opportunistic infections (Swiecki et al., 2011). However, the mechanism of type I IFN-dependent pDC apoptosis is not entirely clear and represents an important subject for future research.

Type I IFNs promote cell death in multiple ways. Balachandran et al. have demonstrated that type I IFN and protein kinase R (PKR) can sensitize cells to apoptosis primarily through the FADD/caspase-8 pathway (Balachandran et al., 2000). In this study, stimulation of mouse cells with IFN-α/β resulted in enhanced apoptosis and reduced viral replication. In a follow up study, Ezelle et al. showed that the poxvirus-encoded protein CrmA was able to inhibit both viral infection- and dsRNA-mediated apoptosis (Ezelle et al., 2001). Both HSV and vaccinia virus affect PKR and RNase L-mediated apoptosis pathways that are activated by dsRNA products released during viral replication (Der et al., 1997; Diaz-Guerra et al., 1997; Kibler et al., 1997). Influenza viruses cause severe lung pathology leading to lung failure and mortality. Type I IFNs have been recognized to mediate induction of pro-apoptotic TRAIL leading to excessive cell death and tissue injury (Hogner et al., 2013). Infection or treatment with type I IFN induces pro-apoptotic genes; IFN-stimulated gene 54 (ISG54) or IFN-induced gene with tetratricopeptide repeats 2 (IFIT2) that promotes apoptosis by mitochondrial-associated BCL2 family proteins (Reich, 2013).

FUNGI

While type I IFNs are widely known as anti-viral factors, which are either protective or detrimental in bacterial infections, their role in fungal infections is poorly defined. Recently, two reports have shown that *Candida* spp. induce IFN-β in mouse bone marrow-derived DCs (BMDCs) and macrophages (Biondo et al., 2008, 2012; Bourgeois et al., 2011). Different forms of fungal glucans and mannans are sensed by a wide range of innate pattern receptors like TLRs, CLRs, dectins, and mannose receptors and initiate MyD88–mediated NF-κB and MAPK pathways or SYK-CARD9 signaling for cytokine induction and cell death (Netea et al., 2008; Brown, 2011). During fungal infections, type I IFNs are also produced by a TLR-independent pathway requiring RNA sensor MAVS and IRF3 (Inglis et al., 2010). In contrast, Del Fresno et al. have recently reported that *Candida albicans* induces type I IFN in DCs through a DECTIN-1, SYK-, and CARD9-dependent pathway that requires IRF5-mediated transcription but not IRF3 or IRF7 (Del Fresno et al., 2013). Jensen et al. have reported that poly(I:C)-induced or exogenously added IFNα and IFNβ treatments of macrophages suppress anti-*Candida* immune responses and cause death of infected mice (Worthington and Hasenclever, 1972; Jensen et al., 1992; Jensen and Balish, 1993). In addition, *Ifnar*$^{-/-}$ mice are extremely resistant to otherwise lethal *Candida* and *Histoplasma* infections (Inglis et al., 2010; Majer et al., 2012). These studies demonstrated that type I IFNs induce severe kidney damage by promoting excessive recruitment and activation of inflammatory monocytes and neutrophils. However, other reports suggest that type I IFN can be beneficial as part of the host immune response to *C. albicans* (Biondo et al., 2011; Del Fresno et al., 2013).

ROLE OF INTERFERONS IN INFLAMMASOME ACTIVATION AND PYROPTOSIS

Type I IFNs are innate immune effector molecules with strong pro-inflammatory activities, and have been shown to contribute to the high mortality rates in septic shock syndromes (Karaghiosoff et al., 2003; Huys et al., 2009). Type I IFNs also contribute to inflammasome-dependent caspase-1 activation leading to pro-inflammatory pyroptotic cell death (**Figure 2**) (Anand et al., 2011; Franchi et al., 2012). There have been multiple different inflammasomes identified that sense a diverse array of microbial- and damage-associated PAMPs. These include the Naip-Nlrc4 inflammasome (Mariathasan et al., 2004; Kofoed and Vance, 2011; Zhao et al., 2011), the Nlrp1b inflammasome

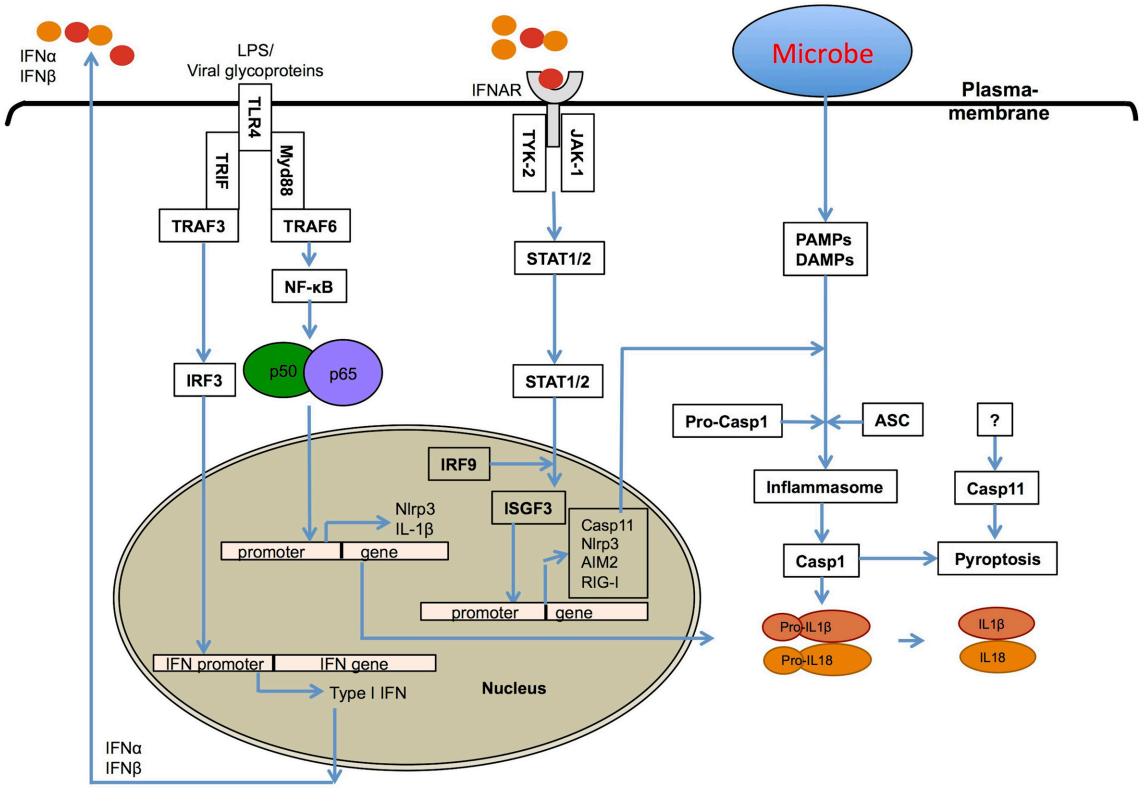

FIGURE 2 | Role of type I interferons in inflammasome activation. Type I IFNs contribute to inflammasome activation through two different mechanisms. First, type I interferons are required for the upregulation of caspase-11, which contributes to activation of a non-canonical NLRP3 inflammasome in response to enteropathogenic bacteria, such as *Citrobacter rodentium* and *Escherichia coli*. Second, they prime the expression of inflammasome-forming NLRP3, RIG-I and AIM2 molecules for potentiating inflammasome activation.

(Boyden and Dietrich, 2006; Masters et al., 2012), the Nlrp3 inflammasome (Kanneganti et al., 2006b; Mariathasan et al., 2006; Sutterwala et al., 2006; Anand et al., 2011), the Nlrp6 inflammasome (Elinav et al., 2011), the Nlrp12 inflammasome (Vladimer et al., 2012), the Aim2 inflammasome (Fernandes-Alnemri et al., 2010; Jones et al., 2010; Rathinam et al., 2010; Sauer et al., 2010), the RIG-I inflammasome (Poeck et al., 2010; Pothlichet et al., 2013), and the IFI16 inflammasome (Kerur et al., 2011). Inflammasome-dependent casapase-1 activation and pyroptosis are associated with the production of mature IL-1β and IL-18 cytokines, which generates a pro-inflammatory environment in host tissues (**Figure 2**). Inflammasome-dependent pyroptosis shares features of both apoptosis and necrosis and is tightly regulated by distinct signaling pathways.

NLRP3

NLRP3 is the most widely studied inflammasome and it requires two signals for its assembly into an active complex (Kanneganti, 2010; Anand et al., 2011). The first signal is TLR-dependent expression of NLRP3, while the second is often a damage related factor such as production of reactive oxygen species (ROS) or membrane damage (Anand et al., 2011). Activated TLRs transfer signals through two major adapters that specify the downstream signaling pathways (Takeuchi and Akira, 2010). The first one, MyD88 is required for NF-κB activation downstream of all TLRs except TLR3. The second, TRIF plays a dominant role in TLR3-dependent NF-κB activation and TLR4-mediated IRF signaling (Fitzgerald et al., 2003a,b; Sato et al., 2003; Yamamoto et al., 2003).

Recently, our lab and other groups have shown that the TLR4-TRIF axis regulates caspase-11 expression and non-canonical Nlrp3 inflammasome-mediated host defense against enteropathogens, *Escherichia coli* (EHEC), *Citrobacter rodentium*, and *Salmonella Typhimurium* (Kayagaki et al., 2011; Broz et al., 2012; Gurung et al., 2012; Rathinam et al., 2012). Consistent with this, Sander et al. have reported that the gram-negative bacterium *E. coli* induces IFN-β and activates the Nlrp3 inflammasome in the absence of virulence factors other than microbial mRNA (Sander et al., 2011). In addition, Rathinam et al. demonstrated that the IRF3-type I IFN-IFNAR-STAT-1 signaling pathway is indispensable for caspase-11 expression and activation of the Nlrp3 inflammasome and pyroptosis (Rathinam et al., 2012). Furthermore, they demonstrated that IFNβ treatment significantly increased pro-caspase-11 expression and that once induced, caspase-11 undergoes spontaneous activation. Broz et al. have observed that IFNAR-STAT-1 axis is important for caspase-11 activation, but not for its expression in *Salmonella* infected macrophages (Broz et al., 2012). In a subsequent review, they speculated that type I IFN-dependent expression of a yet-unidentified host molecule may trigger caspase-11 activation or

that a unknown bacterial signal is required (Broz and Monack, 2013).

Two recent studies have revealed that several gram-negative bacteria, but not gram-positive bacteria, can activate the non-canonical Nlrp3 inflammasome and identified LPS as their common PAMP responsible for the activation of Caspase-11 (Hagar et al., 2013; Kayagaki et al., 2013). Both of these studies have shown that intracellular LPS is sufficient to induce the activation of caspase-11. Furthermore, these studies revealed that a subcomponent of LPS, lipid A is sufficient to activate the casaspe-11-dependent non-canonical Nlrp3 inflammasome. They presented evidence that when transfected, intracellular LPS or lipid A could activate the non-canonical Nlrp3 inflammasome independent of TLR4 or the TLR4-associated lipid A-binding proteins MD1 and MD2 and even the TLR4 downstream signaling molecules TRIF and IFNAR receptor molecules. In contrast, a study by Guarda et al. reported that type I IFNs suppress Nlrp1 and Nlrp3 inflammasomes in a STAT1-dependent manner (Guarda et al., 2011). Further studies are required to clarify the role of type I IFNs and their precise mechanisms in the regulation of inflammasome activation. Of particular importance is that the intracellular sensor of lipid A is yet to be identified.

Together, regardless of the exact mechanisms, type I IFNs have clearly emerged as crucial regulators of inflammasome activation and pyroptosis (**Figure 2**). Physiologically, caspase-11 triggered pyroptosis is required for surveillance against bacteria that enter the cytosol, such as the sifA mutant of *S. Typhimurium*, an sdhA mutant of *Legionella pneumophila* and *Burkholderia* species (Aachoui et al., 2013). However, type I IFNs are known to be exploited by microbial pathogens to induce the death of immune cells and suppress host immune responses. In support of this, caspase-11-mediated cell death is responsible for pathology and detrimental effects *in vivo* (Wang et al., 1998; Kayagaki et al., 2011). For example, Broz et al. showed that wild type *Salmonella* induces caspase-11-mediated cell death in caspase-1-deficient mouse macrophages, and that caspase-11 increases the bacterial virulence and host cell susceptibility to infection (Broz et al., 2012). In addition, *Salmonella* also exploits type I IFN signaling to induce the RIP1-RIP3-dependent necroptotic cell death pathway to kill macrophages (Lindgren et al., 1996; Robinson et al., 2012).

RIG-I AND NLRP3

Viruses are by far the best-known inducers of type I IFNs and have also been recognized to induce activation of distinct inflammasomes, including those comprised of NLRP3, AIM2, and RIG-I. The first evidence of Nlrp3 inflammasome involvement in antiviral responses showed its role in sensing of both viral RNA and its analog poly(I:C) in mouse macrophages (Kanneganti et al., 2006a,b). *In vivo*, Nlrp3 inflammasome activation protected mice from influenza infection (Allen et al., 2009; Thomas et al., 2009). Poeck et al. have reported that Rig-I from mouse macrophages senses cytoplasmic RNA viruses and assembles an inflammasome (Poeck et al., 2010). More recently, RIG-I was reported to induce type I IFN through a MAVS/TRIM25/RNF135 signaling axis following influenza infection, and was shown to have profound effects on NLRP3 inflammasome activation and IL-1β secretion in human lung epithelial cells (Pothlichet et al., 2013). Together, these studies demonstrate that RIG-I can itself assemble an inflammasome and also contributes toward, type I IFN-dependent potentiation of NLRP3 expression (**Figure 2**).

AIM2

The cytosolic bacteria *Francisella novicida* and *Listeria* both induce killing of myeloid and lymphoid cells in a manner dependent on Type I IFN signaling, an effect which has been shown to be detrimental to the host (Auerbuch et al., 2004; Carrero et al., 2004; O'Connell et al., 2004; Henry et al., 2007). Several published studies have clearly established that both *Listeria* (Henry et al., 2007; Warren et al., 2008; Kim et al., 2010; Tsuchiya et al., 2010; Wu et al., 2010) and *Francisella* (Henry and Monack, 2007; Fernandes-Alnemri et al., 2010; Jones et al., 2010; Rathinam et al., 2010) activate the Aim2 inflammasome and pyroptosis in mouse macrophages. Type I IFNs prime AIM2 expression (Kotredes and Gamero, 2013) and potentiate cytosolic bacterial DNA recognition for inflammasome activation (Fernandes-Alnemri et al., 2010). Although mice studies show that type I IFN dependent caspase-11 expression is important for the activation of the non-canonical Nlrp3 inflammasome (Kayagaki et al., 2011; Gurung et al., 2012; Rathinam et al., 2012), it is not known if it is also required for AIM2 and RIG-I mediated caspase-1 activation or cell death.

CONCLUSIONS

IFNs were the first cytokines discovered to have immune regulatory capacity. Despite their clinical application in some treatment regimens, we still do not have a complete understanding of the mechanistic effects of IFNs required to further develop treatments that capitalize their full potential therapeutic effects. A vast amount of past research has been focused on the role of IFNs as anti-viral molecules with a limited number of studies for other microbial infections. Recent studies have clearly indicated a dual role for type I IFNs both in infectious and inflammatory diseases. Despite the potential benefits, it is often challenging to manipulate type I IFNs for therapeutic purposes due to their role in regulating the expression and activation of a huge number of downstream genes, often complicating the conclusions. Part of the problem is that types I IFNs exert differential immunomodulation on diverse cell types, environments, and varying physiological conditions. The pathogen- and host-mediated counter regulatory pathways further complicate IFN-induced responses in cell death and disease.

Recently, type I IFNs were recognized as crucial regulators of non-canonical NLRP3 inflammasome activation and pyroptosis. Although the majority of literature indicates a positive role for inflammasomes in anti-microbial host defense, recent reports indicate detrimental effects due to excessive cell death, inflammation, and collateral tissue damage in vital organs (Lupfer and Kanneganti, 2012). A paradox exists, where inflammasomes intended to defend against cytoplasmic invaders by pyroptosis, which is predominantly protective *in vitro*, however in *in vivo*, if exceeds a certain level, can lead to cell and tissue damage and organ failure resulting in negative outcomes. The paradox for type-I IFNs is that their ability to inhibit microbial spread by inducing cell death is counteracted by apoptotic depletion of immune cells and inhibiting anti-microbial immune responses leading to immune suppression. Future research should explore

the detailed molecular mechanisms that are responsible for type I IFN-dependent cell death and inflammasomes activation in the context of immunity and immunopathology. These findings may lead to better-targeted therapeutic interventions to treat inflammatory and infectious diseases.

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Subversion of inflammasome activation and pyroptosis by pathogenic bacteria

Larissa D. Cunha and Dario S. Zamboni*

Department of Cell Biology, Ribeirão Preto Medical School, University of São Paulo (FMRP/USP), Ribeirão Preto, Brazil

Edited by:
Yongqun He, University of Michigan School of Medicine, USA

Reviewed by:
Mikhail A. Gavrilin, Ohio State University, USA
Eric Ghigo, Centre National de la Recherche Scientifique, France

****Correspondence:***
Dario S. Zamboni, Department of Cell Biology, Ribeirão Preto Medical School, University of São Paulo (FMRP/USP), Av. Bandeirantes 3900, Ribeirão Preto, SP 14049-900, Brazil
e-mail: dszamboni@fmrp.usp.br

Activation of the inflammasome occurs in response to a notably high number of pathogenic microbes and is a broad innate immune response that effectively contributes to restriction of pathogen replication and generation of adaptive immunity. Activation of these platforms leads to caspase-1- and/or caspase-11-dependent secretion of proteins, including cytokines, and induction of a specific form of cell death called pyroptosis, which directly or indirectly contribute for restriction of pathogen replication. Not surprisingly, bona fide intracellular pathogens developed strategies for manipulation of cell death to guarantee intracellular replication. In this sense, the remarkable advances in the knowledge of the inflammasome field have been accompanied by several reports characterizing the inhibition of this platform by several pathogenic bacteria. Herein, we review some processes used by pathogenic bacteria, including *Yersinia* spp., *Pseudomonas aeruginosa*, *Vibrio parahaemolyticus*, *Chlamydia trachomatis*, *Francisella tularensis*, *Shigella flexneri*, *Legionella pneumophila*, and *Coxiella burnetii* to evade the activation of the inflammasome and the induction of pyroptosis.

Keywords: inflammasome inhibition, pyroptosis, infection control, subversion strategies

INTRODUCTION

Host pattern recognition receptors (PRRs) are capable of sensing conserved microbial molecules, referred as Pathogen-Associated Molecular Patterns (PAMPs) as well as cellular disturbances, referred as Damage-Associated Molecular Patterns (DAMPs). PRR activation usually leads to induction of pro-inflammatory signaling networks that facilitate direct elimination of the pathogens but also to alert the immune system. Consequently, successful replication of an intracellular infectious agent relies not only on the arsenal of virulence factors that modulate host cell functions to establish a replicative niche, but also in the development of efficient subversion strategies to evade host recognition and bypass the host mechanisms related to restriction of pathogen replication.

Induction of cell death pathways is a conserved host response to infection. However, different subtypes of cell death can be triggered and they will vary according to many factors, e.g., the type of infected cell and the surrounding environment, the infectious agent and the infection dosage. Interestingly, the same mechanism of cell death can elicit either an immunogenic or a tolerogenic ("silent") effect upon the immune system, however, the factors controlling such plasticity remain elusive (Green et al., 2009). Apoptosis, autophagy, and necrosis are still considered the main types of cell death, but several other subtypes can be distinguished based mostly on biochemical and functional criteria (Galluzzi et al., 2012).

Of note, activation of intracellular PRRs belonging to the family of Nod-like receptors (NLRs) or the nucleic acid receptors AIM2 and IFI16 (members of the PYHIN family) trigger a specific type of potentially pro-inflammatory, caspase-1-dependent cell death program known as pyroptosis (Cookson and Brennan, 2001; Lamkanfi and Dixit, 2012). Upon sensing of pathogens, NLRs and AIM2/IFI16 trigger the formation of the inflammasome, a cytosolic molecular platform that recruits and activates caspase-1, initiating a program of pore formation in the plasma membrane of activated cells, with consequent cell rupture and release of cytosolic contents (Martinon et al., 2002; Fink and Cookson, 2006). The activity of caspase-1 also mediates the activation and controlled secretion of pro-inflammatory cytokines such as IL-1β and IL-18 (Thornberry et al., 1992; Ghayur et al., 1997; Gu et al., 1997). Although pyroptosis and cytokine secretion are both dependent on caspase-1 and occur concomitantly, it is not confirmed that cytokine release is mediated by induction of cell death pathway. Recently, caspase-11 was shown to mediate a non-canonical pathway of inflammasome activation in response to Gram-negative bacteria, leading to pyroptosis and release of cytokines such as IL-1α independently of caspase-1 activation (Kayagaki et al., 2011; Broz et al., 2012; Aachoui et al., 2013; Case et al., 2013; Casson et al., 2013). It was recently demonstrated that intracellular sensing of lipid A motif of lipopolysaccharide (LPS) induces caspase-11-dependent pyroptosis and NLRP3-dependent caspase-1 activation, with subsequent secretion of IL-1β and IL-18 (Hagar et al., 2013; Kayagaki et al., 2013). Most strikingly, detrimental effects of exacerbated inflammation during systemic infectious are possibly mediated by caspase-11, but not caspase-1 (Kang et al., 2002; Kayagaki et al., 2011, 2013; Hagar et al., 2013). This novel caspase-11-mediated inflammasome may operate synergistically with the other caspase-1- mediated inflammasomes for the recognition of pathogenic bacteria encoding type III/type IV secretion systems or escaping the vacuole (**Figure 1**).

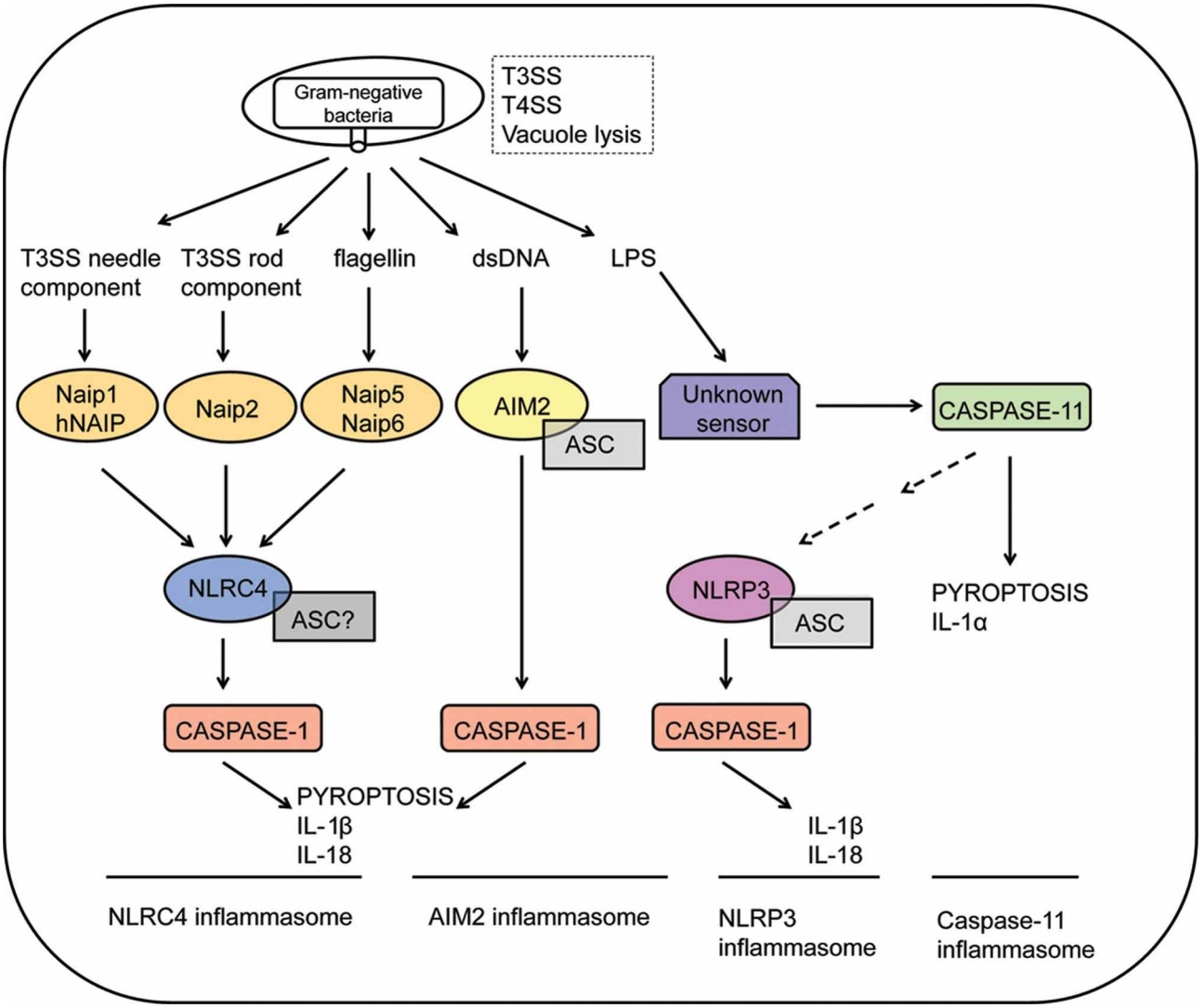

FIGURE 1 | Inflammasome activation in response to Gram-negative bacteria. Intracellular sensing of Gram-negative bacteria that violate cytosolic compartments by expressing type III or type IV secretion systems (T3SS and T4SS, respectively) or by inducing vacuolar lysis. NLRC4 inflammasomes are activated in response to recognition of bacterial flagellin by Naip5, T3SS needle proteins by murine Naip1 or by human NAIP (hNAIP), or T3SS rod proteins by Naip2. Activation of the NLRC4 inflammasomes culminates in caspase-1 activation, leading to IL-1β/IL-18 secretion and pyroptosis. The requirement of ASC to the assembly of the NLRC4 inflammasomes is still controversial. Recognition of cytosolic DNA by AIM2 leads to formation of a AIM2/ASC/Caspase-1 multimeric complex known as the AIM2 inflammasome. Cytosolic LPS of Gram-negative bacteria are recognized by an unknown receptor, triggering activation of caspase-11. This process is independent on ASC, NLRP3 and caspase-1, inducing pyroptosis and secretion of IL-1α. Non-canonical inflammasome activation mediated by caspase-11 also regulates NLRP3 activation by unclear mechanisms. Finally, formation of the NLRP3/ASC/caspase-1 complex leads to the secretion of inflammatory cytokines such as IL-1β and IL-18.

Activation of the inflammasome, with consequent induction of pyroptosis, has been demonstrated for several microbial pathogens (Osawa et al., 2011; Lamkanfi and Dixit, 2012; Lima-Junior et al., 2013; Silva et al., 2013). In the case of bacterial pathogens, pyroptosis is a mechanism that effectively contributes to infection control (Miao et al., 2010a, 2011; Terra et al., 2010). Evolutionary pressure thus, shaped modulation of the inflammasome activation, with consequent inhibition of pyroptosis, as a subversion strategy found among microbial pathogens. Bona-fide intracellular pathogens (such as bacteria and viruses that modulate host cell functions through secretion systems and secreted proteins) use diverse strategies to evade recognition and inflammasome activation. However, the molecular mechanisms of inflammasome inhibition by pathogens remain largely unknown. Herein, we review the current knowledge on the mechanisms of inflammasome and pyroptosis suppression by pathogenic bacteria. We discuss the importance of inflammasome subversion to their pathogenesis and highlight recent findings on the diverse strategies adopted by bacterial pathogens to inhibit the activation of the inflammasome and how they affect pyroptosis.

MECHANISMS OF PYROPTOSIS INHIBITION BY BACTERIAL PATHOGENS

Yersinia spp.

The pathogenic Gram-negative bacteria belonging to the genus *Yersinia* have a tropism to target lymphoid tissues, inducing distinct types of host cell death in the course of infection. The three human pathogens of the genus, *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* share a virulence plasmid encoding a conserved type III secretion system (T3SS) and a few identified effector proteins known as Yops (*Yersinia* outer proteins: YopE, YopT, YopH, YopM, YopA/O, and YopJ/P) (Trosky et al., 2008). The injection of Yops into infected cells allows the modulation of several signaling pathways and immune responses by *Yersinia*, including cell death. However, as the control of *Yersinia* multiplication is affected by a complex interplay of distinct types of cell death in different types of infected cells, it is likely that the demise of infected cells not only contributes to pathogenesis but also signals to mount an effective immune response (Philip and Brodsky, 2012). For instance, the early stage of infection is characterized by induction of apoptotic-like death of macrophages and dendritic cells, and YopJ, YopP (the homologous of YopJ in *Y. enterocolitica*) and YopK have already been implicated in this process, with evidence that apoptosis contributes to bacterial persistence *in vivo* (Mills et al., 1997; Monack et al., 1997, 1998; Ruckdeschel et al., 1998, 2001; Grobner et al., 2007; Peters et al., 2013). Translocation of YopJ is also implicated in late proinflammatory lytic cell death, independently of caspase-1 (Lilo et al., 2008). Although the molecular mechanisms triggering the inflammasome and caspase-1 activation in response to *Yersinia* are largely unknown, recognition of YopJ leads to differential regulation of inflammasome responses. Secretion of IL-1β in response to translocated YopJ requires caspase-1, Nlrp3, and Asc adaptor, whereas caspase-1 activation occurs in the absence of Nlrp3, Nlrc4, and Asc (Brodsky et al., 2010; Zheng et al., 2011). Proinflammatory cell death of infected macrophages mediated by YopJ does not require other inflammasome components such as Nlrp3, Nlrc4, and Asc, corroborating the lack of inflammasome participation in this process (Brodsky et al., 2010; Zheng et al., 2011). Moreover, T3SS recognition also induces caspase-1 activation and IL-1β secretion, requiring the inflammasome adaptor Asc and mediated by both Nlrp3 and Nlrc4, possibly in synergy (Brodsky et al., 2010). Notably, recognition of *Yersinia* T3SS also triggers caspase-1 mediated pyroptosis, independently of the effector YopJ, Nlrp3, Asc, and Nlrc4 (Bergsbaken and Cookson, 2007; Brodsky et al., 2010). Diverse effector proteins secreted by T3SS of *Yersinia* has already been shown to negatively modulate inflammasome activation with associated impairment of pyroptosis in response to *Yersinia* recognition. This accumulating evidence corroborates that although cell death processes might play different roles in the pathogenesis of *Yersinia*, evasion of inflammasome activation and inflammatory burst caused by pyroptosis should be important to bacterial success.

Regulation of cytotoxicity by differential secretion of YopJ is one of the processes that impacts virulence of *Yersinia*. Mutants of *Y. pseudotuberculosis* lacking YopJ do not induce cell death but fail to disseminate, showing that YopJ is required for optimal virulence (Monack et al., 1998). On the other hand, secretion of reduced levels of YopJ contributes to the pathogenesis of *Yersinae in vivo*. In this sense, cytotoxicity of dendritic cells induced by *Yersinia* positively correlated with the level of secretion of YopJ/P, but enhanced cytotoxicity in response to infection with *Y. pseudotuberculosis* ectopically expressing highly secreted YopP reduced virulence *in vivo,* causing an attenuated infection of the oral mucosa (Brodsky and Medzhitov, 2008). However, recent data demonstrated that caspase-1 deficiency does not impair the control of infection by hypercytotoxic *Y. pseudotuberculosis* (ectopically expressing YopP) (Zheng et al., 2012). Thus, it is possible that regulation of the levels of YopJ secretion might not be a subversion strategy to downregulate the induction of pyroptosis mediated by YopJ-dependent activation of the inflammasome.

The effectors YopE and YopT, which inactivate Rho GTPases that regulate cytoskeleton rearrangements (Cdc42, Rac1, Rho), were also shown to inhibit secretion of IL-1β in macrophage-like cells infected with *Y. enterocolitica* (Schotte et al., 2004). YopE also reduced cytotoxicity in these cells. Although YopE and YopT reduce activation of overexpressed caspase-1 and inhibit cell death in response to caspase-1 overexpression, the precise mechanism of inhibition of the inflammasome in macrophages has not been examined in detail. In the case of *Y. pseudotuberculosis*, there is no evidence that YopE and YopT play a role in inflammasome modulation (Larock and Cookson, 2012).

The caspase-1 activation, secretion of IL-1β and pyroptosis mediated by inflammasome recognition of T3SS in infected macrophages was shown to be inhibited by the effector YopK (Brodsky et al., 2010). This protein is secreted into the host cell cytosol and interacts with the T3SS, possibly leading to inhibition of inflammasome activation by impairment of recognition of the bacterial T3SS translocon structure. Activation of the inflammasome by mutants of *Y. pseudotuberculosis* lacking YopK leads to bacterial clearance *in vivo,* indicating a role of inflammasome inhibition by YopK in bacterial pathogenesis, promoting pathogen multiplication and dissemination. However, whether virulence mediated by inhibition of the inflammasome by YopK requires regulation of pyroptosis is yet only suggestive.

A recent report revealed that *Yersinia* also directs inhibition of caspase-1 and consequent inhibition of pyroptosis in infected macrophages through aT3SS-dependent effector (Larock and Cookson, 2012). The effector YopM binds to the active site of caspase-1 through a four amino acid motif similar to the sequence of the caspase-1 substrate YVAD and poxvirus protein CrmA, thus, sequestering the molecule and abrogating its interaction with the molecular platform formed by Nlrp3 and Asc in infected macrophages. Inhibition of caspase-1 activation by YopM impaired induction of pyroptosis, demonstrating that the effector modulates inflammatory cell death during infection. Importantly, absence of YopM impaired virulence of *Y. pseudotuberculosis in vivo*, suggesting that inhibition of caspase-1-dependent cell death and cytokine secretion should play a role in the pathogenesis of *Yersinia.*

How regulation of different types of cell death by *Yersinia*, i.e., apoptosis, pyroptosis and possibly necrosis, determines the balance between promotion of effective immune responses and successful immunomodulation, dissemination, and growth of the pathogen is yet to be understood.

Pseudomonas aeruginosa

The Gram-negative bacterium *P. aeruginosa* is an opportunistic extracellular pathogen ubiquitously found in the environment. Antibiotic-resistance and vast distribution make *P. aeruginosa* a major source of nosocomial acute infection of immunocompromised individuals and infection associated to the use of contaminated medical devices. *P. aeruginosa* is also often associated to the infection of chronic cystic fibrosis patients (Garau and Gomez, 2003). The bacteria express a functional T3SS through which four known effectors, exoenzyme S (ExoS), ExoT, ExoU, and ExoY are secreted into host cell (Engel and Balachandran, 2009). Of note, expression of the exoenzymes varies among the different strains of *P. aeruginosa* (Engel and Balachandran, 2009). Activity of these effectors trigger signaling cascades, such as synthesis of cAMP (ExoY), cleavage of phospholipids (ExoU), and modulation of cytoskeleton dynamics (ExoS, ExoT, ExoU) that potentially can lead to activation of cell death pathways, although cytotoxic effects have been described to ExoS and ExoU only (Pederson and Barbieri, 1998; Sato and Frank, 2004).

The contribution of the inflammasome to recognition of *P. aeruginosa* by macrophages has been extensively described. The Nlrc4 receptor plays a major role in activation of caspase-1 in macrophages infected with pathogenic bacteria. Activation of the Nlrc4 inflammasome is triggered upon recognition of bacterial flagellin and the T3SS secretion system (Franchi et al., 2007; Sutterwala et al., 2007; Galle et al., 2008; Miao et al., 2008). Recognition of the T3SS rod component also occurs, dependent on Nlrc4 in a process mediated by activation of theNaip2 protein (Miao et al., 2010b; Zhao et al., 2011). Moreover, a toxin encoded by the *rhsT* gene of *P. aeruginosa* induces inflammasome activation and cytotoxicity in response to the bacteria, contributing to bacterial clearance *in vivo* (Kung et al., 2012). Activation of Nlrc4 inflammasome in response to *P. aeruginosa* mediates pyroptotic cell death and IL-1β secretion. Both processes have been shown to contribute to control of infection *in vivo*, although recent data argues that neutrophils, instead of macrophages, are the main source of IL-1β in infected mouse. Moreover, the IL-1β production by neutrophils occurs independently of bacterial flagellin, Nlrc4 or caspase-1 (Karmakar et al., 2012; Cohen and Prince, 2013). Thus, the contribution of the inflammasome to control of infection by *P. aeruginosa* may be further investigated.

Importantly, the T3SS-dependent effector proteins of *P. aeruginosa* have been shown to inhibit the inflammasome activation in macrophages both *in vitro* and *in vivo.* From the four described effectors secreted by *P. aeruginosa* T3SS, ExoS, and ExoU inhibit inflammasome-dependent responses, arguing that if not inhibited this pathway can play a pivotal role in immune responses and bacterial clearance.

It has been shown that ExoS deficiency leads to the secretion of cleaved IL-1β in both alveolar macrophages and in the lungs of mice infected with *P. aeruginosa* (Galle et al., 2008). ExoS is a bifunctional protein containing an amino-terminal Rho GTPase Activating Protein (GAP) domain, which modifies host cell targets that control the cytoskeleton, such as Cdc42, Rho and Rac1; and a carboxy-terminal ADP-ribosyltransferase domain (ADPRT) with ribosylation activity causing cytoskeleton rearrangements. Of note, the ADPRT domain, but not the GAP domain of ExoS, is essential to inhibition of IL-1β mediated by the exoenzyme. The importance of ribosylation activity of ExoS in this process allows speculating that modulation of host cell cytoskeleton dynamics is a possible mechanism through which ExoS inhibits the inflammasome. For instance, it has been recently reported that organelle transportation activity of microtubules is critical for activation of the inflammasome mediated by Nlrp3 (Misawa et al., 2013). Finally, ExoS induces caspase-3 dependent apoptotic cell death in response to infection but pro-inflammatory death of macrophages is also increased in the infection of macrophages with mutants lacking ExoS. Whether this effect is due to a putative ExoS-mediated inhibition of caspase-1-dependent pyroptosis or caused by cytotoxic effects independent of inflammasome activation has yet to be addressed.

For ExoU, it has been demonstrated that this exoenzyme inhibits Nlrc4-dependent caspase-1 activation and IL-1β in a process dependent on its phospholipase A2 activity (Sutterwala et al., 2007). However, pyroptosis triggered by Nlrc4 and caspase-1 was not modulated by ExoU, which is suggestive that the cytotoxic effect induced by the exotoxin may be due to non-apoptotic, caspase-1-independent necrosis (Sutterwala et al., 2007).

In summary, whether pyroptosis is involved in the pathogenesis of *P. aeruginosa* or whether it contributes to an efficient immune response by the host is still uncertain. However, differential induction and modulation of specific cell death pathways by the exotoxins of the pathogen, as well as clear inhibition of specific responses of the inflammasome by them, make *P. aeruginosa* a valuable model to investigate the role of different cell death pathways to the outcome of host-pathogen interaction and thus, should be further explored.

Vibrio parahaemolyticus

Pathogenicity of *V. parahaemolyticus*, a Gram-negative extracellular bacterium associated mostly with seafood-borne gastroenteritis, relies on the expression of two thermostable pore-forming hemolysins (TDHs, namely TdhA and TdhS) and two sets of chromosome-encoded T3SS (T3SS-1 and T3SS-2) (Makino et al., 2003). A recent report demonstrated that *V. parahaemolyticus* has been shown to induce robust activation of the inflammasome dependent on multiple mechanisms (Higa et al., 2013). *V. parahaemolyticus* TDHs activate Nlrp3-dependent inflammasome (mainly through recognition of TdhA) and bacterial T3SS-1 induces inflammasome activation mediated by both Nlrp3 and Nlrc4. In addition, bacterial flagellin triggers the Nlrc4 inflammasome. Notably, recognition of TDHs and T3SS-1 were required to induce caspase-1-dependent pyroptosis in response to infection. In addition, inflammatory cell death independent of caspase-1 was also observed, suggesting that other pathways may be involved in the induction of cell death in response to *V. parahaemolyticus*. Notably this same report described a regulatory role for inflammasome activation mediated by the T3SS effectors VopQ and VopS, encoded in the pathogenicity island *h1* of Chromosome I. VopQ and VopS inhibited activation of the Nlrc4-dependent inflammasome upon recognition of T3SS-1. VopQ and VopS synergize to inhibit cleavage of caspase-1 and secretion

of cleaved IL-1β, but any effect on pyroptosis has yet to be determined. In fact, complete deletion of *h1* decreased pyroptosis in response to infection, suggesting that other effectors of the bacteria also encoded in the region *h1* may be important for specific induction of pyroptosis by *V. parahaemolyticus.*

The effector VopQ is also known to be a determinant to the induction of autophagy in HeLa cells infected with *V. parahaemolyticus* (Burdette et al., 2009). Higa et al. (2013) demonstrated that VopQ induced autophagy in murine macrophages in response to infection. In addition, suppression of autophagic pathway by knocking down of Atg5 impaired the inhibition of IL-1β secretion mediated by VopQ, supporting a possible role of autophagy in inflammasome inhibition by VopQ. Importantly, induction of autophagy has been previously shown to negatively regulate inflammasome activation dependent on Nlrp3 (Saitoh et al., 2008). How the induction of autophagy mediated by VopQ could possibly mediate inflammasome suppression by the effector remains elusive, but it is possible that Nlrc4-mediated inflammasome activation may also be regulated by autophagy.

In the case of VopS, it is observed that the effector binds and inactivates endogenous Cdc42, which could account as a mechanism for inhibition of Nlrc4-inflammasome (Higa et al., 2013). As mentioned above, inhibition of inflammasome responses by the effectors ExoS of *P. aeruginosa* and YopE of *Y. enterocolitica* relies on their GAP activity that mediates inactivation of Rho GTPases (Schotte et al., 2004; Galle et al., 2008). No molecular role for regulation of inflammasome activation by active Rho GTPases has been demonstrated yet, but evidence suggests that these molecules may participate in inflammasome activation in response to pathogens. For instance, the SP-1 effector SopE of *Salmonella enterica* serovar Typhimurium, an activator of Rho GTPases induces inflammasome activation by stromal cells in response to bacterial recognition (Muller et al., 2009). Importantly, activation of caspase-1 by SopE requires modulation of Rac1 and Cdc42 by the bacterial effector. Another recent report revealed that activation of Rac1 in response to infection is important to NLRP3/ASC-dependent caspase-1 activation in response to *Chlamydia pneumoniae* by human mononuclear cells (Eitel et al., 2012). Importantly, a recent work showed that type I IFN signaling inhibits Rac1, with consequent repression of Nlrp3 inflammasome in macrophages (Inoue et al., 2012). These evidences reinforce a putative role of signaling pathways controlled by Rho GTPases in the modulation of inflammasome activation in response to pathogens, possibly inducing pyroptosis. How the activity of Rho GTPases in inflammasome activation, as well as modulation as a subversion strategy targeting immune responses may thus, be further explored.

Chlamydia trachomatis

The obligate intracellular Gram-negative pathogen *C. trachomatis* is the causative agent of infections of the conjunctiva and urogenital tract commonly evolving to severe complications such as blindness, pelvic inflammatory disease, ectopic pregnancy, and infertility. The bacteria rely on the expression of a T3SS and secretion of effector proteins to adhere, invade, and establish a replicative inclusion (parasitophorous vacuole) in the target cells (Valdivia, 2008; Betts et al., 2009). Besides the T3SS-dependent effectors protein, the chlamydial protease-like activity factor (CPAF) is pivotal in the molecular pathogenesis of *C. trachomatis*, modulating host responses and stability of bacterial inclusion (Paschen et al., 2008). CPAF is translocated through the general secretory pathway, eventually reaching the host cell cytosol (Zhong et al., 2001). This effector is suggested to regulate by cleavage at least 16 host targets, interfering with several processes such as: proapoptotic signaling (Zhong et al., 2001; Pirbhai et al., 2006); expression of antigen presentation molecules (MHC) (Zhong et al., 2001); organization of host cell cytoskeleton (Dong et al., 2004; Kumar and Valdivia, 2008); control of cell cycle (Paschen et al., 2008) and NF-κB signaling pathway (Christian et al., 2010). Although host substrates were demonstrated to be cleaved by CPAF in cell lysates, enzymatic activity of CPAF may not be necessarily required *in situ* to exert its regulatory functions on host cell proteins (Chen et al., 2012). Nevertheless, CPAF has also been implicated in modulation of chlamydial proteins. It has been demonstrated that CPAF cleaves chlamydial T3SS-dependent effectors in cell-free systems and in infected cells, with evidences that CPAF proteolytic activity toward *C. trachomatis* effectors prevents superinfection and coordinates the formation and the integrity of the inclusion-containing the bacteria (Jorgensen et al., 2011).

In epithelial cells, which are the primary sites of infection by *C. trachomatis*, as well as in human monocytes and dendritic cells, NLRP3 and ASC mediate inflammasome-dependent activation of caspase-1 and secretion of cytokines in response to *C. trachomatis* (Lu et al., 2000; Gervassi et al., 2004; Abdul-Sater et al., 2009, 2010). However, the role of the inflammasome in the control of the infection by the pathogen is still controversial. Asc- and caspase-1-deficient mouse fibroblasts are resistant to infection by *C. trachomatis* (Jorgensen et al., 2011). In addition, in a mouse model of infection with *C. muridarum*, wild-type and caspase-1 deficient mice equally controlled the replication of the bacteria *in vivo* (Cheng et al., 2008). Of note, caspase-1-deficient mice displayed reduced inflammatory damage in the urogenital tract, suggesting that inflammasome activation may contribute to the pathology of infection by *Chlamydia* (Cheng et al., 2008).

Still, *C. trachomatis* regulates caspase-1-dependent cell death through the activity of CPAF. The use of a specific inhibitor of CPAF, design to overcome the refraction of the bacteria to genetic manipulation, revealed that CPAF activity inhibits ASC and caspase-1-dependent cell death in the early times of infection of epithelial cells with *C. trachomatis.* Late activation of caspase-1 occurs in epithelial cells and pharmacological inhibition of caspase-1 reduces bacterial growth in these cells, corroborating the importance of regulation of inflammasome activation to the pathogenesis of *C. trachomatis* (Abdul-Sater et al., 2009). However, a role for pyroptosis for caspase-1-dependent susceptibility to infection is still speculative. The mechanism of inhibition of early pyroptotic cell death by CPAF is yet to be understood and it is not ruled out that the protease directly interferes with inflammasome formation. Still, one interesting possibility proposed by Jorgensen et al. (2011) is that CPAF may function as a metaeffector (Kubori et al., 2010), regulating the pool of T3SS effectors in the host cytosol by proteolysis and also avoiding the accumulation of putative PAMPs to be sensed by cytosolic NLRs, possibly

providing a novel mechanism of pathogenic modulation of the inflammasome.

Francisella tularensis

Tularemia, a life-threatening infectious disease of the respiratory tract, is caused by the Gram-negative intracellular pathogen *Francisella tularensis.* Inside infected macrophages, the main target of infection, *F. tularensis* escapes the vacuole and replicates within the cytosol. However, in contrast to most of the intracellular pathogens, the bacteria do not rely on the activity of exotoxins or encoded T3SS and T4SS secretion systems and related effectors to modulate host cell functions (Larsson et al., 2005), and virulence mechanisms of the pathogen remain largely unknown (Broms et al., 2010; Meibom and Charbit, 2010). The inflammasome plays a pivotal role in recognition and control of infection by *F. tularensis* in experimental models of infection. The bacteria trigger activation of Aim2/Asc-dependent inflammasome in mouse macrophages (Mariathasan et al., 2005; Fernandes-Alnemri et al., 2010; Jones et al., 2010; Rathinam et al., 2010). Recognition of the bacteria by this Aim2/Asc leads to activation of caspase-1, secretion of IL-1β and IL-18, pyroptosis and culminate in the control of bacterial replication in macrophages and *in vivo*. In addition, Aim2 and Asc were proposed to trigger caspase-1-independent, caspase-8, -9, -3-mediated apoptosis of macrophages in response to infection with *F. tularensis*, contributing to restriction of bacterial replication in these cells (Pierini et al., 2012). Finally, whereas Nlrp3 is dispensable for inflammasome activation in murine macrophages, NLRP3 and AIM2 are suggested to play a role in human monocytic cells (Mariathasan et al., 2006; Fernandes-Alnemri et al., 2010; Jones et al., 2010; Atianand et al., 2011).

Absence of Aim2 activation by *mviN* and *ripA* mutants of *F. tularensis* have been initially reported (Huang et al., 2010; Ulland et al., 2010), but the lack of these encoded factors was shown to compromise the integrity of bacteria and enhance intramacrophage lysis of mutant bacteria and release of DNA into the host cell cytosol (Peng et al., 2011). Besides mviN and ripA, mutations on core components of the type VI secretion system of *F. tularensis* also affect activation of the inflammasome (Barker et al., 2009; Broms et al., 2012), therefore, it is still possible that this lack of activation may be due to an inherited defect in phagosome escape of this bacteria.

Recent data, however, suggests that the bacteria actively repress inflammasome signaling by the effector protein encoded by *FTL_0325*, a process that may contribute to repression of IL-1β secretion and bacterial growth *in vivo* (Dotson et al., 2013). The authors observed that virulent *F. tularensis* subsp. *tularensis* and *holarctica* fail to induce a robust activation of the inflammasome in the early times of the infection in comparison to attenuated *F. tularensis* subsp. *novicida*. Mutations in *FTL_0325* gene of *F. tularensis* subsp. *holarctica* (live vaccine strain -LVS) do not alter bacterial fitness whilst it exacerbates the synthesis of pro-IL-1β. In addition, mutants lacking *FLT_0325* also induce higher levels of caspase-1 activation dependent on Aim2 and Tlr2 and secretion of IL-1β dependent on Tlr2, Aim2, and Nlrp3 in the early periods of infection. Importantly, suppression of Aim2-dependent inflammasome activation by FLT_0325 inhibits pyroptosis in response to infection by *F. tularensis* LVS in macrophages (Dotson et al., 2013). Whether pyroptosis repression contributes to pathogenesis *in vivo* is still unclear.

Shigella flexneri

Bacillary dysentery in humans is caused by mucosal infection with the Gram-negative intracellular pathogen *S. flexneri*. The bacteria express a functional T3SS, through which sequential delivery of bacterial effectors into host cell cytosol promotes pathogenesis (Ogawa et al., 2008). In addition, recognition of *S. flexneri* PAMPs elicits immune responses that paradoxically contribute to bacterial success (Phalipon and Sansonetti, 2007). *Shigella* invades the epithelia through the M cells of the mucosa barrier, subsequently infecting resident macrophages and dendritic cells. Once within these cells, the bacteria lyse the vacuole, replicates in the host cell cytosol and eventually triggers inflammatory cell death. This inflammatory burst and consequent neutrophil recruitment promotes basolateral invasion and dissemination of *S. flexneri*, followed by their entry into epithelial cells, renewed replication of bacteria and further dissemination along the epithelia using a cell-to-cell spread mechanism. However, the infection of epithelial cells generates an early genotoxic stress that could potentially cause necrotic death and bacterial control; bacterial replication inside these cells, suggests that *Shigella* also antagonizes cell death. In this way, *S. flexneri* concerted modulation of pro-death and pro-survival signaling pathways potentially allow bacterial circulation among different host compartments, maintenance of a replicative niche and a mechanism to circumvent the innate immune response (Schroeder and Hilbi, 2008; Ashida et al., 2011).

Signaling through Nlrc4inflammasome pathway, mediated by recognition of the rod component MxiI of the T3SS apparatus culminates in caspase-1 activation, pyroptosis and IL-1β and IL-18 secretion (Suzuki et al., 2007; Miao et al., 2010b). The T3SS effector protein IpaB also induces pyroptosis and IL-1β dependent on caspase-1 (Chen et al., 1996; Hilbi et al., 1998). Although physical interaction of caspase-1 and IpaB has been demonstrated, recent data support a mechanism of ion channel formation by oligomerization of IpaB in the host cell membrane, possibly inducing Nlrc4 and Asc-dependent inflammasome activation that culminates into caspase-1 activation and pyroptosis (Senerovic et al., 2012). Inflammasome activation in infected macrophages, caspase-1 activation, pyroptosis and secretion of inflammatory cytokines can correspond to the inflammatory burst associated to shigellosis. Importantly, inflammatory burst induced by *S. flexneri*, associated with bacterial invasion and dissemination as well as resolution of infection by a competent host, requires caspase-1, IL-1β, and IL-18 (Sansonetti et al., 1995, 2000). It is likely that fine modulation by *Shigella* of pyroptosis in infected macrophages could favor bacterial basolateral dissemination but avoid the potential restriction of infection associated with robust immune signaling. Nonetheless, a specific molecular mechanism underlying this putative process has not yet been revealed.

In the case of nonmyleoid epithelial cells, acute infection by *Shigella* induces necrotic cell death pathways as a consequence

of mitochondrial damage as well as due to genotoxic stress through activation of calpain. The activation of calpain is a process mediated by the bacterial effector VirA with complex consequences, promoting bacterial uptake, inhibition of early pro-apoptotic signaling by degradation of p53 but also induction of late necrosis that contributes to invasion (Bergounioux et al., 2012). However, death of infected cells is supposed to be modulated by the bacteria to support intracellular bacterial growth inside epithelial cells thus, favoring primary tissue colonization. In this sense, activation of pro-survival NF-κB signaling pathway through recognition of bacterial PAMPs by Nod1 and Rip2 possibly counterbalances necrotic cell death (Carneiro et al., 2009).

A recent report revealed that mutants of *S. flexneri* lacking the expression of the T3SS effector protein OspC3 induce early pyroptotic cell death upon infection by *S. flexneri* of human epithelial cell lines (Kobayashi et al., 2013). In agreement, Δ*ospC3 S. flexneri* increases mucosal cell death and inflammatory infiltrate in the intestine of infected guinea pigs, with associated reduction of bacterial growth in the epithelia without affecting bacterial invasiveness. Of note, pyroptosis induced by Δ*ospC3 S. flexneri* specifically requires caspase-4, the human homolog of murine caspase-11, but not caspase-1. OspC3 reduces catalytic activity of caspase-4, also decreasing cell death induced by overexpression of p19 catalytic subunit of casp-4. Caspase-4 physically interacts with OspC3 through the catalytic site in the p19 subunit of the active caspase-4. Interaction and inhibition of pyroptosis induced by caspase-4 also requires the C-terminal Ankyrin repeat-containing domain of OspC3 (ANK), a eukaryotic-like domain predicted to mediate protein-protein interactions. Most strikingly, certain motifs in the ANK domain of OspC3 share high similarity to other bacterial and viral proteins, including those encoded by *Legionella pneumophila, Coxiella burnetii, Rickettsia rickettsia,* and vaccinia virus. This first demonstration of a pathogen effector protein that inhibits non-canonical induction of pyroptosis is suggestive that this mechanism might be a common strategy to modulate the induction of inflammatory responses among diverse pathogens. In the case of *Shigella*, it is likely that inhibition of caspase-4-dependent pyroptosis provides both the maintenance of epithelial replicative niche as well as evasion of early immune signaling.

Legionella pneumophila

L. pneumophila is a Gram-negative intracellular bacterial pathogen that accidentally infects humans, causing a pneumonia-like disease in immunocompromised individuals. The pathogen resides within a cytosolic endosomal replicative vacuole (LCV), avoiding fusion with lysosomal vesicles and modulating diverse host cell functions to maintain the replicative niche. To this end, *L. pneumophila* secretes through a type IVB secretion system called Dot/Icm (**D**efective **o**rganelle **t**rafficking/**I**ntra**c**ellular **m**ultiplication) more than 300 effectors proteins into the host cell cytosol, which are mostly involved in the maintenance of the LCV and bear wide function redundancy (Hubber and Roy, 2010).

L. pneumophila is known to induce robust activation of the inflammasome by triggering different pathways. Bacterial flagellin secreted through the Dot/Icm system into host cell cytosol is recognized by the Naip5-Nlrc4-caspase-1 axis, triggering pyroptosis and Asc-dependent secretion of IL-1β (Amer et al., 2006; Molofsky et al., 2006; Ren et al., 2006; Zamboni et al., 2006; Lightfield et al., 2008; Case et al., 2009; Silveira and Zamboni, 2010). Of note, flagellin recognition via Naip5/Nlrc4/caspase-1 account to infection control *in vitro* and *in vivo* (Amer et al., 2006; Molofsky et al., 2006; Ren et al., 2006; Zamboni et al., 2006; Coers et al., 2007; Pereira et al., 2011). Moreover, data suggests that activation of caspase-7 dependent on this inflammasome pathway leads to LCV acidification and fast macrophage death, contributing to bacteria control *in vitro* (Akhter et al., 2009). In addition, Dot/Icm products induce flagellin-independent inflammasome activation regulated by caspase-11. Caspase-11 mediates macrophage pyroptosis and secretion of IL-1α, besides regulating Nlrp3/Asc-dependent secretion of IL-1β (Case et al., 2013). Importantly, evidence suggests that inflammasome-dependent pyroptosis and neutrophil recruitment mediate by IL-1β and IL-1α are important to bacterial clearance (Casson et al., 2013). Recently, secretion of IL-1α independent of caspase-1 and caspase-11 has also been shown to participate in neutrophil recruitment and infection control (Barry et al., 2013).

In contrast to the current knowledge on inflammasome activation by *L. pneumophila,* little is known about mechanisms of inflammasome subversion by the pathogen. Of note, *L. pneumophila* evolved cycling through different unicellular amebae protozoa in freshwater reservoirs, possibly conserving features that allow a broad host-range pathogen instead of those specific to provide resilience in specialized phagocytes (Ensminger et al., 2012). In this sense, the course of pathogen adaptation to adequate host, *L. pneumophila* may have encountered little selective pressure to evade PRRs recognition and immune responses (Massis and Zamboni, 2011). Consequently, it is possible that a reduced number of *L. pneumophila* effectors should be involved in subversion of innate immune responses of host macrophages such as inflammasome activation and pyroptosis, favoring the conservation of tools to hijack vesicles and organelles necessary to constant remodeling of the LCV. On the other hand, the recent description of a putative primitive immune-like system encoded in the genome of *Acanthamoeba castellanii* (Clarke et al., 2013) raises the possibility of existence of environmental pressure that could have favored natural selection of bacteria provided with evasion mechanisms against host immune response.

Of note, upregulation of non-apoptotic genes by activation of NF-κB in a Dot/Icm-dependent manner counterbalance the activation of caspase-3-mediated apoptotic pathway upon infection, indicating that *L. pneumophila* can modulate host cell death (Abu-Zant et al., 2005, 2007). Early activation of caspase-3 is induced by multiple bacterial secreted effectors and plays a role in the arrested maturation of nascent bacteria-containing phagosome through the endocytic pathway (Gao and Abu Kwaik, 1999; Zink et al., 2002; Molmeret et al., 2004; Zhu et al., 2013). Importantly, caspase-3 activation accounts for restriction of bacterial replication in dendritic cells (Nogueira et al., 2009). Although direct inhibition of the inflammasome and pyroptosis by *L. pneumophila* effector proteins has not yet been demonstrated, the Dot/Icm effector SdhA is required for bacterial replication in macrophages (Laguna et al., 2006). SdhA is

important to avoid Aim2-dependent inflammasome activation in response to recognition of *L. pneumophila* DNA (Ge et al., 2012). Macrophages infected with mutants lacking *sdhA* gene trigger Aim2-dependent activation of caspase-1, secretion of IL-1β and pyroptosis, which is reversible by infection with genetically complemented bacteria. However, as in the case of *F. tularensis* and the genes *mviN* and *ripA*, it will be important to determine whether the activation of Aim2 inflammasome in response to infection with *sdhA* mutants is not an indirect effect of bacterial DNA release in the cytosol as a consequence of compromised integrity of LCV and bacterial degradation. Interestingly, a recent report suggested that induction of autophagosome turnover dependent on recognition of virulent flagellate *L. pneumophila* through Naip5/Nlrc4/pro-caspase-1 regulates pyroptosis triggered by the same pathway (Byrne et al., 2013). However, the mechanism by which inflammasome components promote the autophagic flux and how the induction of autophagy regulates pyroptosis remain elusive. Paradoxically, *L. pneumophila* inhibits autophagy through irreversible inactivation of Atg8 mediated by the effector RavZ (Choy et al., 2012). Whether autophagy contributes to infection or boosts immunity in response to *L. pneumophila* should be further explored.

Coxiella burnetii

Similarly to *Legionella pneumophila*, the Gram-negative, obligate intracellular bacteria and human pathogen *Coxiella burnetii* express the unique type IVB Dot/Icm secretion system (McDonough et al., 2012). Although the two pathogens are also closely related in phylogenetic analysis, *C. burnetii* is a bona-fide mammalian pathogen, with a strong tropism for alveolar macrophages in infected humans. Their distinct natural history is evident in the strikingly divergent life style adopted by the bacteria once inside the host cells. *C. burnetii* demands an acidified environment for morphological development, Dot/Icm expression and replication, which is accomplished by active maturation of bacteria-containing vacuole through the endosomal pathway, culminating in fusion with recruited lysosomal vesicles and formation of a large replicative vacuoles (LRV) (Newton and Roy, 2011). Of note, *C. burnetii* is capable of modulating several cellular processes to both remodel the LRV as well as to escape bacterial recognition and control. The genome of *C. burnetii* encodes more than 200 putative candidates for Dot/Icm secretion, of those roughly 25% have been shown to be effectively expressed and secreted in to host cell cytosol, with just a few with a demonstrated functionality (Van Schaik et al., 2013). It is possible that functional redundancy among *C. burnetii* effectors is reduced in comparison to *L. pneumophila*, as diverse genes have already been shown to affect LRV formation (Weber et al., 2013).

The mechanism of recognition and immune response to *C. burnetii* in macrophages remains elusive. Variations in the O-antigen of *C. burnetii* LPS are determinant to the virulence of the bacteria, with avirulent organisms expressing a truncated form of O-antigen in the LPS structure. Besides, differences in antigenic reactivity of *C. burnetii* LPS is determined by variation in the chemical composition of the O-polysaccharide chain

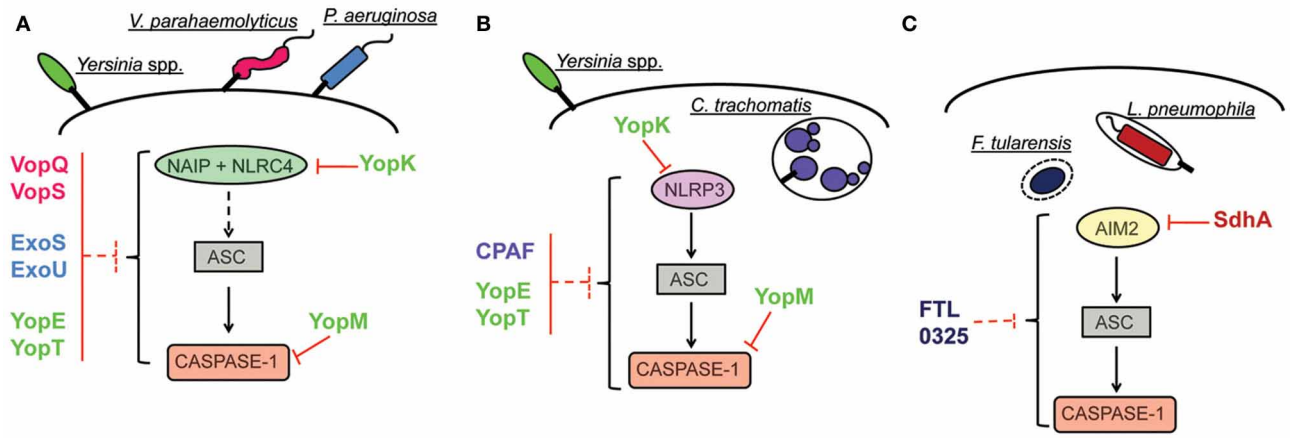

FIGURE 2 | Inhibition of inflammasome activation pathways by pathogenic bacteria. (A) Inhibition of NAIP/NLRC4 inflammasome. The effector YopK of *Yersinia* is secreted into host cell cytosol by the T3SS and interacts with the translocon structure in the host cell cytosol interface; this interaction possibly prevents recognition by cellular receptors. The effectors VopQ/S of *V. parahaemolyticus* and ExoU of *P. aeruginosa* inhibit the NAIP/NLRC4 inflammasome by unknown mechanisms. In the case of the effectors YopE/T of *Yersinia* and ExoS of *P. aeruginosa*, interaction of the bacterial proteins with caspase-1 *in vitro* are suggestive of a putative mechanism for inflammasome inhibition by direct interaction with caspase-1. Inhibition of inflammasome activation by interaction with caspase-1 has been characterized for the effector YopM of *Yersinia*. **(B)** Inhibition of NLRP3 inflammasome. Inhibition of NLRP3-dependent inflammasome by YopK occurs as explained in **(A)**. The protein CPAF of *C. trachomatis* is a protease that can target bacterial effectors secreted into host cell cytosol and inhibits ASC-dependent inflammasome activation (that could be triggered by NLRP3) by unknown mechanisms. In the case of YopE/T, suggestion of inflammasome inhibition by direct interaction with caspase-1 indicates possible subversion of both NLRP3 and NAIP/NLRC4 activation pathways. In the case of YopM, demonstration of caspase-1 inhibition by direct interaction also suggests that both pathways can be subverted. **(C)** Inhibition of AIM2 inflammasome. The effector SdhA of *L. pneumophila*, required for bacterial growth, prevents bacterial DNA release into host cell cytosol, thus, avoiding recognition by host DNA receptor AIM2. In the case of *F. tularensis*, the protein encoded by bacterial gene *FTL_0325* also inhibits AIM2 inflammasome activation by unknown mechanisms.

Table 1 | Summary of bacterial effectors that suppress inflammasome activation and their role on suppression of pyroptosis, as discussed in the main text.

	Bacterial effector	Mechanism of inflammasome inhibition	Inflammasome target	Effect on macrophage pyroptosis	References
Yersinia spp.	YopK	Interaction with YopK possibly prevents recognition of T3SS	Metaeffector	Inhibition	Brodsky et al., 2010
	YopE (*Y. enterocolitica*)	Unknown, YopE interacts with caspase-1 *in vitro*	Unknown	Inhibition	Schotte et al., 2004
	YopM	Direct inhibition of caspase-1 activation	Caspase-1	Inhibition	Larock and Cookson, 2012
	YopT (*Y. enterocolitica*)	Unknown, YopT interacts with caspase-1 *in vitro*	Unknown	Not-described	Schotte et al., 2004
P. aeruginosa	ExoS	Unknown, dependent on ribosylation activity of ExoS	Unknown	Not-described	Galle et al., 2008
	ExoU	Unknown, dependent on phospholipase activity of ExoU	Unknown	No inhibition	Sutterwala et al., 2007
V. parahaemolyticus	VopQ	Unknown, dependent on host cell autophagy	Unknown	Not described	Higa et al., 2013
	VopS	Unknown, dependent on inhibition of Rho GTPase byVopS	Unknown	Not described	Higa et al., 2013
C. trachomatis	CPAF	Unknown, requires proteolytic activity of CPAF	Unknown	Inhibition	Jorgensen et al., 2011
F. tularensis	FTL_0325	Unknown	Unknown	Inhibition	Dotson et al., 2013
S. flexneri	OspC3	Direct inhibition of caspase-4	Caspase-4	Inhibition	Kobayashi et al., 2013
L. pneumophila	SdhA	Inhibition of bacterial DNA release	AIM2	Inhibition	Ge et al., 2012

(Narasaki and Toman, 2012). Although the bacterial lipopeptides are recognized by Tlr2, as demonstrated with infections performed purified molecules and with avirulent phase II *C. burnetii* (Zamboni et al., 2004), the virulent phase I bacteria avoid Tlr2 recognition by forming a protective structure that avoids exhibition of components of the bacteria cell wall for Tlr2 recognition (Shannon et al., 2005). In addition, the structure of the lipid A of the LPS of *C. burnetii* was also revealed and it was

shown that lipid A derived from both virulent and avirulent bacteria antagonizes Tlr4 activation (Zamboni et al., 2004). Of note, antagonic engagement of Tlr4 by *C. burnetii* LPS is possibly a complex process. A recent report showed that virulent bacteria and their LPS trigger an impaired activation of the MAPK pathway in macrophages, which is important to avoid conversion of phagolysosomes hosting bacteria into degradative compartments containing cathepsin D (Barry et al., 2012).

The bacterium is also known to induce pro-survival pathways that sustain bacterial growth. Phase I and phase II *C. burnetii* induce sustained phosphorylation of anti-apoptotic host proteins Akt and Erk1/2 (Voth and Heinzen, 2009). Interaction of Beclin-1, a protein of autophagy, with anti-apoptotic Bcl2 in the membrane of the bacterial LRV prevents apoptosis of cells infected with *C. burnetii* (Vazquez and Colombo, 2010). In addition, *C. burnetii* inhibits caspase-3- dependent intrinsic pathway of apoptosis (Luhrmann and Roy, 2007; Voth et al., 2007), and the Dot/Icm effectors AnkG, CaeA, and CaeB have already been implicated in this process by distinguished mechanisms. The effector AnkG inhibits host cell apoptosis dependent on the interaction with p32, a host cytoplasmic protein implicated in pathogen-induced apoptosis (Luhrmann et al., 2010). Whereas a mechanism for inhibition of apoptosis by CaeA has not yet been demonstrated, the effector CaeB co-localizes with the mitochondria and its overexpression reduces the loss of MOMP (**m**itochondria **o**uter **m**embrane **p**ermeabilization) induced by activation of the apoptosis pathway (Klingenbeck et al., 2012).

A role of NLRs and inflammasome activation in the recognition and control of *C. burnetii* infection has not been demonstrated so far, even though the bacteria is a bona-fide intracellular pathogen that express a functional secretion system, a hallmark for bacterial sensing by macrophages. Still, the capacity of the bacteria to thrive inside the macrophages throughout a slow replicative life cycle suggests that the bacteria might subvert inflammatory responses including the activation of the inflammasome. Future investigations should shed light in a possible role of the inflammasomes in host response to *C. burnetii*, as well as reveal novel mechanisms of bacterial subversion of the inflammasome and pyroptosis.

CONCLUDING REMARKS

Activation of the inflammasome is a broad host response that effectively contributes to innate immune response and infection control of a remarkably high number of infectious agents. Activation of this platform leads to inflammasome-dependent secretion of cytokines, induction of pyroptosis and restriction of pathogen replication, by mechanisms that are still obscure. As reviewed here, targeting inflammasome activation is a common evasion strategy of different species of bacterial pathogens. Importantly, different steps of the signaling cascade that leads to inflammasome activation are targeted by bacterial proteins. However, in most cases, the molecular mechanisms underlying inflammasome inhibition are still not fully understood. Few reports identified a direct interaction with the inflammasome effector molecule caspase-1, whereas others provided evidence of an interference with upstream signaling pathway (**Figure 2**, **Table 1**). Moreover, in some cases the inhibition of the inflammasome was verified in the level of caspase-1 activation and IL-1β secretion, without appropriate assessment of inhibition of pyroptosis. In this scenario, it should be considered that secretion of IL-1β and pyroptosis can be differentially regulated, with caspase-11 emerging as a master regulator of these processes. The contribution of caspase-11 in inflammasome activation in response to pathogens is possibly underscored because published literature on the activation of caspase-1 has been widely assessed using C57BL/6 mice double knockout for both caspase-1 and caspase-11 (Kayagaki et al., 2011). In this scenario, it will be important to reevaluate if the reported suppression of the inflammasome by bacterial proteins reviewed herein occur via inhibition of the canonical (caspase-1-dependent, caspase-11- independent) or non-canonical (caspase-11-dependent only) inflammasome. Finally, it is important to emphasize that dysfunctions on inflammasome signaling is intrinsically connected to the onset of diverse chronic inflammatory metabolically and autoimmune syndromes. Understanding the molecular mechanisms of pathogen subversion strategies for suppression of the inflammasome activation and elucidate how they specifically affect inflammasome responses will be critical to a comprehensive understanding of the bacterial pathogenesis and host response. Importantly, it may provide clues for the advance in the development of effective therapeutics to uncontrolled inflammation associated to systemic infections and chronic inflammatory diseases.

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shown that lipid A derived from both virulent and avirulent bacteria antagonizes Tlr4 activation (Zamboni et al., 2004). Of note, antagonic engagement of Tlr4 by *C. burnetii* LPS is possibly a complex process. A recent report showed that virulent bacteria and their LPS trigger an impaired activation of the MAPK pathway in macrophages, which is important to avoid conversion of phagolysosomes hosting bacteria into degradative compartments containing cathepsin D (Barry et al., 2012).

The bacterium is also known to induce pro-survival pathways that sustain bacterial growth. Phase I and phase II *C. burnetii* induce sustained phosphorylation of anti-apoptotic host proteins Akt and Erk1/2 (Voth and Heinzen, 2009). Interaction of Beclin-1, a protein of autophagy, with anti-apoptotic Bcl2 in the membrane of the bacterial LRV prevents apoptosis of cells infected with *C. burnetii* (Vazquez and Colombo, 2010). In addition, *C. burnetii* inhibits caspase-3- dependent intrinsic pathway of apoptosis (Luhrmann and Roy, 2007; Voth et al., 2007), and the Dot/Icm effectors AnkG, CaeA, and CaeB have already been implicated in this process by distinguished mechanisms. The effector AnkG inhibits host cell apoptosis dependent on the interaction with p32, a host cytoplasmic protein implicated in pathogen-induced apoptosis (Luhrmann et al., 2010). Whereas a mechanism for inhibition of apoptosis by CaeA has not yet been demonstrated, the effector CaeB co-localizes with the mitochondria and its overexpression reduces the loss of MOMP (**m**itochondria **o**uter **m**embrane **p**ermeabilization) induced by activation of the apoptosis pathway (Klingenbeck et al., 2012).

A role of NLRs and inflammasome activation in the recognition and control of *C. burnetii* infection has not been demonstrated so far, even though the bacteria is a bona-fide intracellular pathogen that express a functional secretion system, a hallmark for bacterial sensing by macrophages. Still, the capacity of the bacteria to thrive inside the macrophages throughout a slow replicative life cycle suggests that the bacteria might subvert inflammatory responses including the activation of the inflammasome. Future investigations should shed light in a possible role of the inflammasomes in host response to *C. burnetii*, as well as reveal novel mechanisms of bacterial subversion of the inflammasome and pyroptosis.

CONCLUDING REMARKS

Activation of the inflammasome is a broad host response that effectively contributes to innate immune response and infection control of a remarkably high number of infectious agents. Activation of this platform leads to inflammasome-dependent secretion of cytokines, induction of pyroptosis and restriction of pathogen replication, by mechanisms that are still obscure. As reviewed here, targeting inflammasome activation is a common evasion strategy of different species of bacterial pathogens. Importantly, different steps of the signaling cascade that leads to inflammasome activation are targeted by bacterial proteins. However, in most cases, the molecular mechanisms underlying inflammasome inhibition are still not fully understood. Few reports identified a direct interaction with the inflammasome effector molecule caspase-1, whereas others provided evidence of an interference with upstream signaling pathway (**Figure 2**, **Table 1**). Moreover, in some cases the inhibition of the inflammasome was verified in the level of caspase-1 activation and IL-1β secretion, without appropriate assessment of inhibition of pyroptosis. In this scenario, it should be considered that secretion of IL-1β and pyroptosis can be differentially regulated, with caspase-11 emerging as a master regulator of these processes. The contribution of caspase-11 in inflammasome activation in response to pathogens is possibly underscored because published literature on the activation of caspase-1 has been widely assessed using C57BL/6 mice double knockout for both caspase-1 and caspase-11 (Kayagaki et al., 2011). In this scenario, it will be important to reevaluate if the reported suppression of the inflammasome by bacterial proteins reviewed herein occur via inhibition of the canonical (caspase-1-dependent, caspase-11- independent) or non-canonical (caspase-11-dependent only) inflammasome. Finally, it is important to emphasize that dysfunctions on inflammasome signaling is intrinsically connected to the onset of diverse chronic inflammatory metabolically and autoimmune syndromes. Understanding the molecular mechanisms of pathogen subversion strategies for suppression of the inflammasome activation and elucidate how they specifically affect inflammasome responses will be critical to a comprehensive understanding of the bacterial pathogenesis and host response. Importantly, it may provide clues for the advance in the development of effective therapeutics to uncontrolled inflammation associated to systemic infections and chronic inflammatory diseases.

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Caspase-2 mediates a *Brucella abortus* RB51-induced hybrid cell death having features of apoptosis and pyroptosis

Denise N. Bronner[1], Mary X. D. O'Riordan[1] and Yongqun He[1,2,3]*

[1] *Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI, USA*
[2] *Unit for Laboratory Animal Medicine, University of Michigan Medical School, Ann Arbor, MI, USA*
[3] *Comprehensive Cancer Center, University of Michigan Medical School, Ann Arbor, MI, USA*

Edited by:
Amal O. Amer, The Ohio State University, USA

Reviewed by:
Jianwu Pei, American Animal Health, Inc., USA
Diego J. Comerci, Instituto de Investigaciones Biotecnologicas Dr. Rodolfo A. Ugalde, Argentina

***Correspondence:**
Yongqun He, Unit for Laboratory Animal Medicine and Department of Microbiology and Immunology, University of Michigan Medical School, 018 Animal Research Facility, 1150 W. Medical Dr., Ann Arbor, MI 48109, USA
e-mail: yongqunh@med.umich.edu

Programmed cell death (PCD) can play a crucial role in tuning the immune response to microbial infection. Although PCD can occur in different forms, all are mediated by a family of proteases called caspases. Caspase-2 is the most conserved caspase, however, its function in cell death is ill-defined. Previously we demonstrated that live attenuated cattle vaccine strain *Brucella abortus* RB51 induces caspase-2-mediated and caspase-1-independent PCD of infected macrophages. We also discovered that rough attenuated *B. suis* strain VTRS1 induces a caspase-2-mediated and caspase-1-independent proinflammatory cell death in infected macrophages, which was tentatively coined "caspase-2-mediated pyroptosis". However, the mechanism of caspase-2-mediated cell death pathway remained unclear. In this study, we found that caspase-2 mediated proinflammatory cell death of RB51-infected macrophages and regulated many genes in different PCD pathways. We show that the activation of proapoptotic caspases-3 and -8 was dependent upon caspase-2. Caspase-2 regulated mitochondrial cytochrome *c* release and TNFα production, both of which are known to activate caspase-3 and caspase-8, respectively. In addition to TNFα, RB51-induced caspase-1 and IL-1β production was also driven by caspase-2-mediated mitochondrial dysfunction. Interestingly, pore formation, a phenomenon commonly associated with caspase-1-mediated pyroptosis, occurred; however, unlike its role in *S. typhimurium*-induced pyroptosis, pore formation did not contribute to RB51-induced proinflammatory cell death. Our data suggest that caspase-2 acts as an initiator caspase that mediates a novel RB51-induced hybrid cell death that simulates but differs from typical non-proinflammatory apoptosis and caspase-1-mediated proinflammatory pyroptosis. The initiator role of the caspase-2-mediated cell death was also conserved in cellular stress-induced cell death of macrophages treated with etoposide, naphthalene, or anti-Fas. Caspase-2 also regulated caspase-3 and -8 activation, as well as cell death in macrophages treated with each of the three reagents. Taken together, our data has demonstrated that caspase-2 can play an important role in mediating a proinflammatory response and a hybrid cell death that demonstrates features of both apoptosis and pyroptosis.

Keywords: proinflammatory, caspase-2, programmed cell death, mitochondrial dysfunction, macrophage, Brucella

INTRODUCTION

Programmed cell death (PCD) is a crucial process initiated by the host in response to cellular stress and microbial infections. PCD can occur in a variety of ways (Galluzzi et al., 2012). Apoptosis, pyroptosis, and necroptosis are three pathways of PCD that can occur during microbial infections with substantially different outcomes. Apoptosis is a silent PCD due to a lack of cytokine secretion (Elmore, 2007). Although the plasma membrane remains intact, apoptotic bodies can bleb off and be phagocytosed by other phagocytic cells in the surrounding area. Pyroptosis or "*death by fire*," is inflammatory PCD typically mediated by caspase-1 (Bergsbaken et al., 2009). Caspase-1 processes proinflammatory cytokines (IL-1β and IL-18), and secretion of these cytokines requires pore formation in the plasma membrane, which leads to cell swelling and eventually lysis. Necroptosis is a newly identified type of PCD that includes a proinflammatory response as well as loss of plasma membrane integrity (Vandenabeele et al., 2010). In contrast to apoptosis and pyroptosis, serine/threonine kinases, RIP1 and RIP3, mediate necroptosis. In addition, necroptosis leads to the release of intracellular contents; mostly damage associated molecular patterns (DAMPs).

Many microbes can induce cell death during infection and dissemination (Gao and Abu Kwaik, 2000). Avirulent *Mycobacterium* induces apoptosis in macrophages (Fratazzi et al., 1999).

Neighboring uninfected macrophages, upon phagocytosis, killed *Mycobacterium* in apoptotic bodies released by *Mycobacterium*-infected macrophages. In addition, apoptotic blebs from bacterially infected cells induce a T_H17 response. In contrast, *Shigella* and *Salmonella*-induced pyroptosis leads to the release and exposure of bacteria to reactive oxygen species (ROS) and neutrophils (Hilbi et al., 1998; Brennan and Cookson, 2000; Miao et al., 2010). In addition to bacterial pathogens, parasitic and viral pathogens such as *Trypanosoma cruzi* and Vaccinia virus also have the ability to induce apoptosis and necroptosis, respectively (Cho et al., 2009; Duaso et al., 2011). The outcomes of necroptosis are an increase in cytokine secretion and leukocyte infiltration as well as ROS production. As illustrated from previous studies, PCD can play an important role in controlling microbial infections. Meanwhile, many pathogens can inhibit these PCD pathways in various approaches. For example, virulent wild type (WT) *Brucella* strains typically inhibit PCD of infected macrophages (Chen and He, 2009; Chen et al., 2011; Li and He, 2012). Elucidating the PCD mechanism induced or inhibited by such pathogens is critical to uncovering mechanisms of pathogenesis, as well as protective immunity.

The main executors of the PCD process are caspases, which are divided into two groups: initiators and effectors. Initiator caspases activate effector caspases via cleavage whereas effector caspases initiate cell death by cleaving various downstream apoptotic proteins. *C. elegans* has a single caspase, Ced-3, that mediates all cell death. Of 13 caspases existing in mammalian systems, caspase-2 has the highest sequence homology with Ced-3 (Hengartner, 1997; Geng et al., 2009). Caspase-2 plays important biological roles from oocyte development to aging control, and in intermediary development stages including DNA damage repair, tumor prevention, and infection control (Guo et al., 2002; Ho et al., 2009; Shi et al., 2009; Bouchier-Hayes and Green, 2012). Caspase-2 can play different roles due to its unique domain structure, which resembles an initiator and effector caspase. It contains a caspase activation and recruitment domain (CARD) which is required for auto-activation and binding to other molecules. Caspase-2 also contains a cleavage site (Hofmann et al., 1997) which resembles that of the effector caspase-3 (Talanian et al., 1997). These factors make the classification of caspase-2 difficult. Caspase-2-deficient mice develop without an overt phenotype although only mild apoptotic defects in oocyte and neuron developments were exhibited, suggesting that the function of caspase-2 is largely redundant for cellular homeostasis during development (Bergeron et al., 1998). Caspase-2 has been shown to be instrumental in bacterial infections. Caspase-2 played a role in both caspase-1-dependent and -independent apoptosis of macrophages infected with *Salmonella* (Jesenberger et al., 2000). The various and often controversial roles of caspase-2 in different organisms and experimental conditions have been documented and discussed (Troy and Ribe, 2008; Kitevska et al., 2009). The role of caspase-2 in regulating cell death and the exact mechanism remain unclear.

We previously demonstrated that rough attenuated *Brucella abortus* strain RB51 induces caspase-2-mediated, caspase-1-independent apoptotic and necrotic cell death (Chen and He, 2009). As a licensed cattle vaccine strain, RB51 is able to induce IFNγ and $CD8^+$ T cell mediated cytotoxicity in mice (He et al., 2001). Unlike its virulent counterparts, RB51 does not replicate in macrophages but it induces robust caspase-2-mediated apoptotic and necrotic cell death (Chen and He, 2009). In addition, RB51 induces cell death in dendritic cells (Li and He, 2012). However, the caspase-2-mediated RB51-induced cell death pathway is largely unknown. Previously, we showed that caspase-2 activation as well as decrease of the mitochondrial membrane potential occurred in dying macrophages infected with RB51 (Chen and He, 2009). These characteristics would suggest that apoptosis via the mitochondria-driven intrinsic pathway was the cell death mechanism. We also showed that rough attenuated *B. suis* strain VTRS1 induces caspase-2-mediated proinflammatory cell death, which we tentatively named "caspase-2-mediated pyroptosis" (Chen et al., 2011). It is likely that RB51 also induces proinflammatory response that differs inherently from non-proinflammatory apoptosis. How RB51 induces cell death remains unclear.

Here we investigated which PCD mechanism was responsible for RB51-induced cell death in macrophages. We found that RB51-infected macrophages exhibited mitochondrial dysfunction, activation of the caspase cascade (caspase-3 and caspase-8), IL-1β and TNFα secretion, and pore formation in the plasma membrane—all of which were dependent upon caspase-2. In addition to infection, we found that caspase-2 also mediated cell death as well as caspase-3 and -8 activation in macrophages treated with etoposide, naphthalene, and anti-Fas. These results illustrate that RB51-induced caspase-2-mediated macrophage cell death is unique in that it exhibits characteristics of both non-proinflammatory apoptosis and caspase-1-mediated proinflammatory pyroptosis.

RESULTS

CASPASE-2 MEDIATES RB51-INDUCED CELL DEATH VIA INTRINSIC AND EXTRINSIC APOPTOSIS PATHWAYS

Our previous experiments using a chemical inhibitor and siRNA demonstrated that caspase-2 mediates RB51-induced macrophage cell death (Chen and He, 2009). To confirm the caspase-2 mediated cell death, WT and caspase-2 deficient (*casp2*$^{-/-}$) bone-marrow derived macrophages (BMDMs) were infected with RB51, and cell death was assessed by Annexin V/propidium iodide (PI) staining and lactate dehydrogenase (LDH) release. The RB51 infection was confirmed to induce strong macrophage cell death as shown at 24 h post-infection (h.p.i., **Figures S1A,B**). In contrast, *casp2*$^{-/-}$ BMDMs exhibited little or no cell death throughout infection and only reached 8.5 ± 4.7% and 8.9 ± 5.1% at 24 h.p.i. in Annexin V/PI and LDH release assays, respectively. These results confirmed that RB51-induced cell death is mediated by caspase-2. We also assessed if there was any difference in terms of intracellular RB51 survival inside infected WT or caspase-2 deficient macrophages. Our study showed that the *casp2*$^{-/-}$ BMDMs were able to kill RB51 just as efficiently as WT BMDM (without statistically significant difference) (**Figure S1C**). Therefore, any differences in initiating cell death were not likely due to a lack of infectivity or bacterial killing in *casp2*$^{-/-}$ BMDMs.

Table 1 | Cell death measurements in caspase-2 deficient and caspase-3 and -8 inhibited macrophages.

Condition	% Cell death (±*SD*)
Untreated	0.7 ± 0.9
WT BMDMs	84.7 ± 1.7
Casp2KO BMDMs	12.7 ± 2.5
Z-DEVD-FMK (Casp3 inhibitor)	57.3 ± 2.1
Z-IETD-FMK (Casp8 inhibitor)	65.7 ± 3.7
Z-DEVD-FMK + Z-IETD-FMK	36.3 ± 2.5

Percent ± SD of $n \geq 3$ independent experiments. Cells were counted in randomly selected fields of 100 cells. All conditions except untreated where in the presence of RB51 (MOI 200).

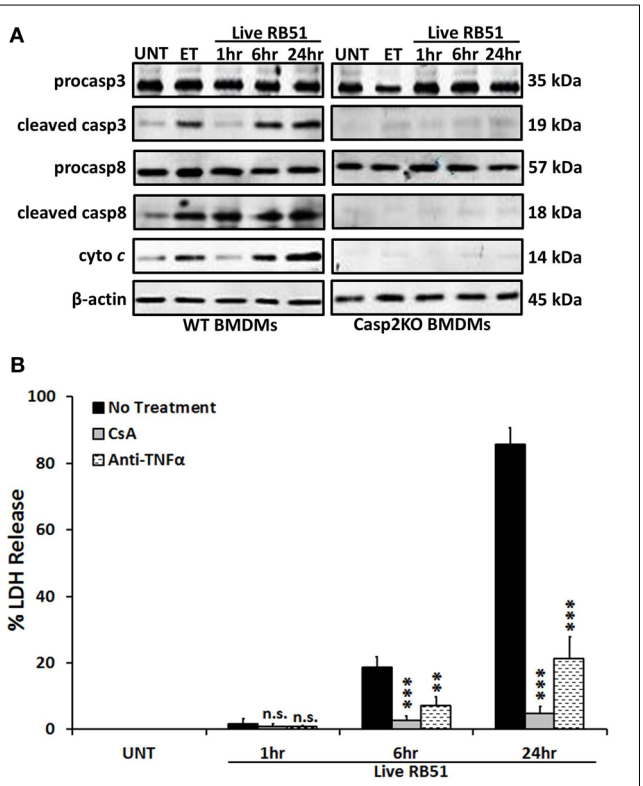

FIGURE 1 | Caspase-2 drives both the intrinsic and extrinsic cell death pathways. (A) caspase-3 and -8 cleavage (activation) as well as cytochrome *c* (cyto *c*) release in Live RB51-infected WT and *casp2*$^{-/-}$ BMDMs. β-actin served as a loading control. UT and ET represent untreated and etoposide (25 μM, 6 hr treatment), respectively. Immunoblots are representatives of $n \geq 3$ independent experiments. **(B)** LDH release in Live RB51-infected RAW264.7 macrophages in the presence of cyclosporin A (CsA; inhibitor of mitochondrial permeability transition pore, 10 μM) and Anti-TNFα (10 μ g/mL). Error bars represent mean ± *SD* of $n \geq 3$ independent experiments. $^{**}p < 0.001$ and $^{***}p < 0.0001$, Student's *t*-test. n.s. = not significant. Immunoblots are representatives of $n \geq 3$ independent experiments.

Since caspase-2 is required for RB51 induced cell death, we assessed which PCD pathway caspase-2 was mediating RB51-induced cell death. Inhibition of caspase-3 and/or caspase-8 activity led to a decrease in cell death, however, it was not as significant as caspase-2 deficiency (**Table 1**). Inhibition of caspase-3 or -8 in RB51-infected macrophages resulted in 57.3 ± 2.1% and 65.7 ± 3.7% cell death, respectively ($P < 0.001$) (**Table 1**). Inhibition of both caspase-3 and -8 led to only 36.3 ± 2.5% cell death, suggesting the two types of inhibitions are likely additive but may also be synergistic. These data suggest that RB51-induced cell death may involve the apoptotic pathways associated with caspase-3 and caspase-8.

Classically, caspase-3 and caspase-8 are linked to intrinsic (intracellular signal driven) and extrinsic (death receptor driven) apoptotic pathways, respectively (Elmore, 2007). Both pathways are linked to caspase-2 by mediating mitochondrial cytochrome *c* release and caspase-8 activation (Lin et al., 2004; Upton et al., 2008). To assess if caspase-2 regulated these pathways during RB51 infection, we investigated caspase-3 and caspase-8 activation in WT and *casp2*$^{-/-}$ BMDMs by measuring cleavage. The cleavage of both caspase-3 and caspase-8 was abolished in RB51-infected *casp2*$^{-/-}$ BMDMs (**Figure 1A**). Inhibition of caspase-3 and -8 did not affect caspase-2 activation (**Figure S2**). Previous studies illustrated that both the intrinsic and extrinsic cell death pathways can propagate signaling by inducing mitochondrial dysfunction, which eventually leads to cell death. Therefore, we investigated if caspase-2 mediated mitochondrial dysfunction in RB51-infected macrophages. Previously we observed that in RB51-infected macrophages, mitochondrial membrane potential decreased over time, suggesting that mitochondrial cytochrome *c* release (a marker of mitochondrial dysfunction) occurs. We found that in WT BMDMs, cytochrome *c* release increased throughout infection, however, in *casp2*$^{-/-}$ BMDMs cytochrome *c* release was abolished (**Figure 1A**).

To assess if mitochondrial dysfunction contributed to RB51-induced cell death, the cytochrome *c* release was blocked with cyclosporin A (CsA) in RB51-infected RAW264.7 (murine) macrophages. CsA prevents opening of the mitochondrial permeability transition pore (MPTP), a pore responsible for the release of mitochondrial contents such as cytochrome *c* (Handschumacher et al., 1984). In the presence of CsA, cell death was significantly reduced ($p < 0.0001$) in RB51-infected macrophages (**Figure 1B**). Seeing that mitochondrial dysfunction occurs and both caspase-3 and caspase-8 are activated, we explored if RB51-induced cell death was acting through the extrinsic pathway. Extrinsic or death receptor mediated cell death can be activated by Fas ligand (FasL) and TNFα. Since live attenuated *B. suis* strain VTRS1 induces a proinflammatory response (Chen et al., 2011), we investigated if TNFα played a role in mediating RB51-induced cell death. We treated RAW264.7 macrophages with anti-TNFα and assessed cell death via LDH release. Anti-TNFα treatment led to a decrease in LDH release when compared to untreated RB51-infected RAW264.7 macrophages (**Figure 1B**, 85.5 ± 5.1% vs. 21.4 ± 6.5%, respectively). These observations suggest that caspase-2 drives the extrinsic cell death pathway in RB51-infected macrophages, and the TNFα cytokine that triggers the extrinsic cell death pathway is likely secreted from RB51-infected macrophages. It is also possible that the production of internal

TNFα from a macrophage cell plays an important role of the PCD of the same macrophage cell.

CASPASE-2 REGULATES CASPASE-1 ACTIVATION AND IL-1β PRODUCTION IN RB51-INFECTED MACROPHAGES

Studies have illustrated that a proinflammatory response can be the trigger or product of cell death (Elmore, 2007; Bergsbaken et al., 2009). In addition to trigger extrinsic apoptotic cell death pathway, TNFα is also an important proinflammatory cytokine. Since TNFα played a role in RB51-induced cell death, we assessed if caspase-2 mediated TNFα production. Over time, TNFα levels increased in RB51-infected WT BMDMs. However, in *casp2*$^{-/-}$ BMDMs, TNFα was reduced to untreated levels (**Figure 2A**). In addition, CsA treatment led to a decrease in TNFα production.

Another cytokine associated with proinflammatory cell death is IL-1β. During pyroptosis, caspase-1 processes IL-1β and aids in its secretion. Interestingly, caspase-2 contains a CARD domain and has been shown to mediate caspase-1 activation during *Salmonella* infections (Jesenberger et al., 2000). Similar to TNFα, IL-1β levels increased above untreated levels starting at 6 h.p.i., however, in *casp2*$^{-/-}$ and caspase-1 inhibited BMDMs, IL-1β production was abolished (**Figure 2B**). In the presence of CsA, IL-1β levels were significantly reduced in RB51-infected macrophages. A decrease in IL-1β levels in *casp2*$^{-/-}$ BMDMs suggested that either caspase-2 regulates caspase-1 activation or caspase-2 is directly responsible for the processing of IL-1β. To evaluate these two possibilities, caspase-1 cleavage in RB51-infected WT and *casp2*$^{-/-}$ BMDMs was measured. RB51 induced caspase-1 cleavage in WT BMDMs starting at 6 h.p.i., however, cleaved caspase-1 was absent in *casp2*$^{-/-}$ BMDMs (**Figure 2C**). These data suggest that caspase-2, via mitochondrial dysfunction, mediates caspase-1 activation and IL-1β production in RB51-infected macrophages.

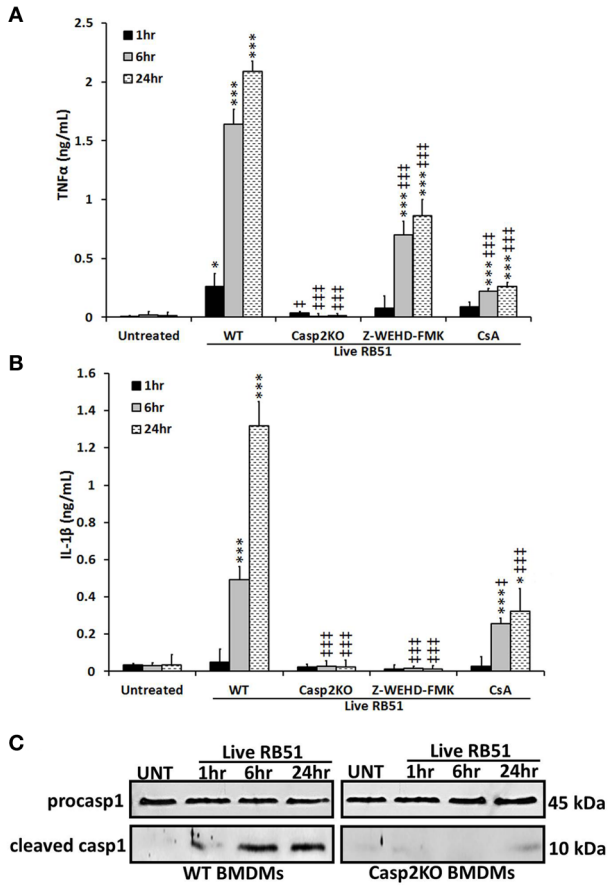

FIGURE 2 | Caspase-2 drives proinflammatory responses in RB51-infected macrophages. (A) TNFα and **(B)** IL-1β levels in WT, casp2$^{-/-}$, CsA (cyclosporin A, inhibitor of mitochondrial permeability transition pore, 10 μM) and Z-WEHD-FMK (caspase-1 inhibitor, 20 μM) treated BMDMs. Error bars represent mean ± *SD* of $n \geq 3$ independent experiments. ‡, $^{*}p < 0.01$ and ‡‡‡, $^{***}p < 0.0001$, Student's *t*-test. (*, ***) and (‡, ‡‡‡) represent comparison to untreated and WT + Live RB51, respectively. **(C)** caspase-1 cleavage (activation) in Live RB51-infected WT and casp2$^{-/-}$ BMDMs. UT represents untreated (negative control). Immunoblots are representatives of $n \geq 3$ independent experiments.

PORE FORMATION IS NOT REQUIRED FOR RB51-INDUCED CELL DEATH

Caspase-1 activation and IL-1β production are key indicators of pyroptosis. During pyroptosis, pores form in the plasma membrane, leading to a change in ionic gradient and water influx. The consequence of the pores is cell swelling, due to water influx, and eventually cell lysis. *Salmonella enterica* serovar Typhimurium SL1344 is a pathogen known for inducing pyroptosis in macrophages (Fink and Cookson, 2006). We hypothesized that RB51 induced pore formation in macrophages. To test this, SL1344- and RB51-infected RAW264.7 macrophages were separately stained with two membrane impermeant dyes, ethidium bromide (EtBr, 394 Da) and the larger sized ethidium homodimer-2 (EthD2, 1293 Da). Uptake of EtBr and EthD2 would represent discrete pore formation and loss of plasma membrane integrity, respectively. Gliotoxin (apoptosis inducer) treated RAW264.7 macrophages excluded both impermeant dyes (**Figure 3A**), indicative of an intact plasma membrane. In SL1344- and RB51-infected WT BMDMs, uptake of EtBr began at 1 h.p.i. and increased throughout infection (**Figure 3A**). Uptake of EthD2 was significantly less than EtBr and did not occur until 6 h.p.i. and 24 h.p.i. in RB51- and SL1344-infected macrophages, respectively (**Figure 3A** and **Figure S3A**). Since pore formation has been shown to be dependent upon caspase-1, it was not surprising to see that uptake of both EtBr and EthD2 were abolished in RB51-infected *casp2*$^{-/-}$ and caspase-1 inhibited WT BMDMs (**Figure 3A** and **Figure S3B**).

We further assessed if pore formation in RB51-infected macrophages contributed to RB51-induced cell death. RB51-infected macrophages were treated with glycine, a cytoprotective agent. Glycine prevents cell death by stabilizing the ion gradient (Frank et al., 2000). Glycine treatment decreased SL1344-induced macrophage cell death, however, no effect was seen with RB51 (**Figure 3B**). These findings demonstrate that although pore formation occurs in RB51-infected macrophages, it is not the cause of the RB51-induced macrophage cell death.

CASPASE-2 MEDIATES CELL DEATH IN OTHER CONTEXTS

Since caspase-2 can regulate cell death in the context of a *Brucella* infection, we investigated if caspase-2 also plays a critical

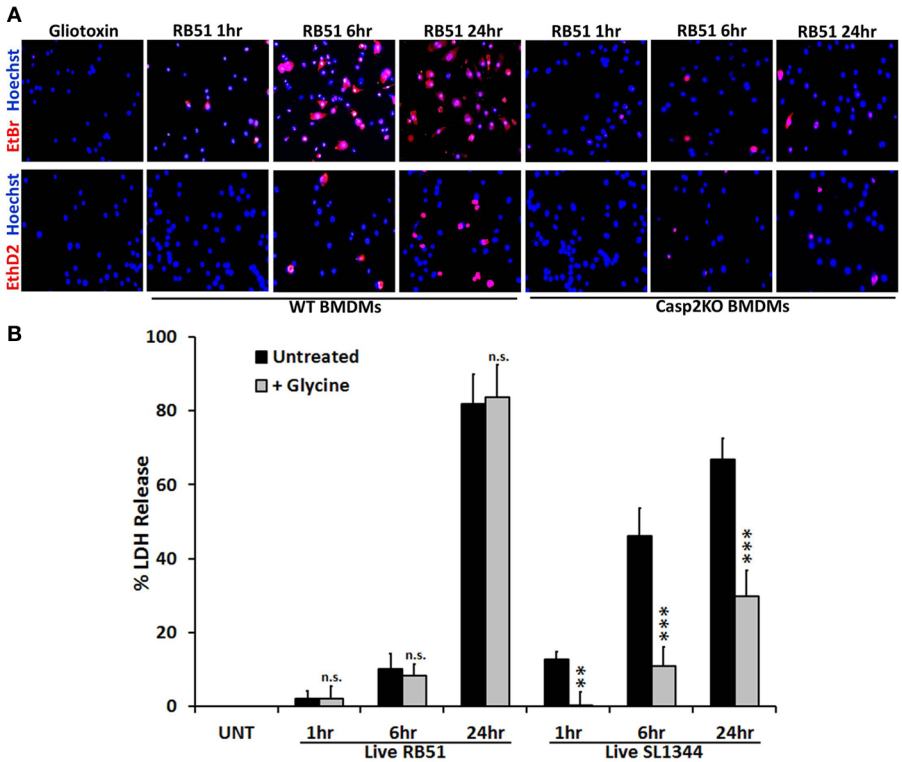

FIGURE 3 | Pore formation does not contribute to RB51-induced cell death. (A) WT and casp2$^{-/-}$ BMDMs treated with Gliotoxin (10 μM, 6 h treatment) or infected with RB51 were stained with the membrane permeable dye Hoechst 33342 (blue) and the membrane impermeant dyes (red), EtBr (MW 394) or EthD2 (MW 1293). Adherent cells were visualized by fluorescence microscopy (100x). Images are representatives of n ≥ 3 independent experiments. **(B)** LDH release in Live RB51 and SL1344-infected RAW264.7 macrophages in the absence or presence of glycine (5 mM). Error bars represent mean ± *SD* of $n \geq 3$ independent experiments. $^{**}p < 0.001$ and $^{***}p < 0.0001$, Student's *t*-test. n.s. = not significant.

role in the presence of different cell death chemical inducers—etoposide, naphthalene, anti-Fas, and gliotoxin. Etoposide and naphthalene are DNA damaging agents that trigger mitochondrial ROS production, inducing cell death via the intrinsic pathway (Gorman et al., 1997; Bagchi et al., 1998, 2001; Pham and Hedley, 2001). Treatment with anti-Fas antibody mimics activation of death receptor signaling by FasL through the extrinsic pathway. (Aggarwal and Gupta, 1999). Gliotoxin mediates Bak activation leading to cytochrome *c* release from the mitochondria and cell death (Pardo et al., 2006). To assess the role of caspase-2 in the presence of these chemical inducers, we first assessed caspase-2 activation. In macrophages treated with etoposide, naphthalene, and anti-Fas, caspase-2 was activated. However, gliotoxin did not induce caspase-2 activation (**Figure 4A**). Next, we investigated if caspase-2 mediated cell death in the presence of these inducers via Annexin V/PI staining. All four chemicals induced cell death after 6 h of treatment. The caspase-2 deficiency led to a significant decrease in cell death in etoposide, naphthalene, and anti-Fas treated but not gliotoxin treated macrophages (**Figure 4B**).

Knowing the mechanism by which these chemical inducers mediate cell death, we assessed whether caspase-3 and -8 activation occurred and whether caspase-2 mediated their activation as seen in RB51-infected macrophages. Caspase-3 was activated in etoposide, naphthalene, anti-Fas, and gliotoxin treated WT BMDMs (**Figure 4C**). Caspase-3 activation was abolished in *casp2*$^{-/-}$ BMDMs treated with etoposide, naphthalene, and anti-Fas but not gliotoxin. Caspase-8 activation was observed in etoposide, naphthalene, and anti-Fas treatment yet absent during gliotoxin treatment (**Figure 4C**). Similar to caspase-3, caspase-8 activation was abolished in etoposide, naphthalene, anti-Fas treated *casp2*$^{-/-}$ BMDMs. These data indicate that caspase-2 can exhibit a crucial role in mediating cell death initiated by other stimuli besides microbial infection.

DISCUSSION

Here we report that RB51-induced cell death is driven by the caspase-2/mitochondrial axis. Our results demonstrated that RB51-infected macrophages induce the caspase-2 activation, and the activated caspase-2 mediates caspase-1, -3, and -8 activations, and TNFα and IL-1β secretions. Mitochondrial release of cytochrome *c* was associated with caspase-3 activation and contributed to RB51-induced cell death. Although pores formed in the plasma membrane upon infection, they did not contribute to cell death. In addition to cell death, we showed that the RB51-induced proinflammatory response was mediated by caspase-2 and mitochondrial dysfunction. In addition

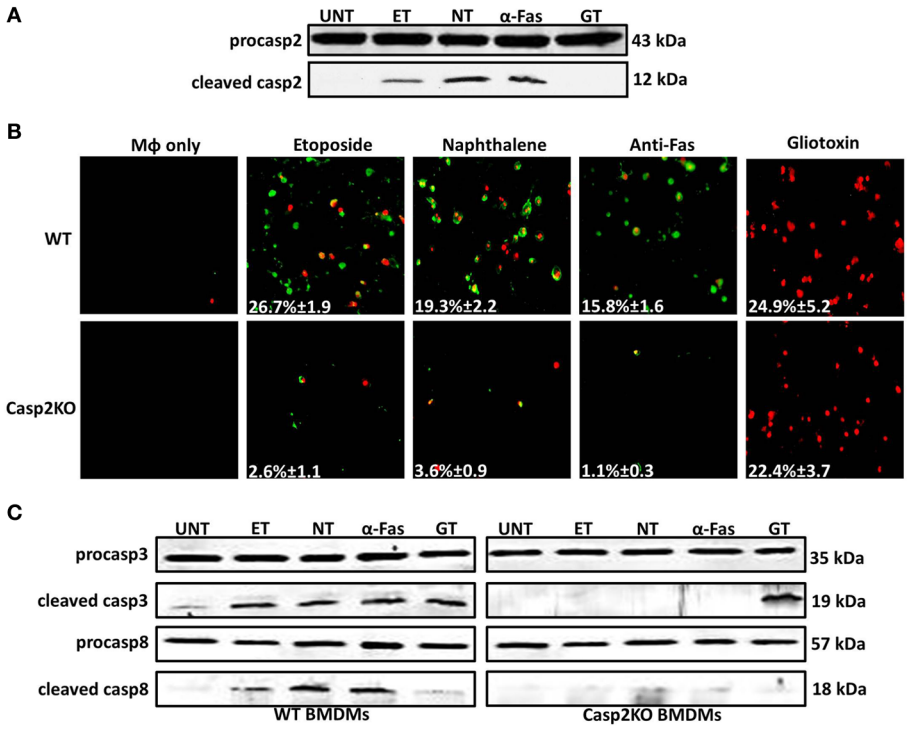

FIGURE 4 | Caspase-2 mediates caspase-3 and -8 activation in other contexts. (A) Caspase-2 cleavage (activation) in Live RB51-infected RAW264.7 macrophages. UT, ET, NT, α-Fas, and GT represent untreated, etoposide (25 μM, 6 h treatment), Naphthalene (100 μM, 6 h treatment), Anti-Fas (1 μg/mL, 6 h 4 treatment), and Gliotoxin (10 μM, 6 h treatment), respectively. **(B)** ET, NT, α-Fas, GT treated WT and casp2^{-}/$^{-}$ BMDMs were stained with Annexin V/propidium iodide (PI). Adherent cells were visualized by fluorescence microscopy (100x). Cells were counted in randomly selected fields of 100 cells. Images are representatives of $n \geq 3$ independent experiments. **(C)** Caspase-3 and −8 cleavage (activation) in ET, NT, α-Fas, GT treated WT and casp2−/− BMDMs. Immunoblots are representatives of $n \geq 3$ independent experiments.

to microbial infections, caspase-2 also demonstrated a contributing role in mediating cell death with different cell death inducers.

Rough *Brucella* species are known to induce PCD (Freeman et al., 1961; Fernandez-Prada et al., 2003; Pei et al., 2006) as well as a robust immune response (Rittig et al., 2003). Here we show that proinflammatory cytokine TNFα plays a critical role in RB51-induced macrophage cell death, however, TNFα appears not to play an important role in macrophage cell death induced by the infection of strain CA180, another *B. abortus* rough strain (Pei et al., 2006). Although both RB51 and CA180 share a rough *Brucella* lipopolysaccharide (LPS) phenotype (i.e., containing deficient O antigen on their LPS), the LPS phenotype patterns in these two strains are likely different. CA180 has a truncated core polysaccharide due to a mutation in the phosphomannomutase gene (Allen et al., 1998). Phosphomannomutase is needed to the elongation of the polysaccharide core. The polysaccharide core has been shown to elicit cytokine production, any changes (e.g., shortening) can decrease cytokine production (Otterlei et al., 1993; Berntzen et al., 1998). The rough phenotype of RB51 is primarily due to the mutation of a gene *wboA* that encodes for a glycosyltransferase required for O-side chain sysnthesis (Vemulapalli et al., 1999). Additional mutation also exists in RB51 (Marianelli et al., 2004). Rough mutants with different gene mutations are known to possibly induce different phenotypes (Gonzalez et al., 2008). Therefore, the different gene mutations in RB51 and CA180 may explain the difference in the role of TNFα in the induced macrophage cell death.

Although caspase-2 has been linked to many different cellular processes, caspase-2 is still considered primarily a proapoptotic caspase. Here we show that caspase-2 can mediate IL-1β production, as well as caspase-1 activation. In addition to *Brucella* infection, caspase-2 has also been shown to regulate caspase-1 activation in *Salmonella* infected macrophages. We speculate that caspase-2 might regulate caspase-1 by interacting with the inflammasome complexes responsible for caspase-1 activation or by inducing an inflammasome trigger (e.g., ER stress, mitochondrial DNA release, or ROS production). Caspase-1 interacts with different inflammasome proteins and accessory proteins via its CARD domain. Inflammatory caspases (caspase-1, −4, −5, −11) interact with inflammasome and accessory proteins via their CARD domain. Caspase-2 also contains a CARD domain. Further investigation into whether caspase-2 can interact with different inflammasome components is needed. Another possible mechanism for caspase-2 regulation of caspase-1 activation is through mitochondrial dysfunction. As seen with CsA treatment, there was a sharp decrease in IL-1β production suggesting that the mitochondria can aid in inflammasome activation during RB51 infection. Recent studies have

linked NLRP3 and AIM2 activation to mitochondrial dysfunction (Nakahira et al., 2011; Misawa et al., 2013; Subramanian et al., 2013). It is possible that caspase-2-induced mitochondrial dysfunction leads to release of mitochondrial danger associated molecular patterns (DAMPs). Mitochondrial DAMPs (e.g., mtDNA or cardiolipin exposure) can lead to NLRP3 and AIM2 inflammasome activation (Nakahira et al., 2011). Caspase-2 has been linked to mediating mitochondrial dysfunction—caspase-2 cleaves Bid leading to mitochondrial outer membrane pore formation and eventually release of mitochondrial content (e.g., cytochrome *c*). Whether caspase-2-mediated release of mitochondrial DAMPs is the mechanism by which the inflammasome and caspase-1 activation occurs remains to be elucidated.

The caspase-2-mediated cell death pathway seen in RB51-infected macrophages differs from classical apoptosis, caspase-1-mediated pyroptosis, and necroptosis (**Table 2**). RB51-induced cell death is not mediated by caspase-1. Our data suggest that RIP1 and RIP3 (mediators of necroptosis) are not active because both caspase-2 and -8 activated by RB51 infection can cleave RIP1 and RIP3—the cleavage of these two prevents necroptosis from occurring (Lin et al., 1999; Lamkanfi et al., 2005). In addition, RB51-infected macrophages secrete proinflammatory cytokines starting at 6 h.p.i., a characteristic absent in apoptosis. Caspase-1 is active and contributes to IL-1β production. However, unlike the phenomenon seen in caspase-1-mediated pyroptosis, neither caspase-1 nor the pore in the plasma membrane contributes to cell death. Our data suggest that RB51-induced caspase-2-mediated cell death does not fall into any of the three classical PCD pathways. The term "pyroptosis" was originally coined to indicate proinflammatory (="pyro") PCD ("=ptosis") (Bergsbaken et al., 2009). Owing to the common feature of caspase-mediated proinflammatory cell death between caspase-1-mediated pyroptosis and caspase-2-mediated cell death, previously we tentatively labeled the caspase-2-mediated proinflammatory cell death as "caspase-2-mediated pyroptosis" (Chen et al., 2011). Considering that current study also clearly shows that caspase-2 also mediates apoptosis-like features (e.g., caspase-3 and -8 activation and loss of mitochondrial membrane potential) (**Table 2**), caspase-2 appears to mediate a hybrid cell death that includes partial features of both non-proinflammatory apoptosis and caspase-1-mediated proinflammatory pyroptosis. Although distinct, caspase-2-mediated cell death and necroptosis appear to also share some similarities as well (**Table 2**).

Table 2 | Comparison of caspase-2-mediated cell death to classical apoptosis and pyroptosis.

	Apoptosis	Casp-1 pyroptosis	Necroptosis	*Casp2*-mediated cell death
Plasma membrane pore formation	−	+	−	+
Loss in plasma membrane integrity	−	+ (swelling = lysis)	+	+ (6 and 24 h.p.i.)
Loss of mitochondrial membrane potential	+	−	+	+
Cytochrome *c* release	+	−	+	+
Proinflammatory	−	+	+	+
Caspase-1 mediated	−	+	−	−
Caspase-2 mediated	+/−	−	−	+
Caspase-3 activation	+	−	−	+
Caspase-8 activation	+/− (extrinsic)	−	−	+
ROS production	+	−	+	+
Release of DAMPs	−	+	+	+
RIP1/3-mediated	−	−	+	?

+, occurs; −, does not occur; +/−, may occur but not required; ?, unknown.

In addition to RB51, rough attenuated *B. abortus* strain RA1 (Chen and He, 2009) and *B. suis* strain VTRS1 also induces caspase-2-mediated macrophage cell death (Chen et al., 2011). However, their parent WT strains 2308 and 1330 do not induce macrophage cell death. Our recent study found that caspase-2 also mediates PCD of dendritic cells infected with RB51 and its WT strain 2308 (Li and He, 2012). The induction of caspase-2-mediated cell death in strain 2308-infected dendritic cells but not in strain 2308-infected macrophages facilitates the survival of the virulent strain inside macrophages and avoidance of *Brucella* antigen presentation inside dendritic cells (Li and He, 2012). It is likely that such caspase-2-mediated host cell death is also induced or inhibited by other infectious microbes, such as live attenuated vaccine strains or virulent pathogens of intracellular bacteria and viruses.

Our studies on the relationship between caspase-2 and cell death induced by different cell death inducers further illustrate the critical and conserved role and the mechanism of caspase-2 in triggering PCD. Both etoposide and naphthalene are DNA damage inducers, a stimulus known for activating caspase-2. Interestingly, both etoposide and naphthalene induced caspase-8 activation. Previous studies have illustrated a connection between DNA damage, caspase-8, and caspase-2. Both caspase-2 and caspase-8 can be activated by p53 (Seth et al., 2005; Liu et al., 2011); this suggests that upon etoposide and naphthalene treatment, p53 activates caspase-2 leading to caspase-8 activation. Anti-Fas works through the extrinsic pathway and leads to cell death via caspase-8 dependent activation of caspase-3. Seeing that caspase-2 aided in caspase-8 activation (outside of DNA damage) clearly suggests that caspase-2 can act as an initiator caspase to mediate activation of other caspases. Whether caspase-2 mediates caspase-8 activation directly or indirectly remains unclear. Gliotoxin induced cell death via the intrinsic pathway. Only caspase-3 was activated in gliotoxin-treated macrophages. Cytochrome *c* can aid in caspase-2 activation,

however, (Slee et al., 1999), in the context of gliotoxin intoxication this was not the case because caspase-2 activation did not occur. Gliotoxin acts on Bak and its downstream target t-Bid [a known target cleaved by caspase-2, (Upton et al., 2008)]; therefore, it is possible that gliotoxin short-circuits the classical intrinsic pathway and does not require signaling components upstream of the mitochondria. In the case of etoposide and naphthalene, the data suggest that caspase-2 regulates caspase-3 activation via the mitochondria. Caspase-3 is activated via the apoptosome, a multiprotein complex dependent upon cytochrome *c* release. Caspase-2 was previously reported to act upstream of caspase-8 during ceramide-induced mitochondrial apoptosis in T cells (Lin et al., 2004). It appears that the caspase-2 regulation of caspase-3 and -8 can occur in different cell types with different treatments, so this type of regulation is neither cell specific nor context specific. These observations suggest that caspase-2 can play a critical role in initiating PCD.

The caspase-2-mediated cell death pathway is likely critical to microbial pathogenesis and host immunity. In the context of RB51, cell death and the proinflammatory response may have synergistic effects on host immune responses. Cell death may result in the exposure of RB51 to a more hostile extracellular environment (as seen in pyroptosis and necroptosis). In addition, neighboring macrophages and dendritic cells may recognize processed RB51 antigens leading to cross priming of $CD8^+$ T cells (important for RB51-induced protective immunity). We recently showed that RB51 induced cell death in bone marrow derived dendritic cells and aided in maturation as well as priming of T cells—all of which were dependent upon caspase-2 (Li and He, 2012). These observations demonstrate the importance for caspase-2, as well as cell death, in initiating the immune response. Utilizing this PCD pathway may ensure that the host triggers a potent immune response. Prevention of this pathway may aid in enhancing survival of virulent *Brucella* in macrophages. Our previous work suggested prevention—virulent strain *B. abortus* S2308 did not induce caspase-2 activation nor cytochrome *c* release in infected macrophages (Chen and He, 2009).

Caspase-2 is also implicated in other processes (cancer regulation and metabolism) and may take on a regulatory role in these processes as well. We have made an original observation that caspase-2 plays a non-redundant role in triggering the proinflammatory cell death of RB51-infected macrophages and in macrophages treated with various drugs. After the evolution of complex caspase-cascade cell death signaling pathways in advanced animals, it is suggestive that the protein functions of the highly conserved caspase-2 have been preserved during evolution and serve as safeguards to regulate various cell death pathways. It is likely that intracellular pathogens with similar lifestyles to *Brucella* (e.g., *Salmonella*, *Mycobacterium*, *Listeria*, *Francisella*, and *Legionella*) may utilize caspase-2 during infection. Further understanding of caspase-2-mediated pyroptosis can aid in supplying a blueprint for effective brucellosis vaccines (both animals and humans) as well as effective therapeutics against cancers and other diseases.

MATERIALS AND METHODS

MICE

The caspase-2 knockout (Casp2KO) mice were originally generated by Junying Yuan and kindly provided by Dr. Brian Herman of the University of Texas Health Science Center at San Antonio with Dr. Yuan's consent (Bergeron et al., 1998). The deletion inactivates both the long and short form of caspase-2. The mice were backcrossed with C57BL/6 once in the Unit for Laboratory Animal Medicine at the University of Michigan Medical School, and then used as founders. Casp2KO and WT C57BL/6 (Jackson) mice with similar ages were applied in the experiments. The University Committee on Use and Care of Animals (UCUCA) at the University of Michigan approved the protocol (#09695) to use mice for studies described here.

BACTERIAL STRAINS AND REAGENTS

RAW264.7 macrophages and bone marrow derived macrophages (BMDMs) were infected with *Brucella abortus* strain RB51 (from Dr. G. Schurig, Virginia Polytechnic Institute and State University) and *Salmonella typhimurium* SL1344 (from Mary O'Riordan, University of Michigan). The following inhibitors and inducers were used: CsA (Sigma-Aldrich), etoposide (Sigma-Aldrich), naphthalene (Sigma-Aldrich), Anti-Fas (BioVision), gliotoxin (-Aldrich), glycine (Sigma-Aldrich), Z-WEHD-FMK (Caspase-1 inhibitor, R&D Systems), Z-DEVD-FMK (Caspase-3 inhibitor, R&D Systems), Z-IETD-FMK (Caspase-8 inhibitor, R&D Systems), and anti-TNFα (mouse specific, BioVision).

The following antibodies were used: anti-cytochrome *c* (cat#:4272S, Cell Signaling), anti-caspase-3 (cat#: 9662S, Cell Signaling), anti-caspase-8 (cat#: 4927S, Cell Signaling), anti-caspase-2 (cat#: 3027-100, BioVision), anti-caspase-1 (cat#: sc-514, Santa Cruz), and anti-actin (cat#: MS1295P1, Thermo Scientific).

CELL CULTURE AND INFECTION

BMDMs were isolated from WT and *casp2*$^{-/-}$ mice on a C57BL/6 background. Isolated BMDMs were differentiated in DMEM (GIBCO) supplemented with 20% heat-inactivated FBS (GIBCO), 1% L-glutamine (200 mM), 1% sodium pyruvate (100 mM), 0.1% β-mercaptoethanol (55 mM), and 30% L-929 fibroblast conditioned medium. BMDMs were cultured in non-TC treated plates, fed fresh media on Day 3, and harvested on Day 6. BMDMs were maintained at 37°C under 5% CO_2.

Four million RAW264.7 macrophages and BMDMs were seeded in 6 well plates 18 h prior to infection. The following day, where indicated cells were pretreated with CsA (10 μM), Z-WEHD-FMK (20 μM), Z-DEVD-FMK (20 μM), Z-IETD-FMK (20 μM) and Anti-TNFα (10 μ g/mL) for 1 h prior to infection. Untreated and pretreated cells were infected with RB51 (MOI 200) or SL1344 (MOI 25) for 30 min, after which the inoculum was removed and cells were washed with PBS. Medium containing 50 μ g/ml of gentamicin was added to kill extracellular bacteria. To synchronize infection, cells were spun at 1200 rpm for 3 min after adding inoculum. Cells were treated with etoposide (25 μM), naphthalene (100 μM), anti-Fas (1 μ g/mL), and gliotoxin (10 μM) for 6 hr. At the indicated times, cells were lysed

in buffer containing 1% NP-40 on ice for 15 min and spun at 13,000 rpm for 15 min to pellet the insoluble fraction. Soluble fractions were used for immunoblot assays.

IMMUNOBLOT ASSAY

Cytosolic extracts collected at various time points (1, 6, and 24 h pi) were separated by SDS-PAGE, transferred to PVDF membranes (Millipore), blocked with 5% milk in TBS-Tween20 (TBS-T), and incubated overnight at 4°C with primary antibodies stated above. Membranes were washed with TBS-T and incubated with secondary HRP conjugated to either goat anti-rabbit IgG (cat#: 12–348, Millipore) or goat anti-mouse IgG (cat#: 1034-05, Southern Biotech) at room temperature for 1 h. Bands were visualized using the ECL Western Blotting Substrate Kit (Pierce). Immunoblots in the figures are representative of $n \geq 3$ independent experiments.

CYTOKINE DETECTION

Culture supernatants were collected at different time points (1, 6, and 24 h pi) from macrophages infected as described above. IL-1β and TNFα levels were determined by sandwich enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions (BioLegend). A minimum of three technical replicates per experiment and three experimental replicates were analyzed for each condition.

CELL DEATH ASSAY

RAW264.7 macrophages were seeded in 6 well plates at a concentration 9.6×10^4 per well and infected with RB51 as stated above. Cells were stained with Annexin V and PI using the Annexin V-FLUOS staining kit (Roche Diagnostics Corporation). Cells were washed with PBS and incubated with the fluorescent dyes for 15 min in the dark at room temperature. Fluorescence was observed with a Nikon TK-2000-S microscope and photographed with a RT Slide Spot digital camera and QCapture Pro software. Uninfected macrophages served as negative controls.

ETHIDIUM BROMIDE (EtBr) AND ETHIDIUM HOMODIMER-2 (EthD2) STAINING

Macrophages were grown in 6-well plates and infected as described above. At different time points post-infection, cells were washed with PBS (GIBCO) and stained with Hoechst 33342 (5 μ g/mL) and either EtBr or EthD2 (25 μ g/mL) according to the manufacturer's instructions. Cell were analyzed with a Nikon TK-2000-s microscope and photographed with a RT slide spot digital camera and QCapture Pro Software.

LACTATE DEHYDROGENASE (LDH) RELEASE ASSAY

Macrophages were seeded in 96-well plates and infected with RB51 or SL1344 as stated above. Supernatants were analyzed for the presence of LDH enzyme using the CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega) as directed by the manufacturer's instructions. Percentage of LDH release was calculated as 100 × [(Experimental LDH Release—Culture Medium Background)/(Maximum LDH Release—Culture Medium Background)].

STATISTICAL ANALYSIS

All p-values were generated between identified samples using unpaired two-tailed Student's t-tests and represent analysis of ≥3 replicates per condition. ‡, $^*p < 0.01$ ‡ ‡, $^*p < 0.001$ and ‡ ‡ ‡, $^{***}p < 0.0001$.

AUTHOR CONTRIBUTIONS

Denise N. Bronner and Yongqun He designed the experiments. Denise N. Bronner performed the experiments. Denise N. Bronner, Mary X. D. O'Riordan and Yongqun He analyzed the data. Yongqun He and Mary X. D. O'Riordan contributed reagents and materials. Denise N. Bronner and Yongqun He wrote the paper. All authors edited and approved the manuscript.

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SUPPLEMENTARY METERIAL

The Supplementary material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fcimb.2013.00083/abstract

Figure S1 | Caspase-2 mediates RB51-induced macrophage cell death. (A) Annexin V/propidium iodide (PI) staining of RB51-infected WT and *casp2*$^{-/-}$ BMDMs at 100X magnification. Images are representatives of $n \geq 3$ independent experiments. **(B)** LDH release in Live RB51-infected WT and *casp2*$^{-/-}$ BMDMs. Cells were counted in randomly selected fields of 100 cells. **(C)** CFU analysis of RB51 in WT and *casp2*$^{-/-}$BMDMs, Error bars represent mean ± *SD* of $n \geq 3$ independent experiments. $^{**}p < 0.001$ and $^{***}p < 0.0001$, Student's t-test. n.s. = not significant.

Figure S2 | Caspase-3 and -8 are involved in RB51-induced cell death. (A) Caspase-2 cleavage (activation) in Live RB51-infected RAW264.7 macrophages with or without Z-DEVD-FMK (20 μM, Caspase-3 inhibitor). **(B)** Caspase-2 cleavage (activation) in Live RB51-infected RAW264.7 macrophages with or without Z-IETD-FMK (20 μM, Caspase-8 inhibitor). UNT and ET represent untreated and etoposide (25 μM, 6 h treatment), respectively. Immunoblots are representatives of $n \geq 3$ independent experiments.

Figure S3 | Caspase-1 aids in RB51-induced pore formation. RB51-infected RAW264.7 macrophages treated with Z-YVAD-FMK (20 μM, caspase-1 inhibitor) were stained with the membrane permeable dye Hoechst 33342 (blue) and the membrane impermeant dyes (red), EtBr (MW 394) or EthD2 (MW 1293). Adherent cells were visualized by fluorescence microscopy (100x). Images are representatives of $n \geq 3$ independent experiments.

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ESX-1-induced apoptosis during mycobacterial infection: *to be or not to be, that is the question*

Nacho Aguiló[1,2]*†, Dessislava Marinova[1,2], Carlos Martín[1,2] and Julián Pardo[3]*†

[1] Grupo de Genética de Micobacterias, Department of Microbiología, Medicina Preventiva y Salud Pública, Universidad de Zaragoza, Zaragoza, Spain
[2] CIBER Enfermedades Respiratorias, Instituto de Salud Carlos III, Madrid, Spain
[3] Cell Immunity in Cancer, Inflammation and Infection group, Biomedical Research Centre of Aragon, Nanoscience Institute of Aragon, Aragon I+D Foundation, IIS Aragon/University of Zaragoza, Zaragoza, Spain

Edited by:
Yongqun He, Virginia Tech, USA

Reviewed by:
Galina Mukamolova, University of Leicester, UK
Michael L. Vasil, University of Colorado School of Medicine, USA

***Correspondence:**
Nacho Aguiló, Department of Microbiología, Medicina Preventiva y Salud Pública, Universidad de Zaragoza, C/ Domingo Miral s/n, 50009 Zaragoza, Spain
e-mail: naguilo@unizar.es;
Julián Pardo, Biomedical Research Centre of Aragon, Avda. San Juan Bosco 13, 50009 Zaragoza, Spain
e-mail: pardojim@unizar.es
† *These authors share senior authorship.*

The major *Mycobacterium tuberculosis* virulence factor ESAT-6 exported by the ESX-1 secretion system has been described as a pro-apoptotic factor by several independent groups in recent years, sustaining a role for apoptosis in *M.*tuberculosis pathogenesis. This role has been supported by independent studies in which apoptosis has been shown as a hallmark feature in human and mouse lungs infected with virulent strains. Nevertheless, the role of apoptosis during mycobacterial infection is subject to an intense debate. Several works maintain that apoptosis is more evident with attenuated strains, whereas virulent mycobacteria tend to inhibit this process, suggesting that apoptosis induction may be a host mechanism to control infection. In this review, we summarize the evidences that support the involvement of ESX-1-induced apoptosis in virulence, intending to provide a rational treatise for the role of programmed cell death during *M. tuberculosis* infection.

Keywords: *Mycobacterium tuberculosis*, apoptosis, virulence, necrosis, attenuated strains, cell death

INTRODUCTION

Host cells can recognize any self-damage (aging, pathogen infection, DNA damage, etc.) and as a result can activate the extrinsic or the intrinsic apoptotic program that leads to programmed cell death. This process is regulated by a family of cysteine proteases (caspases) (Hotchkiss and Nicholson, 2006), the Bcl-2 family proteins consisting of pro-apoptotic (Bid, Bak, Bax, Bim, PUMA, etc) and anti-apoptotic (Bcl-2, Bcl-XL, Mcl-1, etc) members (Adams and Cory, 2007) and the p53 family (p53, p63, and p73) involved in cell cycle control and induction of apoptosis following DNA damage (Levrero et al., 2000). The major effect of apoptosis is the generation of specific signals to attract phagocytes to remove dying cells and avoid tissue damage (Ravichandran, 2011). Resultantly, for some time apoptosis was considered a silent form of cell death. However, in recent years, it has become evident that apoptotic cell death is not always silent, but can induce activation of the immune system against tumoral or pathogen-derived antigens contained within dying cells (Kono and Rock, 2008) by a process known as cross-priming (Bevan, 1976). This process involves the activation of MHC-I restricted naive $CD8^+$ T cells by dendritic cells that have engulfed exogenous antigens (den Haan et al., 2000) including dying cells (Ronchetti et al., 1999).

Intracellular pathogens can modulate programmed cell death by blocking or promoting host cell apoptosis to favor infection outcome (Finlay and McFadden, 2006). Some intracellular pathogens such as *Salmonella* (Guiney, 2005), *Shigella* (Zychlinsky et al., 1992), or *Yersinia* (Monack and Falkow, 2000) use apoptosis as a colonization mechanism to infect new host cells, thus, avoiding exposure to extracellular host defence mechanisms (Finlay and McFadden, 2006). The different lifestyle and replication adaptation of obligate vs. facultative intracellular pathogens could explain the paradox of programmed cell-death modulation by different intracellular pathogens. Moreover, the same microorganism can inhibit or induce apoptosis depending on the stage of infection, as described in the case of Chlamydia (Byrne and Ojcius, 2004). In continuation, we summarize the experimental evidences supporting either inhibition or activation of apoptosis as mechanisms of *M. tuberculosis* virulence with the aim to provide a rational explanation of how apoptosis modulation can affect mycobacterial pathogenesis.

ESX-1 DEPENDENT APOPTOSIS

The role of apoptosis in *M. tuberculosis* infection has been a matter of intense debate over the last years. Conflicting results supporting either inhibition (Balcewicz-Sablinska et al., 1998; Keane et al., 2000; Chen et al., 2006; Gan et al., 2008; Divangahi et al., 2009; Behar et al., 2010) or induction (Rojas et al., 1997; Schaible et al., 2003; Gao et al., 2004; Derrick and Morris, 2007; Leong et al., 2008; Davis and Ramakrishnan, 2009; Seimon et al., 2010; Aporta et al., 2012) of apoptosis as a virulence strategy to establish and spread mycobacterial infection have been reported.

One of the strongest experimental findings supporting the ability of virulent *M. tuberculosis* to induce apoptosis in host

macrophages is the expression of the major virulence factor 6 kDa early secretory antigenic target (ESAT-6), secreted through the ESX-1 export system. Different groups have independently reported that ESAT-6 secretion is essential for apoptosis induction on infected cells (Derrick and Morris, 2007; Choi et al., 2010; Aporta et al., 2012; Aguilo et al., 2013). Moreover, provided that loss of ESAT-6 is linked to attenuation of different mycobacterial strains (Pym et al., 2002), it is tempting to speculate that ESAT-6-induced cell death could represent a viable mechanism of virulence for *M. tuberculosis*. Attenuated mycobacterial strains, like BCG or the live-attenuated *phoP*-/DIM-deficient *M. tuberculosis* strain MTBVAC (Arbues et al., 2013), which lack a functional ESX-1 system have lost the ability to induce apoptosis and cell death (Rojas et al., 1997; Schaible et al., 2003; Aporta et al., 2012; Aguilo et al., 2013). Indeed, Winau et al induced apoptosis externally on BCG-infected macrophages by serum deprivation to demonstrate that apoptosis is linked to cross-priming of mycobacterial antigen-specific CD8+ T-cells (Winau et al., 2006). Remarkably, RD1-complemented BCG, which fully restores ESAT-6 secretion and virulence, results highly pro-apoptotic *in vitro* and *in vivo* (Aguilo et al., 2013).

HOW CAN APOPTOSIS CONTRIBUTE TO VIRULENCE?

Data from different works provide evidence that ESX-1-induced apoptosis can contribute to virulence by spreading infection. RD1-defficient H37Rv, which is unable to trigger apoptosis (Derrick and Morris, 2007), has shown impaired capacity to colonize new uninfected cells (Gao et al., 2004; Guinn et al., 2004), suggesting that apoptosis favors cell-to-cell bacterial spread. Confirming the role of apoptosis in host colonization by virulent mycobacteria, we recently reported that *in vitro* apoptosis induction by several virulent strains promotes bacterial spread into bystander macrophages. Conversely, ESX-1-deficient strains have lost cell-to-cell colonization capacity, indicating that this mechanism is dependent on ESAT-6 secretion (Aguilo et al., 2013). Confirming these data *in vivo*, the importance of ESX-1 dependent apoptosis for bacterial spread has been shown in the Zebra fish model (Davis and Ramakrishnan, 2009).

Supporting the hypothesis of apoptosis induction as an advantageous cell-to-cell spread mechanism for pathogenic mycobacteria, Schaible et al showed that virulent *M. tuberculosis* Erdman strain induces apoptosis in both macrophages and dendritic cells and cell death is accompanied by the generation of typical apoptotic bodies (Schaible et al., 2003). In a series of elegantly controlled experiments the authors showed that these apoptotic bodies were engulfed by bystander macrophages using classical phagocytic receptors for apoptotic cells.

HOW DOES ESAT-6 INDUCE APOPTOSIS ON THE HOST CELL?

Previous works suggest that endoplasmic reticulum (ER)-stress associated pathways are activated and induce apoptosis during *M. tuberculosis* infection in an ESAT-6-dependent fashion (Choi et al., 2010; Grover and Izzo, 2012). Lim et al. (2011) demonstrated the activation of classical ER-stress markers in macrophages during *M. tuberculosis* infection *in vitro*. Co-localization of ER-stress and apoptotic markers has also been found in both mouse and human infected lungs indicating that these signaling routes are activated by *M. tuberculosis* under physiological conditions (Seimon et al., 2010).

Different intracellular events can trigger activation of ER-stress associated pathways leading to the activation of the intrinsic apoptotic pathway (Gorlach et al., 2006). ESAT-6 has been reported to increase intracellular Ca^{2+} concentration and reactive oxygen species (ROS) (Choi et al., 2010), which are classical ER-stress activators. A mechanism involving ER-stress and ROS induction has been described for *M. kansasii*-induced apoptosis (Lim et al., 2013). Interestingly, RD1-deficient H37Rv mutant is unable to cause intracellular Ca^{2+} increment and subsequent calpain activation (Yang et al., 2013), suggesting that ESAT-6 is responsible for triggering the initial events that would lead to cell death through ER-stress. One of the main downstream regulators of ER-stress-induced apoptosis is the ASK1-p38MAPK route (Matsuzawa et al., 2002). ASK1-deficient macrophages are not able to phosphorylate p38MAPK after *M. tuberculosis* infection and as a consequence are highly resistant to apoptosis induced by *M. tuberculosis* (Kundu et al., 2009). Additionally, p38MAPK inhibition has also been described to profoundly abrogate *M. tuberculosis*-induced apoptosis (Aleman et al., 2004; Kundu et al., 2009; Aguilo et al., 2013).

Ultimately, the mitochondrial apoptotic pathway is activated in *M. tuberculosis*-infected macrophages involving the release of cytochrome *c* (Abarca-Rojano et al., 2003; Chen et al., 2006) and the subsequent activation of caspases 9 and 3 (Uchiyama et al., 2007; Aporta et al., 2012; Lim et al., 2013). Accordingly, inhibition of caspase 9, which is the initiating caspase of the intrinsic apoptotic pathway, impairs *M. tuberculosis*-induced apoptosis Martin et al. ASK1-induced cell death has also been described to depend on the activation of mitochondrial apoptotic pathway (Hatai et al., 2000), possibly linking ER stress induced by virulent *M. tuberculosis* with the activation of the intrinsic apoptotic pathway.

In addition to its ability to directly induce apoptosis, interaction of ESAT-6 with the host cell has been shown to interfere with different signaling cascades, such as the inflammatory NF-κB pathway (Pathak et al., 2007) and autophagy (Romagnoli et al., 2012). Remarkably, these pathways are naturally associated with cell survival and it is possible that by interfering with them, ESAT-6 could be sensitizing cells to undergo programmed cell death by down-modulating anti-apoptotic cellular mechanisms. A similar mechanism has been described for other microorganisms, such as *Yersinia*, where virulence factor YopJ abrogates MAPK and NF-κB to favor apoptosis induction (Zhang et al., 2005).

Importantly, different groups have found that ESAT-6 possesses pore-forming and membrane lysing capacities (de Jonge et al., 2007; Smith et al., 2008). Thus, virulent mycobacteria can cause phagosome membrane disruption in an ESAT-6-dependent fashion, reaching the cytosol and triggering cell death (van der Wel et al., 2007; Houben et al., 2012; Simeone et al., 2012). Consequently, host macrophage death is concurrent with contact of *M. tuberculosis* with the cytosol, suggesting that *M. tuberculosis* needs to physically reach the cytosol to trigger the pro-apoptotic signaling cascade.

M. tuberculosis INHIBITS APOPTOSIS AND PROMOTES NECROSIS

In discrepancy with data discussed above, different groups maintain that the ability to trigger apoptosis is more evident in attenuated strains, while virulent mycobacteria tend to inhibit this process (Keane et al., 1997, 2000; Balcewicz-Sablinska et al., 1998; Danelishvili et al., 2003; Hinchey et al., 2007), sustaining a role for apoptosis induction as a host mechanism to control infection Martin et al., rather than a virulence mechanism of infectivity. Several works indicate that TNFα is the main inductor of apoptosis by attenuated strains (Keane et al., 1997; Balcewicz-Sablinska et al., 1998). Some anti-apoptotic genes (e.g., *nuoG*) have been described to exert their function by inhibiting TNFα secretion (Miller et al., 2010). Conversely, virulent strains would promote necrotic-like cell death, which would allow bacteria to be released to the extracellular milieu, restarting the cycle of re-infection (Chen et al., 2006; Gan et al., 2008; Lee et al., 2011). Virulent *M. tuberculosis* has been reported to favor necrotic cell death by interfering with the plasma membrane repair mechanisms (Divangahi et al., 2009) thus, blocking the synthesis of prostaglandin E_2 (PGE_2), which is important for lysosome-dependent membrane repair (Divangahi et al., 2010).

A possible reason for the discrepancies regarding the cell death phenotype induced by *M. tuberculosis* could lie in that most of the studies in this field have been conducted under *in vitro* conditions, where the use of a single procedure to differentiate between apoptotic and necrotic phenotype is common. Nevertheless, the use of parallel methodologies to accurately define a cell death phenotype is recommended (Galluzzi et al., 2009). In this regard, some works that show virulent *M. tuberculosis* to induce necrosis have reported the appearance of typical apoptotic features, such as DNA fragmentation and nuclear fragmentation and/or condensation (Lee et al., 2011) in addition to plasma membrane permeability, a necrotic cell death characteristic (Butler et al., 2012). A common methodology to discern apoptosis from necrosis is to measure phosphatidylserine exposure together with plasma membrane integrity. This procedure can result confusing since under *in vitro* conditions the appearance of secondary necrosis is usual in cells which might have undergone apoptosis at earlier time points (Krysko et al., 2008). Finally, different experimental procedures and absence of standardized protocols could contribute to varying and discrepant results. For example, in the same experimental design, apoptotic- or necrotic-like phenotype can be observed depending on whether low or high multiplicity of infection (MOI) is used, respectively (Aporta et al., 2012). These observations indicate that the type of cell death induced by *M. tuberculosis in vitro* can vary depending on the experimental conditions. As such, it is difficult to define an absolute cell death phenotype *in vitro* that can be extrapolated to what would be observed under real physiological situations.

LESSONS FROM *in vivo* DATA

Based on *in vitro* results, several authors have attributed bactericidal properties to mycobacteria-induced apoptosis (Lee et al., 2006; Martin et al., 2012) in a process that depends on efferocytosis, where phagocytosed mycobacteria contained within efferosomes are unable to arrest phagosome acidification leading to loss of bacterial viability (Martin et al., 2012). However, experiments *in vivo* with an *M. tuberculosis nuoG* mutant, characterized by an enhanced capacity to induce apoptosis in mouse lungs, did not show loss of viability of the mutant as compared

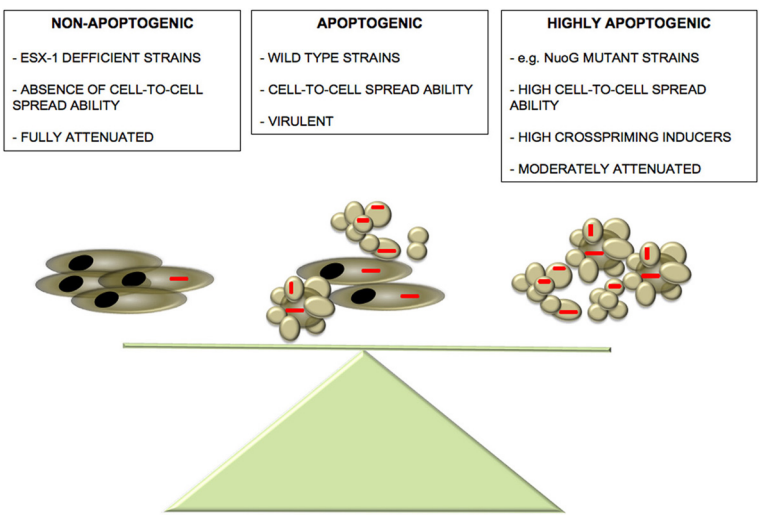

FIGURE 1 | Balance between *M. tuberculosis*-induced apoptosis and virulence. We propose the existence of a delicate balance between mycobacteria-induced apoptosis and pathogenesis. Absence of apoptosis observed in ESX-1-deficient strains leads to a sound attenuation phenotype accompanied by abrogated cell-to-cell spread capacity (Aguilo et al., 2013). On the other hand, although highly apoptogenic strains show an increased cell-to-cell spread capacity compared to wild-type strains, they might present higher visibility to the host favoring an accelerated establishment of host immune defences, thus, tipping the balance to the favor of the host. In the "golden mean" between the two extremes of apoptosis induction, virulent wild-type strains have active both pro- and anti-apoptotic mechanisms that even though partially limit cell-to-cell infectivity, they impair the rapid establishment of host immune response, thus, favoring *M. tuberculosis* pathogenesis.

to wild-type strain following low-dose aerosol infection in mice (Blomgran et al., 2012). These data suggest that the described bactericidal capacity of *M. tuberculosis*-induced apoptosis *in vitro* is not observed *in vivo* and more importantly, they indicate that enhancing the pro-apoptotic potential of a virulent strain does not reduce its virulence in a physiological infection.

Unlike discrepant *in vitro* results, *in vivo* data seem to be more consensual. Different independent works have shown the presence of apoptotic markers such as active caspase 3 or TUNEL in murine and human lungs following virulent mycobacterial infection (Keane et al., 1997; Klingler et al., 1997; Leong et al., 2008; Seimon et al., 2010; Aporta et al., 2012; Blomgran et al., 2012; Aguilo et al., 2013). In the zebra fish model of piscine tuberculosis, *M. marinum* triggers apoptosis in an ESAT-6-dependent manner as a spread mechanism of infection (Davis and Ramakrishnan, 2009). Supporting an *in vivo* role for apoptosis in cell-to-cell bacterial spread, Blomgran et al showed that pulmonary infection of mice with the *nuoG* mutant correlates increased apoptosis induction with a higher cell-to-cell transmission capacity as compared to wild-type strain (Blomgran et al., 2012). Conversely, attenuated strains BCG and MTBVAC, with a defective ESX-1 system, do not trigger apoptosis in lungs of mice (Aporta et al., 2012; Aguilo et al., 2013). Despite discrepant data on the role of mycobacteria-associated apoptosis *in vitro*, these results suggest that *in vivo* apoptosis is a feature associated with ESAT-6 secretion and virulence.

Different authors have suggested that apoptosis is a host defence mechanism as it is an effective cross-priming inducer, favoring cross-presentation of mycobacterial antigens contained in apoptotic bodies in the local lymph nodes (Schaible et al., 2003; Winau et al., 2006; Hinchey et al., 2007; Divangahi et al., 2010; Blomgran et al., 2012). These data could be in apparent discrepancy with the possible role of apoptosis as a virulence mechanism used by mycobacteria to favor cell-to-cell spread. Nonetheless, virulent *M. tuberculosis* strains, shown to trigger apoptosis *in vivo*, also elicit a strong specific immune response (Cooper, 2009) indicating that these two events are not necessarily exclusive. Indeed, data from experimental mouse models indicate that one of the best strategies of *M. tuberculosis* to successfully colonize the host is to delay the establishment of an effective adaptive immune response during the early phases of infection (Cooper, 2009). It is estimated that the adaptive response takes around 2–3 weeks to be triggered in the local lymph nodes and to migrate to the lungs (Wolf et al., 2008). This is enough time for *M. tuberculosis* to replicate without host resistance, allowing mycobacteria to reach critical bacterial burden against which the adaptive immune response could only exert a bacteriostatic effect (Cooper, 2009). Hence, if mycobacteria-loaded apoptotic bodies must migrate to the lymph nodes for cross-presentation (Winau et al., 2006), this would imply valuable time before the host could establish an effective response in the early stages to control the infection. During this critical period, *M. tuberculosis* would induce apoptosis in host phagocytes allowing bacterial spread and gain of new replication niches, while maintaining the intracellular environment. In line with this model, mouse infection with the highly apoptogenic *M. tuberculosis nuoG* mutant showed higher cell-to-cell spread capacity together with increased efficiency to trigger specific adaptive immune response as compared to wild-type *M. tuberculosis* (Blomgran et al., 2012).

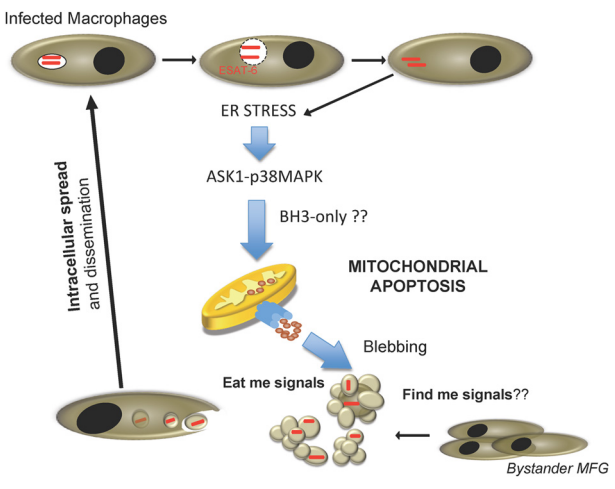

FIGURE 2 | Proposed cell-to-cell spread model for *M. tuberculosis* during host cell infection. Internalized *M. tuberculosis* disrupts phagosome in an ESAT-6 dependent manner. After reaching the cytosol, mycobacteria trigger ER stress, and activate pro-apoptotic cellular routes involving the ASK1-p38MAPK signaling axis, and causing host cell apoptosis by activation of the mitochondrial apoptotic pathway. Consequently, fresh macrophages are recruited and *M. tuberculosis*-containing apoptotic bodies are phagocytosed by new cells, re-starting the infection cycle.

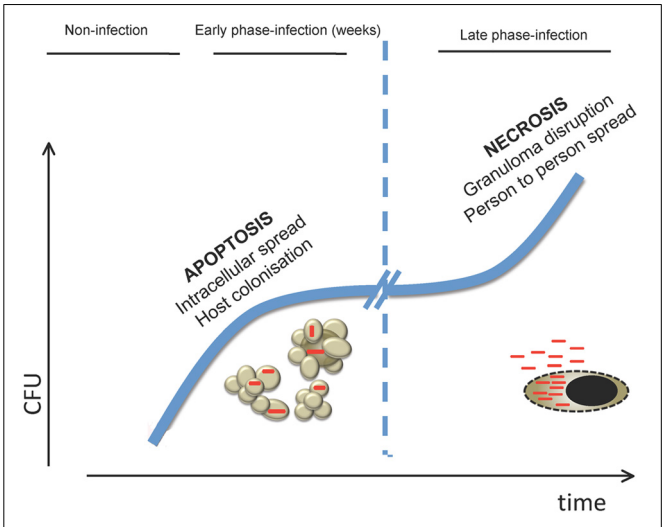

FIGURE 3 | Model of how cell death is involved in the different stages of *M. tuberculosis* host infection. In the early stages of infection, killing of macrophages by apoptosis would allow *M. tuberculosis* to spread cell-to-cell in the lung in the absence of inflammatory mediators associated with necrotic release of intracellular content. This would permit bacteria to replicate in the preferred intracellular environment prior to establishment of an effective adaptive immune response. In the latest stages of infection, when bacterial burden is high, *M. tuberculosis* would induce necrosis on infected cells leading to mycobacterial release to the extracellular medium. As a consequence, granuloma is disrupted allowing aerosol transmission to new hosts.

Thenceforth, which could be the physiological significance of cross-priming of specific T-cell responses by apoptotic bodies during *M. tuberculosis* infection? We speculate that apoptosis could have dual and opposing roles during the interaction of mycobacteria with the host. The pathogen could favor cell-to-cell bacterial spread at early stages, as well as induction of mycobacteria-specific host immune response, a process that would be accelerated if the levels of apoptosis were excessive. This way, during co-evolution with the host, *M. tuberculosis* could have developed pro-apoptotic ESX-1-dependent mechanisms essential for successful cell-to-cell infection spread and in parallel, molecular mechanisms (e.g., *nuoG, secA2*) to restrict excessive apoptosis that would otherwise result in an accelerated generation of host immunity that could impair propagation of infection in the lungs. (Hinchey et al., 2007; Blomgran et al., 2012). This hypothesis is summarized in **Figure 1**.

CONCLUDING REMARKS

Can apoptosis and necrosis be mutually exclusive processes in the context of *M. tuberculosis* infection? If we consider only the available *in vitro* data in the literature, the answer to this question seems to be affirmative. However, existing *in vivo* data suggests that both processes can occur during *M. tuberculosis* infection in different spatiotemporal stages. Data indicate that apoptosis is a common feature associated with virulent strains crucial to promote dissemination and host colonization. Thus, ESX-1-mediated apoptosis could be a critical step during the early stages of host-pathogen interaction, when bacterial load is low and few macrophages are infected. *M. tuberculosis* contained within apoptotic bodies would recruit and infect bystander macrophages, allowing infection of new host cells while maintaining an intracellular environment (**Figure 2**). *M. tuberculosis* is a successful intracellular pathogen, which in its co-evolution with the human host has developed multiple effective mechanisms to prevent intracellular defences. In this context, little evidence exists for mycobacterial strategies targeting extracellular antimicrobial barriers. Apoptosis induction could allow mycobacteria to propagate in the absence of inflammatory reactions normally associated with release of cytosolic material extracellularly, a typical feature of necrotic cell death. Efferocytosis of apoptotic bodies by bystander macrophages has been shown to create an anti-inflammatory environment due to IL-10 and PGE2 release known to inhibit macrophage function which could contribute to delayed establishment of the adaptive immune response (Medeiros et al., 2009).

Conversely, during active tuberculosis disease high bacterial burden would induce massive necrosis in host cells breaking the granuloma and reaching the respiratory tract to infect new individuals. Macrophages infected with high MOIs have been shown to die in a necrotic-like way (Lee et al., 2006). Probably, an exacerbated immune response also participates in this process. A model proposed in zebra fish indicates that during *M. marinum* infection, high levels of TNFα production lead to necroptosis events (Roca and Ramakrishnan, 2013). Indeed, caseation and necrosis are usual events observed in granulomas *in vivo*. It is possible that depending on the different environments encountered during the various phases of infection, *M. tuberculosis* is able to modulate the way that the host cell dies, favoring a successful infection and disease outcome (**Figure 3**).

The debate about whether apoptosis is beneficial for the bacteria or the host during mycobacterial infection remains open. Unlike *in vitro* observations, which tend to attribute an only role to apoptosis in mycobacterial pathogenesis, *in vivo* data seem to indicate that the answer to this question is neither black nor white. The available experimental evidence indicates that mutant strains without a functional ESX-1 system, which are not able to induce apoptosis/cell death, are much more attenuated than mutants in which apoptosis is enhanced (e.g., by deletion of *nuoG*), suggesting that ESX-1-mediated apoptosis is eminently a virulence mechanism that favors cell-to-cell mycobacterial spread and host colonization. Nevertheless, excessive apoptosis induction could result beneficial for the host as cross-priming is favored. Accordingly, it seems that apoptosis could have dual and opposing roles during infection where both the host and the pathogen attempt to use this process to tip the balance to their benefit. Thus, the fundamental question *to be or not to be* during mycobacterial infection results highly complex and does not seem to have a single answer.

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Apoptosis-associated uncoupling of bone formation and resorption in osteomyelitis

Ian Marriott*

Department of Biology, University of North Carolina at Charlotte, Charlotte, NC, USA

Edited by:
Yongqun "Oliver" He, Virginia Tech, USA

Reviewed by:
Glen C. Ulett, Griffith University, Australia
George Hajishengallis, University of Pennsylvania, USA

****Correspondence:***
Ian Marriott, Department of Biology, University of North Carolina at Charlotte, 9201 University City Boulevard, Charlotte, NC 28223, USA
e-mail: imarriot@uncc.edu

The mechanisms underlying the destruction of bone tissue in osteomyelitis are only now being elucidated. While some of the tissue damage associated with osteomyelitis likely results from the direct actions of bacteria and infiltrating leukocytes, perhaps exacerbated by bacterial manipulation of leukocyte survival pathways, infection-induced bone loss predominantly results from an uncoupling of the activities of osteoblasts and osteoclasts. Bacteria or their products can directly increase osteoclast formation and activity, and the inflammatory milieu at sites of infection can further promote bone resorption. In addition, osteoclast activity is critically regulated by osteoblasts that can respond to bacterial pathogens and foster both inflammation and osteoclastogenesis. Importantly, bone loss during osteomyelitis is also brought about by a decline in new bone deposition due to decreased bone matrix synthesis and by increased rates of osteoblast apoptosis. Extracellular bacterial components may be sufficient to reduce osteoblast viability, but the causative agents of osteomyelitis are also capable of inducing continuous apoptosis of these cells by activating intrinsic and extrinsic cell death pathways to further uncouple bone formation and resorption. Interestingly, bacterial internalization appears to be required for maximal osteoblast apoptosis, and cytosolic inflammasome activation may act in concert with autocrine/paracrine death receptor-ligand signaling to induce cell death. The manipulation of apoptotic pathways in infected bone cells could be an attractive new means to limit inflammatory damage in osteomyelitis. However, the mechanism that is the most important in bacterium-induced bone loss has not yet been identified. Furthermore, it remains to be determined whether the host would be best served by preventing osteoblast cell death or by promoting apoptosis in infected cells.

Keywords: osteomyelitis, apoptosis, osteoblasts, osteoclasts, inflammation, osteoimmunology, bacterial infection

INTRODUCTION

Osteomyelitis is a severe infection of bone tissue that is associated with significant morbidity and typically leads to bone resorption, dysfunction, and progressive inflammatory destruction (Sax and Lew, 1999). Such infections are characterized in rodent models by the rapid production of inflammatory mediators, followed by infiltration of leukocytes at 3–7 days after infection, and subsequent bone resorption and adjacent areas of new bone deposition at 14–28 days (Yoshii et al., 2002). *Staphylococcus aureus* and *Salmonella* spp. are the most common causative agents of osteomyelitis. *S. aureus* accounts for approximately 80% of all osteomyelitis cases (Lew and Waldvogel, 2004; Labbé et al., 2010) while *Salmonella* species represent one of the most serious pathogens of bone in sickle cell patients and immunosuppressed patients (Anand and Glatt, 1994; Workman et al., 1996; Koehler et al., 1998; Overturf, 1999). *S. aureus* has a propensity to colonize broken skin and so a history of trauma or skin infection is a significant risk factor for bone and joint infections caused by this organism (Barton et al., 1987; Dubey et al., 1988). The majority of bone infections in children are caused by hematogenous spread of bacteria from distant infection foci through the bloodstream, while most cases in adults result from external sources such as post-traumatic wounds and post-operative infections (Mousa, 2003; De Boeck, 2005). Indeed, implant-related infection is such a feared complication in orthopedic surgery that perioperative administration of antibiotics is routinely used to reduce this risk (Davis, 2005). However, despite prophylaxis and improvements in the diagnosis of osteomyelitis, the incidence and severity of these bone infections appear to be increasing (Jensen et al., 1997; Arnold et al., 2006).

While osteomyelitis is associated with progressive inflammatory tissue destruction, such infections also result in marked bone resorption at sites of infection and proximal abnormal bone formation. The continual process of bone remodeling requires the coordinated regulation of the genesis and activity of osteoblasts and osteoclast lineages. Osteoclasts drive the resorption of bone by acidification and release of lysosomal enzymes (Teitelbaum et al., 1997). In contrast, osteoblasts produce components of bone, principally type I collagen, and catalyze the calcification process. As such, any interference with these integrated cell types can result in abnormal bone remodeling. Bacteria such as *S. aureus* and their products can be potent stimulators of resorptive bone loss (Nair et al., 1995, 1996). While bacteria can directly damage bone by producing acids and proteases, they can also stimulate osteoclastogenesis. For example, the site of infection in animal models of *S. aureus* osteomyelitis contains high

numbers of macrophages and osteoclasts (Wiggers et al., 2011), and *S. aureus* surface-associated proteins can stimulate osteoclast formation and activity (Meghji et al., 1998; Lau et al., 2006). Similarly, systemically administered lipopolysaccharide (LPS) or local application of LPS derived from *Aggregatibacter actinomycetemcomitans* can reduce bone volume (Ochi et al., 2010; Madeira et al., 2013) and macrophages and osteoclast-like cells respond to this Gram-negative bacterial product by releasing cytokines and nitric oxide (NO) (Wiggers et al., 2011). However, it is unclear whether such effects are due to a direct action on osteoclasts and/or their progenitors, or are secondary to the production of other mediators, such as inflammatory cytokines, which modulate osteoclast formation and activity (Meghji et al., 1998).

Importantly, it is becoming increasingly apparent that, in addition to increased osteolysis and direct inflammatory degradation of bone matrix, tissue damage during osteomyelitis progression is associated with significant bone cell death. Again, cultured macrophages and osteoclasts can respond to LPS and *S. aureus* by releasing factors such as NO that can promote cell death (Wiggers et al., 2011). Importantly, osteonecrosis of the jaw is characterized by high-grade inflammation and large lesions coupled with osteocyte apoptosis (Lesclous et al., 2009). The death of this cell type is especially important given that this bone matrix embedded cell type regulates the bone remodeling activities of bone surface osteoclasts and osteoblasts via the lacuna-canaliculi network (as discussed in Matsuo, 2009). Furthermore, apoptotic cells accompany trabecular osteolysis in porcine models of osteomyelitis, in a similar manner to that typically observed in osteomyelitic lesions in following hematological *S. aureus* spread (Jensen et al., 2010). In the present review, we provide an overview of the mechanisms that can directly or indirectly induce bone cell death following infection and we discuss the possible significance of modulated programmed cell death in the progression of osteomyelitis.

ROLE OF RESIDENT BONE CELLS IN INFLAMMATORY BONE LOSS ASSOCIATED WITH OSTEOMYELITIS

Current treatment for osteomyelitis involves expensive and prolonged (even lifelong) parenteral antibiotic treatment, surgically debridement of necrotic bone, and often amputation. While these strategies are effective in reducing or eliminating the ability to isolate culturable bacteria from affected tissue, osteomyelitis is often refractory with a recent retrospective study revealing that a bacteriological cure was not achieved in up to 17% of cases (Priest and Peacock, 2005). Possible explanations for this persistence include the increasing incidence of antibiotic resistance in *S. aureus* and *Salmonella* spp. (Workman et al., 1996; Heseltine, 2000), with the appearance of isolates that are even resistant (Boneca and Chiosis, 2003) or less susceptible (Tenover et al., 2001) to vancomycin, and the formation of staphylococcal bacterial biofilms that may render these organisms less susceptible to host immune attack (as reviewed in Brady et al., 2008; Hanke and Kielian, 2012). However, recent *in vitro* studies have demonstrated that neutrophils can recognize biofilms and respond by phagocytosis and release of lactoferrin, elastase, and DNA suggesting that staphylococcal biofilms are not inherently resistant to neutrophil-mediated destruction, although it remains to be determined how effectively these cells attack biofilms *in vivo* (Meyle et al., 2010). Furthermore, these explanations fail to fully account for the observation that up to 50% of "cured" osteomyelitis cases display infection-related sequelae. Indeed, patients can have recurrent attacks of osteomyelitis following the completion of treatment regimens even when causative organisms can no longer be isolated from infection sites (Priest and Peacock, 2005).

An alternative explanation for these phenomena may lie in the ability of the causative agents of osteomyelitis to invade and reside within resident bone cells. An intracellular lifestyle provides advantages to a variety of microbes that include access to host cell metabolites (as discussed in Ray et al., 2009; Wright and Nair, 2010; Friedrich et al., 2012). Importantly, internalization also provides a means of protection against neutrophil and antibody-mediated immune responses, and mitigates therapeutic interventions by limiting exposure to antibiotics. *Salmonella* species are intracellular bacterial pathogens and this organism is recognized to invade epithelial cells (Rosenshine et al., 1994; Jones and Falkow, 1996; Ohl and Miller, 2001). Importantly, we have demonstrated that this bacterium can invade primary osteoblasts (Bost et al., 2000). In contrast, staphylococci have traditionally been regarded as non-invasive pathogens that damage host bone cells after adhering to the extracellular matrix (Nair et al., 1996). However, it is becoming increasingly apparent that these organisms can be internalized by cultured osteoblasts and can persist intracellularly (Hudson et al., 1995; Ellington et al., 1999, 2003, 2006; Jevon et al., 1999; Ahmed et al., 2001). Osteoblasts appear to be active in this invasion process as evidenced by the requirement that these cells, but not bacteria, be alive for internalization to occur (Hudson et al., 1995; Ellington et al., 1999). Furthermore, reorganization of the osteoblast cytoskeleton and receptor-mediated endocytosis are utilized in the internalization process (Ellington et al., 1999; Jevon et al., 1999). It is important to note that bacteria released following lysis or trypsinization of *S. aureus*-containing human osteoblasts are viable and are capable of invading other osteoblasts (Ellington et al., 2003). In addition, significant time-dependent changes in the structure of *S. aureus* can occur after as little as 12 h exposure to an intracellular environment that render the organisms less sensitive to antibiotics capable of penetrating eukaryotic cells (Ellington et al., 2006). Interestingly, the ability of bacteria to invade osteoblasts may also direct the ability of this organism to form biofilms and it has been suggested that invasion promotes a biofilm-like surface on *S. aureus* that correlates with antibiotic resistance (Brady et al., 2008). As such, these host evasion mechanisms may be intimately related (Brady et al., 2008). Such findings may explain why antibiotic treatment can reduce the number of viable bacteria in animal models of staphylococcal osteomyelitis but does not reliably sterilize infected bone (Monzón et al., 2001). Bacteria sequestered inside the osteoblast may therefore provide a reservoir of bacteria and contribute to recurrent chronic osteomyelitis that often occurs despite the presence of antibiotics and a seemingly adequate humoral response (Lew and Waldvogel, 2004).

In addition to the synthesis of new bone matrix, osteoblasts produce soluble factors that regulate the formation and activity of osteoclasts. For example, osteoblasts are an important source of receptor activator of NF-kB ligand (RANKL) and osteoprotegerin

(OPG), critical regulators of osteoclast development and function (Wada et al., 2006). RANKL interacts with its receptor RANK on osteoclast progenitor cells and plays a central role in promoting osteoclast formation and activity (Hsu et al., 1999; Kong et al., 1999a,b; Kim et al., 2000; Li et al., 2000; Wada et al., 2006). In contrast, OPG functions as a decoy receptor for RANKL and limits osteoclastogenesis (Wada et al., 2006). Interestingly, causative agents of periodontal infections such as *Porphyromonas gingivalis* have been found to induce the RANKL expression in osteoblasts (Okahashi et al., 2004) and elevated levels of this molecule have been found in osteomyelitis-associated bone lesions (Montonen et al., 2006). While such RANKL production may be attributable to the activity of resident bone cells, it should be noted that activated T-lymphocytes also produce this cytokine (Anderson et al., 1997; Horwood et al., 1999; Hsu et al., 1999). RANKL production by osteoblasts and T-cells can be stimulated by the inflammatory cytokines interleukin (IL)-1β and tumor necrosis factor-α TNF-α (Walsh and Choi, 2003) that are detectable in infected bone tissues of osteomyelitis patients (O'Keefe et al., 1997). In addition, both of these inflammatory mediators have been demonstrated to concomitantly depress OPG expression in osteoblasts and we have shown that *S. aureus* challenge similarly inhibits osteoblast production of this RANKL decoy receptor (Young et al., 2011). This then provides another mechanism that can favor bone resorption at sites of infection. Finally, Choi et al. (2005) have shown that cyclooxygenase-2 (COX2) inhibitors can inhibit *P. gingivalis*-induced RANKL expression by osteoblasts and restore OPG levels. This observation is in agreement with our own recent studies demonstrating that *S. aureus* elevates RANKL production by osteoblasts and this effect is similarly abolished by a specific COX2 inhibitor (Somayaji et al., 2008). These findings suggest that osteoblasts produce PGE2 that acts in a paracrine or autocrine manner to induce RANKL release and limit OPG production, creating a microenvironment that favors bone resorption (Choi et al., 2005). Together, these data suggest that bacterially challenged osteoblasts can promote the formation and activity of osteoclasts leading to a net loss of bone at sites of infection (as summarized in **Figure 1**).

But the activities of osteoblasts may not be limited to the synthesis of bone matrix components and the regulation of osteoclast activity. There is increasing evidence that osteoblasts may directly contribute to inflammation in infected bone tissue. Studies from our group and others have demonstrated that isolated osteoblasts utilize members of the toll-like receptor (TLR) and nucleotide-binding oligomerization domain-containing (NOD)-like receptor (NLR) families of innate immune receptors to detect the presence of microbial products (Gasper et al., 2002; Madrazo et al., 2003; Marriott et al., 2005; Chauhan and Marriott, 2010) (**Figure 1**). The activation of these sensors precipitates transcription factor activation and leads to the release of inflammatory cytokines and the expression of cell surface antigen presenting and co-stimulatory molecules (Barton and Medzhitov, 2003). Consistent with this observation, our *in vitro* studies show that *S. aureus* is a potent stimulus for the production of soluble and cell surface molecules by isolated osteoblasts that could play key roles in the initiation and/or progression of inflammatory immune responses (Marriott, 2004). Such molecules include

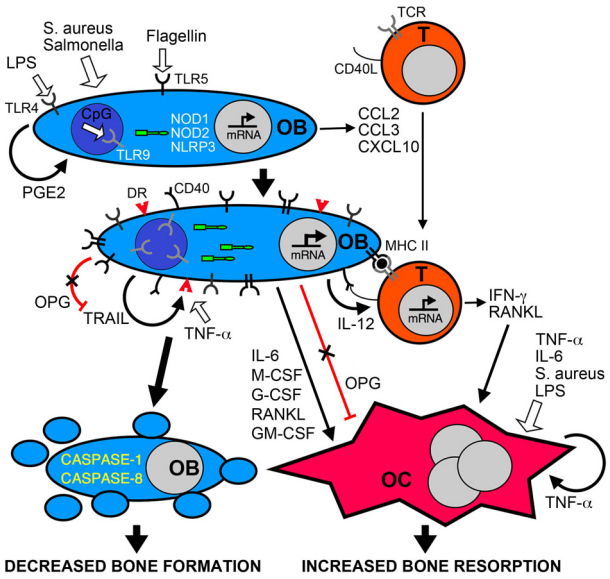

FIGURE 1 | Bacterial infection leads to inflammatory bone loss resulting from increased formation and/or activity of bone resorbing osteoclasts, and a decrease in the number of bone forming osteoblasts. Osteoblasts (OB) can recognize bacteria and their components such as LPS, flagellin, or unmethylated CpG DNA motifs (CpG) via cell surface pattern recognition receptors including TLRs 4, 5, and 9, and the cytosolic sensors NOD1, NOD2, and NLRP3. These microbial sensors can directly or indirectly (via PGE2 autocrine/paracrine signaling) induce the rapid release of chemokines such as CCL2, CCL3, and CXCL10 to recruit leukocytes including T-lymphocytes (T) to the site of infection. Bacterially stimulated osteoblasts show elevated levels of these bacterial sensors and can also express cell surface molecules including MHC class II and CD40 that can elicit antigen-specific activation of infiltrating T-cells. Bacteria and their components can directly induce the formation and activity of osteoclasts (OC) or indirectly activate these cells via the autocrine/paracrine actions of released TNF-a. In addition, activated T-cells and osteoblasts produce an array of molecules including RANKL, IL-6, IFN-γ and colony stimulating factors (M-CSF, G-CSF, GM-CSF) that can promote osteoclastogenesis and bone resorption, and activation of osteoblasts decreases the production of the RANKL decoy receptor OPG. Finally, bacteria can induce the apoptotic death of osteoblasts either directly via the NLRP3 inflammasome, or indirectly via the autocrine/paracrine actions of released TRAIL interacting with death receptors (DR) expressed on the surface of challenged cells. Since OPG also functions as a decoy receptor for TRAIL, the decreased production of this molecule by infected osteoblasts could exacerbate TRAIL-induced apoptosis leading to decreased numbers of this bone forming population and greater bone loss at sites of infection.

CXCL10 (Gasper et al., 2002) and CCL2 (Bost et al., 2001) that are potent chemoattractants for T-cells and macrophages, leukocytes frequently observed in bone tissue following infection (Stashenko et al., 1992; Bremell et al., 1994), and IL-6 (Bost et al., 1999) an often inflammatory cytokine that can directly or indirectly modulate the activity of bone-resorptive osteoclasts (Lowik et al., 1989; Ishimi et al., 1990; de la Mata et al., 1995; Greenfield et al., 1995; Hofbauer and Heufelder, 1996). Furthermore, our laboratory has demonstrated that primary osteoblasts can produce growth factors such as granulocyte macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor

(M-CSF) and granulocyte colony stimulating factor (G-CSF) following *S. aureus* exposure (Bost et al., 2000) that could increase osteoclastogenesis and promote bone resorption (Lorenzo et al., 1987; Kodama et al., 1991; Takahashi et al., 1991; Liggett et al., 1993; Povolny and Lee, 1993), and augment immune responses to this bacterial pathogen (Frenck et al., 1990; Freund and Kleine, 1992; Dale, 1995). As such, the production of these mediators by bacterially challenged osteoblasts may significantly contribute to involucrum and sequestrum formation during osteomyelitis, and exacerbate damaging inflammation.

MODULATION OF LEUKOCYTE APOPTOSIS IN OSTEOMYELITIS

The interaction between *Salmonella* species and host cell types often results in the death of mammalian cells but these bacteria appear to do so by host cell-type specific mechanisms (as reviewed in Fink and Cookson, 2007) and the mechanisms underlying host cell death by such Gram-positive bacterial pathogens have been extensively reviewed elsewhere (Ulett and Adderson, 2006). While *Salmonella* induces apoptosis in epithelial cells, invasion of macrophages rapidly triggers caspase-1-dependent programmed cell death, or pyroptosis, in a salmonella pathogenicity island-1 type III secretion system and flagella dependent manner (Fink and Cookson, 2007). Since caspase-1-deficient mice are more susceptible to salmonellosis, such pyroptotic death has generally been considered to be a protective response to infection (Fink and Cookson, 2007). In contrast, *Brucella abortus*, an intracellular zoonotic pathogen that causes osteomyelitis in humans, elicits host macrophage apoptosis following invasion (Cha et al., 2013). However, apoptosis only occurs after a period of bacterial replication and so such cell death appears to favor the pathogen by eliminating immune cells. Furthermore, it should be noted that some Gram-positive pathogens of bone including streptococci also show a capacity to escape in a viable form from macrophages in an NO-dependent manner (Ulett and Adderson, 2005).

While several causative agents of osteomyelitis can induce programmed cell death of infiltrating macrophages, delayed neutrophil apoptosis is a characteristic feature of human osteomyelitis arising from either Gram-negative or Gram-positive organisms (as discussed in Ocaña et al., 2008). Neutrophils from osteomyelitis patients exhibit less spontaneous apoptosis than that seen in cells from healthy donors (Asensi et al., 2004). Interestingly, the serum of osteomyelitis patients has been found to significantly reduce apoptosis rates over a 12 h time period in isolated neutrophils (Asensi et al., 2004). This effect has been attributed to the elevated IL-6 levels seen in patient's sera as these anti-apoptotic effects could be reversed with IL-6 neutralizing antibodies or mimicked with exogenous IL-6 (Asensi et al., 2004). Such cytokine-induced neutrophil apoptosis inhibition has been demonstrated following infection with the Gram-positive organism, *S. aureus*, or the Gram-negative bacterium, *Escherichia coli*, and has also been observed following challenge with lipoteichoic acid (LTA) or LPS, bacterial ligands for TLR2 and TLR4 pattern recognition receptors, respectively (Ocaña et al., 2008). This auto-induced reduction in neutrophil apoptosis correlates with an altered ratio of pro-apoptotic B-cell lymphoma (Bcl)2–associated X protein (Bax) to anti-apoptotic Bcl-extra large (Bcl-xL) expression in these cells, and this is notable since the loss-of-function Bax promoter polymorphism A allele has been found to be more frequent in osteomyelitis patients (Ocaña et al., 2008). The neutrophils in such patients express less Bax and, accordingly, lower rates of apoptosis. Similarly, polymorphisms in the gene encoding TLR4, but not TLR2, have been identified as risk factors for chronic Gram-negative bacterial osteomyelitis (Montes et al., 2006). Neutrophils isolated from osteomyelitis patients with TLR4 polymorphisms show less LPS-induced activation of the key pro-inflammatory transcription factor NF-kB, reduced IL-6 production, and a lower induced reduction in apoptosis (Montes et al., 2006). It is conceivable that inflammatory cytokine-induced lengthening of neutrophil lifespan represents an attempt by the host to augment bacterial killing at the site of infection. However, limiting leukocyte apoptosis in this manner may ultimately be detrimental to the host due to the exacerbation of inflammatory damage (Ocaña et al., 2007).

MODULATION OF OSTEOCLAST FORMATION AND SURVIVAL IN OSTEOMYELITIS

While bacteria or their products can directly destroy bone tissue, they can also indirectly elicit bone loss by increasing the formation and function of bone-resorbing osteoclasts (Chung et al., 2006; Maruyama et al., 2006). Indeed, osteoclast responses in *S. aureus*-infected tissue differ markedly from those seen in sterile bone trauma in that these cells behave as acute inflammatory responders with substantial activity at the margins of the infected site and adjacent uninjured tissue (Pesanti and Lorenzo, 1998). Osteoclastogenesis and osteoclast activity can be up-regulated by soluble mediators produced by infiltrating leukocytes such as macrophages and neutrophils. For example, the capsular-like polysaccharide antigen of *A. actinomycetemcomitans*, an organism implicated in juvenile periodontitis, stimulates the production of IL-1α that, in turn, promotes osteoclast formation and bone resorption (discussed in Yamamoto et al., 1999). Similarly, surface-associated material extracted from *S. aureus* has been shown to stimulate osteoclastogenesis and pit formation on dentine slices in a TNF-α and IL-6 dependent manner (Meghji et al., 1998). Such induction by cytokines generally considered to be inflammatory could, in part, explain the large numbers of osteoclasts that are typically associated with infarcted bone in osteomyelitis.

However, the formation and bone resorbing functions of osteoclasts are regulated in large part by osteoblasts and a substantial body of evidence has accumulated to show that bacterially challenged osteoblasts produce an array of soluble mediators that can promote osteoclastogenesis and osteoclast activity (as summarized in **Figure 1**). Recently, Gram-negative bacterial LPS has been demonstrated to induce osteoclastogenesis and reduce bone volume following in vivo administration, and this effect was blocked by co-administration of OPG (Ochi et al., 2010). While most cultured mononuclear osteoclasts die within 24 h in the absence of stimuli, both RANKL and bacterial LPS support survival and induce differentiation into multinuclear cells, providing a potential mechanism underlying LPS-induced bone loss (Suda et al., 2002). Such an effect does not appear to be limited to Gram-negative bacterial products, as *S. aureus* binding to

osteoblasts can induce the release of soluble RANKL by these cells (Widaa et al., 2012). Furthermore, biofilm components from a clinical *S. aureus* strain have recently been demonstrated to induce osteoblast RANKL production and increase the RANKL/OPG ratio in culture media (Sanchez et al., 2013). In particular, staphylococcus protein A (SpA) has been reported to bind to TNF receptor 1 (TNFR1) on osteoblasts (Claro et al., 2013), and *S. aureus*-induced increases in osteoblast RANKL expression are not seen with SpA mutants (Claro et al., 2011). However, one study suggests that *S. aureus* surface-associated material can promote osteoclast formation *in vitro* in a RANKL independent manner (Lau et al., 2006), while another indicates that LPS-induced bone resorption can occur independent of RANKL or the inflammatory mediators IL-1β or TNF-α (Suda et al., 2002). As such, it is possible that bacterial products can have both direct and indirect effects on osteoclast activity in inflammatory bone loss.

It is important to note that some bacterial pathogens appear to manipulate bone homeostasis in a species-specific manner. For example, *P. gingivalis* culture products including hemoglobin receptor protein can suppress RANKL-induced *in vitro* osteoclastogenesis (Fujimura et al., 2006). It seems likely that differences in the manipulation of bone tissue reflect distinct bacterial survival and/or dissemination strategies. While net resorption of bone may favor *S. aureus* and *Salmonella* infections, rapid and extensive destruction of alveolar bone would be anticipated to result in tooth loss and the elimination of the gingival crevice that is an anatomical niche for periodontal pathogens such as *P. gingivalis*. A suppressive effect of this organism on osteoclastogenesis may, therefore, permit chronic infection (Fujimura et al., 2006).

INDUCTION OF APOPTOSIS IN BONE FORMING OSTEOBLASTS DURING OSTEOMYELITIS

In addition to the increased formation and activity of bone resorbing osteoclasts in osteomyelitis, inflammatory bone loss may also result from the elimination of the cells responsible for new bone matrix deposition following infection. In support of this notion, reduced expression of markers associated with differentiated osteoblasts including alkaline phosphatase activity, collagen type I production, and mineralized nodules, is seen in alveolar bone tissue from patients with periodontal disease (Mori et al., 2007). Importantly, work from our laboratory and our collaborators have demonstrated that bone-forming osteoblasts undergo apoptotic cell death following infection with the principle causative agents of osteomyelitis, *S. aureus* and *Salmonella* (Tucker et al., 2000; Alexander et al., 2003). We have shown that *Salmonella enterica* can elicit significant apoptotic cell death as rapidly at 24 h post-infection. Interestingly, we have found that intracellular invasion is required for maximal induction of apoptosis in osteoblasts as evidenced by the sensitivity of this response to a pharmacological inhibitor of endocytosis and a significantly smaller apoptotic response in this cell type to invasion deficient *Salmonella* strains (Marriott et al., 2005; McCall et al., 2008). As such, inflammatory bone loss during osteomyelitis may result from the elimination of the cells responsible for new bone matrix deposition in addition to the increased numbers and activity of bone resorbing osteoclasts at sites of bacterial infection (Nair et al., 1996). Along these lines, it is becoming apparent that the causative agents of osteomyelitis are capable of inducing continuous apoptosis of bone lining osteoblasts by activating intrinsic and extrinsic cell death pathways to further uncouple bone formation and resorption.

DIRECT INDUCTION OF OSTEOBLAST CELL DEATH FOLLOWING BACTERIAL CHALLENGE

Clinical *S. aureus* strain biofilm components have been shown to inhibit osteoblast differentiation and reduce viability with increased rates of apoptosis (Sanchez et al., 2013). Similarly, *S. aureus* binding to osteoblasts inhibits de novo bone formation by preventing expression of key products including alkaline phosphatase, collagen type I, osteocalcin, and osteopontin (Widaa et al., 2012). SpA appears to underlie this effect as this product can directly bind osteoblasts, inhibit proliferation and mineralization, and initiate apoptosis in this cell type (Claro et al., 2011). A role for SpA binding to osteoblasts in inducing apoptosis is supported by the observation that these effects are not seen with SpA deficient bacterial mutants (Claro et al., 2011; Widaa et al., 2012). Similarly, *A. actinomycetemcomitans* extracts can also induce the death of osteoblast-like cells (Morimoto et al., 1999). Again, a bacterial protein product appears to underlie this phenomenon as cycloheximide-treated *A. actinomycetemcomitans* fails to induce osteoblast death, and heat or trypsin treatment of bacterial extracts neutralizes this effect (Morimoto et al., 1999). Although, it should be noted that at least one study suggests that *A. actinomycetemcomitans*-induced osteoblast apoptosis can be mediated by a capsular-like polysaccharide (Yamamoto et al., 1999). Interestingly, while *P. gingivalis* lipids can inhibit osteoblast differentiation and activity, they do not significantly alter osteoblast viability (Wang et al., 2010), but binding between the fimbriae of this organism and the host cell integrin alpha5beta1 can initiate apoptosis in this cell type (Zhang et al., 2013). However, such induction appears to be a delayed effect as *P. gingivalis* prolongs cell life prior to host cell destruction (Zhang et al., 2013), presumably to permit bacterial replication within the osteoblast.

We have demonstrated that osteoblasts express members of the TLR family of pattern recognition receptors and that these cell surface sensors can detect the presence of extracellular bacteria and their products (Kikuchi et al., 2001; Gasper et al., 2002; Madrazo et al., 2003). However, our studies indicate that bacterial components, UV-inactivated bacteria and invasion defective strains of bacteria are far less effective in eliciting the osteoblast immune functions than viable wild type bacteria (Bost et al., 1999, 2000, 2001). Furthermore, Thammasitboon et al. (2006) have demonstrated that bacterial components such as LPS fail to elicit apoptosis in osteoblasts. Consistent with these findings, we have shown that cytochalasin significantly attenuates inflammatory cytokine production by invasive *Salmonella* (McCall et al., 2008). These observations indicate that invasion is required for optimal osteoblast responses and suggest that the cell surface TLRs are not the only means by which these cells perceive bacterial pathogens. This phenomenon might be explained by our finding that osteoblasts express NOD1 and NOD2 (Marriott et al., 2005), two members of the NLR family of receptor proteins that can serve as intracellular sensors for bacterial peptidoglycans and

initiate pro-inflammatory mediator production (as reviewed in Ting and Davis, 2005; Strober et al., 2006).

The importance of bacterial invasion in the initiation of osteoblast apoptosis is underscored by the demonstration that the presence of intracellular *S. aureus* causes rounding of this cell type and chromatin condensation, DNA laddering, and DNA strand breaks in the nuclei (Tucker et al., 2000). Furthermore, *S. aureus*-induced apoptosis in human osteoblasts is markedly attenuated following inhibition of cellular invasion (Ning et al., 2011). Similarly, the Gram-negative bacteria *P. gingivalis* and *B. abortus*, causative agents of periodontitis and osteomyelitis, respectively, are capable of invading osteoblasts, although these organisms appear to elicit delayed apoptosis thereby permitting intracellular replication (Wang et al., 2010; Scian et al., 2012; Baldi and Giambartolomei, 2013; Zhang et al., 2013). Finally, similar to inflammatory mediator production, our studies indicate that invasive bacteria are more effective at inducing osteoblast apoptosis with *S. aureus* or *S. typhimurium* SB300 being significantly more potent stimuli for murine osteoblast cell death than the invasion defective species, *S. carnosus* and *S. typhimurium* SB136 (Marriott et al., 2005).

While NOD1 and NOD2 can initiate, augment, or reduce inflammatory mediator production by a variety of cell types (as reviewed in Inohara and Nunez, 2003), these cytosolic proteins are not widely recognized to participate in the induction of apoptosis. In contrast, two related receptors, NLR family CARD domain containing 4 (NLRC4) and NLR family pyrin domain containing 3 (NLRP3), have been implicated in the induction of cell death in response to bacteria and/or their components (Gumucio et al., 2002; Mariathasan et al., 2004, 2006; Sutterwala et al., 2006). Both of these molecules can associate with an adaptor protein, apoptosis-associated speck-like protein (ASC) to elicit caspase-1 and caspase-8 activation (Hasegawa et al., 2005; Mariathasan, 2007), enzymes that demonstrate elevated activity in osteoblasts following bacterial challenge (Marriott et al., 2002; Alexander et al., 2003). We have recently investigated the functional expression of NLRP3 and NLRC4 in resting and *Salmonella* exposed cultures of primary murine and human osteoblasts in an attempt to identify the mechanisms linking intracellular invasion to bacterially-induced cell death. We demonstrated that osteoblasts constitutively express NLRP3, but not NLRC4, and such expression was modestly increased following infection with wild type *S. enterica* (McCall et al., 2008). This finding was in contrast with the significant decrease in NLRP3 protein levels seen in osteoblasts following exposure to an invasion defective strain. In addition to showing that osteoblasts possess robust constitutive levels of NLRP3, we demonstrated that these cells also express ASC. While constitutive levels of ASC were low in resting osteoblasts, a marked elevation in expression was observed in cells following *Salmonella* infection (McCall et al., 2008).

Taken together, the conserved expression of NLRP3 and ASC in both mouse and human osteoblasts, and the sensitivity of such expression to bacterial challenge, provides circumstantial evidence of a role for these cytosolic proteins in osteoblast responses to intracellular pathogens. However, we have more directly verified NLRP3 functionality in bacterially challenged osteoblasts (McCall et al., 2008). First, we have shown that NLRP3 associates with ASC following exposure to *Salmonella* as determined by co-immunoprecipitation. Second, we have demonstrated that NLRP3 expression knockdown by siRNA attenuates *Salmonella*-induced changes in transcription factor activity. Third, we have found that osteoblasts derived from NLRP3 deficient animals produce less IL-6 than cells derived from wild type mice. As such, these findings confirm that NLRP3 mediates, at least in part, osteoblast responses to this intracellular bacterial pathogen.

Given that osteoblasts constitutively express NLRP3 and ASC, and the finding that NLRP3 can associate with ASC to elicit caspase-1 (Gumucio et al., 2002; Dowds et al., 2004; Kanneganti et al., 2006; Mariathasan et al., 2006) and perhaps caspase-8 activation (Hasegawa et al., 2005), enzymes that demonstrate elevated activity in osteoblasts following bacterial challenge (Marriott et al., 2002; Alexander et al., 2003), this cytosolic NLR may represent an important mechanism underlying osteoblast apoptosis following exposure to intracellular bacterial pathogens. This hypothesis is supported by our observation that *Salmonella*-induced decreases in NF-kB activity are markedly attenuated in osteoblasts following siRNA-induced NLRP3 knockdown (McCall et al., 2008), since this transcription factor has been reported to mediate anti-apoptotic effects in a variety of cell types (You et al., 2001; Lu et al., 2004; Munshi et al., 2004). Furthermore, we have shown that *Salmonella*-induced caspase-1 activation is absent in osteoblasts derived from NLRP3 deficient animals (McCall et al., 2008). More importantly, we have demonstrated that such cells exhibit significantly less apoptotic cell death following *Salmonella* infection than osteoblasts derived from wild type animals (McCall et al., 2008). These findings differ from studies in macrophages (Mariathasan et al., 2004, 2006) where NLRP3 is not required for *Salmonella*-induced caspase-1 activation, IL-1β production, or cell death. Instead, these functions are predominantly mediated by NLRC4 (Mariathasan et al., 2004, 2006). In osteoblasts, NLRP3 is required for caspase-1 activation and for a portion of *Salmonella*-induced cell death, while NLRC4 is not detectable in these cells. Furthermore, earlier work from our laboratory has indicated that neither the precursor nor the mature form of IL-1β is produced by infected osteoblasts (Marriott et al., 2002). Together these studies underscore the differences between osteoblasts and macrophages and show that at least one of these is a differential reliance on NLRP3 vs. NLRC4 in cell death following *Salmonella* infection. This illustrates an additional level of complexity in the control of cellular functions by NLR family members.

While further experimentation is required to definitively conclude that ASC and alterations in NF-kB activity are essential mechanistic elements in NLRP3-mediated apoptosis in bacterially challenged osteoblasts, these studies indicate that this NLR represents an important component underlying the direct initiation of apoptosis in this bone-forming cell type following challenge with intracellular bacterial pathogens and could, therefore, be a major contributory factor to bone loss at sites of infection.

INDIRECT INDUCTION OF OSTEOBLAST CELL DEATH VIA LEUKOCYTE/BONE CELL DERIVED MEDIATORS

The extrinsic pathway of programmed cell death begins with ligation of a particular group of cell surface receptors and leads to

recruitment of adaptor molecules and the activation of a caspase cascade that results in cellular disassembly. This apoptotic pathway is critically regulated by members of the TNF family of cytokines and receptors in many cell types (Cosman, 1994; Lynch et al., 1994; Smith et al., 1994). Ligands such as TNF-α and Fas ligand have the ability to interact with death domain containing receptors to initiate apoptosis and conditioned medium from LPS-treated macrophage-like cells has been shown to induce osteoblast apoptosis in a TNF-α dependent manner (Thammasitboon et al., 2006). Similarly, the supernatants from *B. abortus*-infected macrophages can induce osteoblast apoptosis in a TNF-α-dependent manner (Scian et al., 2012), which could therefore contribute to the bone and joint destruction seen in patients with osteoarticular complications of brucellosis. However, it is important to note that the events that occur in vivo are likely to be more complex as illustrated by the observation that LPS-induced TNF-α production only provokes in situ osteoblast apoptosis when the TNFR1 signal is negated, suggesting that this receptor can also regulate osteoblast survival (Ochi et al., 2010).

TNF-related apoptosis inducing ligand (TRAIL) is also a member of the TNF superfamily and this soluble protein has the unique ability to interact with five different receptors. TRAIL can interact with death receptor (DR)4 (also known as TRAIL R1) (Pan et al., 1997), DR5 (also known as TRAIL R2 and TRAIL receptor inducer of cell killing-2) (Schneider et al., 1997), DcR1 (also known as TRAIL R3 and TRAIL receptor without an intracellular domain) (Degli-Esposti et al., 1997), DcR2 (also known as TRAIL R4 and TRAIL receptor with a truncated death-domain) (Pan et al., 1998), and finally soluble OPG (Emery et al., 1998). Of these receptors, only DR4 and DR5 in humans and DR5 in mice have functional cytoplasmic death-domains and are capable of inducing apoptosis, while DcR1 and DcR2 appear to function as decoy receptors (Clancy et al., 2005). Death domain-containing receptors trimerize to allow TRAIL to bind and trigger caspase 8 activation that directly activates caspases 3 and 7 via the extrinsic pathway (Clancy et al., 2005). Although it should be noted that that TRAIL has been also been suggested to activate the intrinsic pathway through the activation of BH3 interaction-domain death agonist (Bid) (Suliman et al., 2001; Sinicrope and Penington, 2005). Both of these pathways lead to caspase 3 activation and cell death (Suliman et al., 2001). In contrast, OPG appears to function as a soluble decoy receptor for both TRAIL and the potent osteoclastogenic factor, RANKL. As such, excessive TRAIL secretion in bone tissue would be anticipated to decrease the amount of OPG available to bind RANKL, leading to increased osteoclastogenesis and tissue loss (as illustrated in **Figure 1**).

In a recent study (Young et al., 2011), we have demonstrated that murine osteoblasts constitutively express mRNA encoding the sole murine death-domain containing TRAIL receptor, DR5, and we have shown that resting cultures of these cells contain robust levels of the DR5 protein. These observations are consistent with previous reports that human osteoblast-like cells express mRNA encoding DR4 and DR5 (Atkins et al., 2002). However, we have shown that DR5 expression on the surface of murine osteoblasts is restricted to cells exposed to either *S. aureus* or *Salmonella* (Young et al., 2011). Similarly, we confirmed that both of the death-domain containing TRAIL receptors on human cells are only present on primary human osteoblasts following bacterial challenge and the conserved nature of this response supports the biological significance of such expression on infected cells (Young et al., 2011). In the same work, we showed that the robust constitutive production of the decoy TRAIL receptor OPG by resting osteoblasts was markedly attenuated following infection with either *S. aureus* or *Salmonella* (Young et al., 2011). While other studies indicate that serum OPG levels increase following systemic bacterial LPS administration (Maruyama et al., 2006), our results are consistent with reports that the causative agents of periodontitis and LPS can similarly decrease OPG expression by murine osteoblasts (Chung et al., 2006; Mori et al., 2007). Furthermore, it should be noted that bone tissue is relatively poorly vascularized and bone infections are more commonly associated with Gram-positive bacterial species such as *S. aureus*, infections that do not result in the development of endotoxic shock. As such, changes in OPG expression by resident bone cells are likely to be more physiologically relevant in local bone infections than the systemic effects elicited by bacterial endotoxins. As such, these data suggest that the principle causative agents of osteomyelitis can elicit an imbalance in the expression of death-inducing and decoy TRAIL receptors by osteoblasts, with the induced expression of DR5 and/or DR4 on the surface of infected cells and a concomitant decrease in the production of the decoy receptor, OPG.

Osteoblasts isolated from alveolar bone of patients with periodontal disease have been demonstrated to have higher sensitivity to the apoptosis-inducing actions of TRAIL than cells isolated from healthy donors (Mori et al., 2009). In our recent work, we tested the sensitivity of primary osteoblasts to exogenously administered recombinant TRAIL at rest or following challenge with either *S. aureus* or *Salmonella* (Young et al., 2011). Consistent with the absence of death-inducing TRAIL receptors on uninfected osteoblasts and previous reports with unstimulated human osteoblast-like cells (Atkins et al., 2002; Bu et al., 2003), we showed that TRAIL fails to activate apoptosis signaling pathways in uninfected primary osteoblasts (Young et al., 2011). However, acute bacterial exposure sensitizes osteoblasts to TRAIL-mediated cell death as shown by the ability of exogenous TRAIL to augment apoptotic volume decreases and caspase-8 activation following *S. aureus* or *Salmonella* infection (Alexander et al., 2003; Young et al., 2011). While increased TRAIL-induced apoptosis is only correlative with the observed induction of cell surface death receptor expression, we have previously documented that TRAIL neutralizing antibodies can significantly inhibit bacterially-induced osteoblast apoptosis as assessed by caspase-8 activation and annexin V staining (Alexander et al., 2003). Together, these results support the contention that the induced expression of death receptors renders these cells sensitive to the apoptotic actions of TRAIL, and provide a potential mechanism underlying the reported increase in TRAIL sensitivity of human alveolar osteoblasts isolated from patients with periodontitis (Mori et al., 2009) and transformed osteoblasts (Atkins et al., 2002).

Several studies have shown that human osteoblast-like cells express TRAIL (Atkins et al., 2002; Bu et al., 2003) and work from our laboratory has demonstrated that exposure of mouse

and human primary osteoblasts to *S. aureus* or *Salmonella* induces TRAIL expression by these cells (Alexander et al., 2001). Furthermore, we have also shown that bacterial infection enhances RANKL expression and release by osteoblasts (Somayaji et al., 2008). Since RANKL binds OPG with a similar binding affinity to that of TRAIL (23 and 45 nM for RANKL and TRAIL, respectively (Vitovski et al., 2007), this molecule could further reduce levels of OPG available to bind and neutralize TRAIL. Accordingly, a scenario could be envisaged in which bacterially challenged osteoblasts express TRAIL and RANKL while concomitantly decreasing the production of the decoy receptor OPG and upregulating cell surface death receptor expression. The increased production of this death receptor ligand, in concert with reduced OPG production and bioavailability, would facilitate TRAIL activity and result in the death of infected osteoblasts, providing an additional mechanism whereby bacterial pathogens elicit bone destruction during diseases such as osteomyelitis (as shown in **Figure 1**).

CONCLUDING REMARKS

The inflammatory bone loss associated with osteomyelitis can result from the direct destruction of bone tissue by bacteria and their products. Furthermore, significant bone loss is likely to result from the recruitment and activation of infiltrating leukocytes such as neutrophils and macrophages. The direct activation of these cells by bacteria via an array of cell surface and cytosolic pattern recognition receptors, and the presence of an inflammatory milieu at the site of infection, results in the production of degradative enzymes and cytotoxic molecules. Consistent with host-pathogen interactions at other anatomical sites, the microbes responsible for bone infections can manipulate leukocyte survival pathways in a bacterium specific manner with some organisms inducing apoptosis and/or pyroptotic cell death, while other organisms appear to promote the survival of innate immune cells, particularly neutrophils. While lengthening the life span of these cells might represent an attempt by the host to clear the infection, the unintended consequence of such a vigorous response can be extensive inflammatory bone destruction.

However, bone loss at sites of infection is primarily due to an uncoupling of the control of bone forming osteoblasts and bone resorbing osteoclasts. Bacteria or their products can directly increase the osteoclast formation and activity. In addition, inflammatory mediators produced at the site by infiltrating leukocytes or bacterially challenged osteoclasts themselves can further promote osteoclastogenesis and bone resorption. Importantly, the production and activity of osteoclasts is critically regulated by osteoblasts, and these cells have recently been found to respond to the principle causative agents of osteomyelitis via TLR and NLR family members. Osteoblasts exposed to bacteria express an array of soluble and cell surface molecules that have the potential to promote immune responses and directly or indirectly enhance osteoclast activity at sites of infection.

In addition to the increased number and activity of bone resorbing osteoclasts, it is becoming apparent that bone loss during conditions such as osteomyelitis is also brought about by a decline in new bone deposition. This occurs as a result of decreased production of matrix components by osteoblasts and by increased rates of apoptosis in this cell type. While the presence of certain extracellular bacterial components may be sufficient to directly reduce osteoblast viability, there is considerable evidence that internalization of the principal causative agents of osteomyelitis is required to maximally induce apoptosis in these cells. At least a component of such cell death is attributable to osteoblast-derived TRAIL acting in an autocrine and/or paracrine manner via death domain-containing receptors that are concomitantly and exclusively expressed by infected cells. However, TRAIL-induced death is unlikely to be the sole means by which osteoblasts are eliminated in infected tissue. Indeed, rapid activation of apoptotic pathways is observed in microbe challenged osteoblasts prior to TRAIL production, and at least a proportion of this cell death is unaffected by anti-TRAIL antibodies (Alexander et al., 2001, 2003). Such cell death may result from the direct activation of cytosolic NLRP3 that is constitutively expressed by osteoblasts (McCall et al., 2008), which can activate caspase cascades and initiate apoptosis. As such, NLRP3 may act in concert with TRAIL-mediated pathways to eliminate osteoblasts in infected bone tissue.

It might be anticipated that new therapies that target the perception and initiation of inflammatory immune responses and bone-forming cell death via pattern recognition receptors would be of considerable benefit in reducing the inflammatory damage and cell death associated with bone infections. However, it is important to note that many of these observations have been made in reductive *in vitro* systems and have yet to be confirmed in vivo. Also, it is clear that important issues regarding bacteria-induced inflammatory bone loss remain unresolved. For example, it is not known whether osteocytes embedded within calcified bone can respond to bacteria, an important question given the critical role of cell type in controlling bone surface cells via the lacuna-canaliculi network (Matsuo, 2009). Furthermore, the mechanism that is the most important in the pathophysiology of bacterium-induced bone loss has not been identified, and this issue is further complicated by the disparate nature of the bacterial species that are responsible for bone diseases. As such, it is not presently known whether inhibition of osteoclast formation/activity or the prevention of osteoblast destruction would be most efficient in eliminating bacterially-induced bone loss. Finally, given that infected osteoblasts may harbor intracellular bacteria such as *S. aureus* and promote inflammatory bone damage, it is not clear whether the host would be best served by preventing osteoblast cell death or by eliminating these cells via the induction of apoptosis. Clearly, further work is warranted to resolve these issues.

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CONCLUDING REMARKS

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In addition to the increased number and activity of bone resorbing osteoclasts, it is becoming apparent that bone loss during conditions such as osteomyelitis is also brought about by a decline in new bone deposition. This occurs as a result of decreased production of matrix components by osteoblasts and by increased rates of apoptosis in this cell type. While the presence of certain extracellular bacterial components may be sufficient to directly reduce osteoblast viability, there is considerable evidence that internalization of the principal causative agents of osteomyelitis is required to maximally induce apoptosis in these cells. At least a component of such cell death is attributable to osteoblast-derived TRAIL acting in an autocrine and/or paracrine manner via death domain-containing receptors that are concomitantly and exclusively expressed by infected cells. However, TRAIL-induced death is unlikely to be the sole means by which osteoblasts are eliminated in infected tissue. Indeed, rapid activation of apoptotic pathways is observed in microbe challenged osteoblasts prior to TRAIL production, and at least a proportion of this cell death is unaffected by anti-TRAIL antibodies (Alexander et al., 2001, 2003). Such cell death may result from the direct activation of cytosolic NLRP3 that is constitutively expressed by osteoblasts (McCall et al., 2008), which can activate caspase cascades and initiate apoptosis. As such, NLRP3 may act in concert with TRAIL-mediated pathways to eliminate osteoblasts in infected bone tissue.

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Apoptosis-associated uncoupling of bone formation and resorption in osteomyelitis

Ian Marriott*

Department of Biology, University of North Carolina at Charlotte, Charlotte, NC, USA

Edited by:
Yongqun "Oliver" He, Virginia Tech, USA

Reviewed by:
Glen C. Ulett, Griffith University, Australia
George Hajishengallis, University of Pennsylvania, USA

****Correspondence:***
Ian Marriott, Department of Biology, University of North Carolina at Charlotte, 9201 University City Boulevard, Charlotte, NC 28223, USA
e-mail: imarriot@uncc.edu

The mechanisms underlying the destruction of bone tissue in osteomyelitis are only now being elucidated. While some of the tissue damage associated with osteomyelitis likely results from the direct actions of bacteria and infiltrating leukocytes, perhaps exacerbated by bacterial manipulation of leukocyte survival pathways, infection-induced bone loss predominantly results from an uncoupling of the activities of osteoblasts and osteoclasts. Bacteria or their products can directly increase osteoclast formation and activity, and the inflammatory milieu at sites of infection can further promote bone resorption. In addition, osteoclast activity is critically regulated by osteoblasts that can respond to bacterial pathogens and foster both inflammation and osteoclastogenesis. Importantly, bone loss during osteomyelitis is also brought about by a decline in new bone deposition due to decreased bone matrix synthesis and by increased rates of osteoblast apoptosis. Extracellular bacterial components may be sufficient to reduce osteoblast viability, but the causative agents of osteomyelitis are also capable of inducing continuous apoptosis of these cells by activating intrinsic and extrinsic cell death pathways to further uncouple bone formation and resorption. Interestingly, bacterial internalization appears to be required for maximal osteoblast apoptosis, and cytosolic inflammasome activation may act in concert with autocrine/paracrine death receptor-ligand signaling to induce cell death. The manipulation of apoptotic pathways in infected bone cells could be an attractive new means to limit inflammatory damage in osteomyelitis. However, the mechanism that is the most important in bacterium-induced bone loss has not yet been identified. Furthermore, it remains to be determined whether the host would be best served by preventing osteoblast cell death or by promoting apoptosis in infected cells.

Keywords: osteomyelitis, apoptosis, osteoblasts, osteoclasts, inflammation, osteoimmunology, bacterial infection

INTRODUCTION

Osteomyelitis is a severe infection of bone tissue that is associated with significant morbidity and typically leads to bone resorption, dysfunction, and progressive inflammatory destruction (Sax and Lew, 1999). Such infections are characterized in rodent models by the rapid production of inflammatory mediators, followed by infiltration of leukocytes at 3–7 days after infection, and subsequent bone resorption and adjacent areas of new bone deposition at 14–28 days (Yoshii et al., 2002). *Staphylococcus aureus* and *Salmonella* spp. are the most common causative agents of osteomyelitis. *S. aureus* accounts for approximately 80% of all osteomyelitis cases (Lew and Waldvogel, 2004; Labbé et al., 2010) while *Salmonella* species represent one of the most serious pathogens of bone in sickle cell patients and immunosuppressed patients (Anand and Glatt, 1994; Workman et al., 1996; Koehler et al., 1998; Overturf, 1999). *S. aureus* has a propensity to colonize broken skin and so a history of trauma or skin infection is a significant risk factor for bone and joint infections caused by this organism (Barton et al., 1987; Dubey et al., 1988). The majority of bone infections in children are caused by hematogenous spread of bacteria from distant infection foci through the bloodstream, while most cases in adults result from external sources such as post-traumatic wounds and post-operative infections (Mousa, 2003; De Boeck, 2005). Indeed, implant-related infection is such a feared complication in orthopedic surgery that perioperative administration of antibiotics is routinely used to reduce this risk (Davis, 2005). However, despite prophylaxis and improvements in the diagnosis of osteomyelitis, the incidence and severity of these bone infections appear to be increasing (Jensen et al., 1997; Arnold et al., 2006).

While osteomyelitis is associated with progressive inflammatory tissue destruction, such infections also result in marked bone resorption at sites of infection and proximal abnormal bone formation. The continual process of bone remodeling requires the coordinated regulation of the genesis and activity of osteoblasts and osteoclast lineages. Osteoclasts drive the resorption of bone by acidification and release of lysosomal enzymes (Teitelbaum et al., 1997). In contrast, osteoblasts produce components of bone, principally type I collagen, and catalyze the calcification process. As such, any interference with these integrated cell types can result in abnormal bone remodeling. Bacteria such as *S. aureus* and their products can be potent stimulators of resorptive bone loss (Nair et al., 1995, 1996). While bacteria can directly damage bone by producing acids and proteases, they can also stimulate osteoclastogenesis. For example, the site of infection in animal models of *S. aureus* osteomyelitis contains high

(M-CSF) and granulocyte colony stimulating factor (G-CSF) following *S. aureus* exposure (Bost et al., 2000) that could increase osteoclastogenesis and promote bone resorption (Lorenzo et al., 1987; Kodama et al., 1991; Takahashi et al., 1991; Liggett et al., 1993; Povolny and Lee, 1993), and augment immune responses to this bacterial pathogen (Frenck et al., 1990; Freund and Kleine, 1992; Dale, 1995). As such, the production of these mediators by bacterially challenged osteoblasts may significantly contribute to involucrum and sequestrum formation during osteomyelitis, and exacerbate damaging inflammation.

MODULATION OF LEUKOCYTE APOPTOSIS IN OSTEOMYELITIS

The interaction between *Salmonella* species and host cell types often results in the death of mammalian cells but these bacteria appear to do so by host cell-type specific mechanisms (as reviewed in Fink and Cookson, 2007) and the mechanisms underlying host cell death by such Gram-positive bacterial pathogens have been extensively reviewed elsewhere (Ulett and Adderson, 2006). While *Salmonella* induces apoptosis in epithelial cells, invasion of macrophages rapidly triggers caspase-1-dependent programmed cell death, or pyroptosis, in a salmonella pathogenicity island-1 type III secretion system and flagella dependent manner (Fink and Cookson, 2007). Since caspase-1-deficient mice are more susceptible to salmonellosis, such pyroptotic death has generally been considered to be a protective response to infection (Fink and Cookson, 2007). In contrast, *Brucella abortus*, an intracellular zoonotic pathogen that causes osteomyelitis in humans, elicits host macrophage apoptosis following invasion (Cha et al., 2013). However, apoptosis only occurs after a period of bacterial replication and so such cell death appears to favor the pathogen by eliminating immune cells. Furthermore, it should be noted that some Gram-positive pathogens of bone including streptococci also show a capacity to escape in a viable form from macrophages in an NO-dependent manner (Ulett and Adderson, 2005).

While several causative agents of osteomyelitis can induce programmed cell death of infiltrating macrophages, delayed neutrophil apoptosis is a characteristic feature of human osteomyelitis arising from either Gram-negative or Gram-positive organisms (as discussed in Ocaña et al., 2008). Neutrophils from osteomyelitis patients exhibit less spontaneous apoptosis than that seen in cells from healthy donors (Asensi et al., 2004). Interestingly, the serum of osteomyelitis patients has been found to significantly reduce apoptosis rates over a 12 h time period in isolated neutrophils (Asensi et al., 2004). This effect has been attributed to the elevated IL-6 levels seen in patient's sera as these anti-apoptotic effects could be reversed with IL-6 neutralizing antibodies or mimicked with exogenous IL-6 (Asensi et al., 2004). Such cytokine-induced neutrophil apoptosis inhibition has been demonstrated following infection with the Gram-positive organism, *S. aureus*, or the Gram-negative bacterium, *Escherichia coli*, and has also been observed following challenge with lipoteichoic acid (LTA) or LPS, bacterial ligands for TLR2 and TLR4 pattern recognition receptors, respectively (Ocaña et al., 2008). This auto-induced reduction in neutrophil apoptosis correlates with an altered ratio of pro-apoptotic B-cell lymphoma (Bcl)2–associated X protein (Bax) to anti-apoptotic Bcl-extra large (Bcl-xL) expression in these cells, and this is notable since the loss-of-function Bax promoter polymorphism A allele has been found to be more frequent in osteomyelitis patients (Ocaña et al., 2008). The neutrophils in such patients express less Bax and, accordingly, lower rates of apoptosis. Similarly, polymorphisms in the gene encoding TLR4, but not TLR2, have been identified as risk factors for chronic Gram-negative bacterial osteomyelitis (Montes et al., 2006). Neutrophils isolated from osteomyelitis patients with TLR4 polymorphisms show less LPS-induced activation of the key pro-inflammatory transcription factor NF-kB, reduced IL-6 production, and a lower induced reduction in apoptosis (Montes et al., 2006). It is conceivable that inflammatory cytokine-induced lengthening of neutrophil lifespan represents an attempt by the host to augment bacterial killing at the site of infection. However, limiting leukocyte apoptosis in this manner may ultimately be detrimental to the host due to the exacerbation of inflammatory damage (Ocaña et al., 2007).

MODULATION OF OSTEOCLAST FORMATION AND SURVIVAL IN OSTEOMYELITIS

While bacteria or their products can directly destroy bone tissue, they can also indirectly elicit bone loss by increasing the formation and function of bone-resorbing osteoclasts (Chung et al., 2006; Maruyama et al., 2006). Indeed, osteoclast responses in *S. aureus*-infected tissue differ markedly from those seen in sterile bone trauma in that these cells behave as acute inflammatory responders with substantial activity at the margins of the infected site and adjacent uninjured tissue (Pesanti and Lorenzo, 1998). Osteoclastogenesis and osteoclast activity can be up-regulated by soluble mediators produced by infiltrating leukocytes such as macrophages and neutrophils. For example, the capsular-like polysaccharide antigen of *A. actinomycetemcomitans*, an organism implicated in juvenile periodontitis, stimulates the production of IL-1α that, in turn, promotes osteoclast formation and bone resorption (discussed in Yamamoto et al., 1999). Similarly, surface-associated material extracted from *S. aureus* has been shown to stimulate osteoclastogenesis and pit formation on dentine slices in a TNF-α and IL-6 dependent manner (Meghji et al., 1998). Such induction by cytokines generally considered to be inflammatory could, in part, explain the large numbers of osteoclasts that are typically associated with infarcted bone in osteomyelitis.

However, the formation and bone resorbing functions of osteoclasts are regulated in large part by osteoblasts and a substantial body of evidence has accumulated to show that bacterially challenged osteoblasts produce an array of soluble mediators that can promote osteoclastogenesis and osteoclast activity (as summarized in **Figure 1**). Recently, Gram-negative bacterial LPS has been demonstrated to induce osteoclastogenesis and reduce bone volume following in vivo administration, and this effect was blocked by co-administration of OPG (Ochi et al., 2010). While most cultured mononuclear osteoclasts die within 24 h in the absence of stimuli, both RANKL and bacterial LPS support survival and induce differentiation into multinuclear cells, providing a potential mechanism underlying LPS-induced bone loss (Suda et al., 2002). Such an effect does not appear to be limited to Gram-negative bacterial products, as *S. aureus* binding to

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Intestinal epithelial cell apoptosis and loss of barrier function in the setting of altered microbiota with enteral nutrient deprivation

Farokh R. Demehri, Meredith Barrett, Matthew W. Ralls, Eiichi A. Miyasaka, Yongjia Feng and Daniel H. Teitelbaum*

Section of Pediatric Surgery, Department of Surgery, University of Michigan Health System, Ann Arbor, MI, USA

Edited by:
Yongqun He, Virginia Tech, USA

Reviewed by:
Glen C. Ulett, Griffith University, Australia
Eric Ghigo, CNRS, France

Correspondence:
Daniel H. Teitelbaum, Section of Pediatric Surgery, Mott Children's Hospital, University of Michigan, 1540 E. Hospital Dr., SPC 4211, Ann Arbor, MI 48109-4211, USA
e-mail: dttlbm@umich.edu

Total parenteral nutrition (TPN), a commonly used treatment for patients who cannot receive enteral nutrition, is associated with significant septic complications due in part to a loss of epithelial barrier function (EBF). While the underlying mechanisms of TPN-related epithelial changes are poorly understood, a mouse model of TPN-dependence has helped identify several contributing factors. Enteral deprivation leads to a shift in intestinal microbiota to predominantly Gram-negative Proteobacteria. This is associated with an increase in expression of proinflammatory cytokines within the mucosa, including interferon-γ and tumor necrosis factor-α. A concomitant loss of epithelial growth factors leads to a decrease in epithelial cell proliferation and increased apoptosis. The resulting loss of epithelial tight junction proteins contributes to EBF dysfunction. These mechanisms identify potential strategies of protecting against TPN-related complications, such as modification of luminal bacteria, blockade of proinflammatory cytokines, or growth factor replacement.

Keywords: small intestine, parenteral nutrition, epithelial barrier function, epithelial cell apoptosis, epithelial call proliferation, microbiome

PARENTERAL NUTRITION: A NECESSARY RISK

Maintaining appropriate nutrition in patients is a clinical necessity that is at times difficult to accomplish. Particularly devastating complications are seen in the perioperative period where malnourishment increases complications, delays wound healing, decreases immune function, prolongs hospitalizations, and lowers patient quality of life (Peter et al., 2005; Martindale et al., 2013). The ideal and most physiologic form of nutrition is via the gastrointestinal tract (Moore et al., 1992; Braunschweig et al., 2001; Mirtallo et al., 2004; Peter et al., 2005; Zaloga, 2006). Unfortunately, due to mechanical, functional, or postoperative deficits, some patients are unable to use their intestinal tract for nutritious gain. For these patients who are enterally deprived, total parenteral nutrition (TPN) is an alternative method of nutrition which provides nutrients and calories intravenously (Abunnaja et al., 2013). According to the United States Healthcare Cost and Utilization Project, greater than 352,000 patients received TPN in the United States in 2010 (Pfuntner et al., 2006). It thus serves as a life sustaining treatment for many patients.

A recent adjunct to the nutritional armamentarium, TPN has been used regularly for patients since the mid-twentieth century. Through groundbreaking work by Rhoads and colleagues in the 1960s, challenges regarding access and formulation were overcome and TPN was able to be prescribed to those who would have previously had no means of nutritional access (Dudrick et al., 1968). Efforts to optimize the composition, administration, and patient selection criteria for TPN have since continued, as codified in recent guidelines issued by the American Society for Parenteral and Enteral Nutrition (Mirtallo et al., 2004).

TPN itself is a solution composed of amino acids, sugars, fat emulsions, electrolytes, vitamins, and trace elements (Zaloga, 2006). Due to its hypertonicity, it is recommended that central venous catheters are used for TPN delivery (Mirtallo et al., 2004). Assessment of the need for continued TPN utilization should be performed regularly as TPN, though life preserving, is not without significant risk. In fact, complications of TPN have been extensively described in the literature (The Veterans Affairs Total Parenteral Nutrition Cooperative Study Group, 1991; Moore et al., 1992; Buzby, 1993; Braunschweig et al., 2001; Peter et al., 2005; Heneghan et al., 2013). Blood stream infection, hepatic dysfunction, metabolic derangements, bacterial translocation, immunologic compromise, and enterocyte atrophy are well known sequelae of TPN administration. In a historic multihospital Veterans Affairs study, Buzby et al. (Dudrick et al., 1968) found patients with mild malnutrition received no benefit from TPN and suffered from infectious complications "not explained by the presence of a catheter"—including pneumonia, urinary tract infection, and surgical site infection. In a recent study, Casaer et al. expanded upon these findings, demonstrating not only a lack of benefit with early administration of TPN in critically-ill patients, but in fact poorer outcomes compared to later and more judicious initiation of TPN (Casaer et al., 2011). Such complications have led to cautious administration of TPN and utilization of enteral feeding whenever possible (Braunschweig et al., 2001).

PHYSIOLOGIC AND IMMUNOLOGIC CHANGES WITH TPN IN A MOUSE MODEL

While the etiology of the increased rate of infectious complications with TPN is not fully established, it is known that many of the organisms responsible for these infections predominantly originate from enteric microbiota, suggesting a TPN-associated loss of intestinal epithelial barrier function (EBF) (Feng et al., 2012). TPN-induced changes allow translocation of endotoxins and enteric microbiota across the epithelial barrier, leading to endotoxemia, bacteremia and sepsis (Alverdy et al., 1988) (**Figure 1**). A mouse model of TPN has allowed the identification of regulatory pathways which are involved in these TPN-induced physiologic changes. In this model, mice receive TPN via an internal jugular catheter and no enteral nutrition. These are compared to control mice which also have an intravenous catheter place, but receive only saline (electrolyte solution) and are allowed enteral food. Using such a model, our laboratory and others have identified several contributing factors which drive this diminished EBF with TPN, such as loss of local growth factors, increased levels of pro-inflammatory mucosal cytokines, and alterations in intraluminal microbiota. These TPN-induced changes result in a pro-inflammatory state within the intestinal mucosa, leading to villous atrophy, an increase in epithelial cell (EC) apoptosis, and a decrease in EC proliferation (Wildhaber et al., 2002)—as also demonstrated by the reduction in overall length of the small and large bowel (**Figure 2**).

INFLAMMATORY CYTOKINE DYSREGULATION: IFN-γ AND TNF-α

Inflammatory cytokines linked to loss of EBF include interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), both of which are increased in animal models of TPN-dependence. IFN-γ is a cytokine which, when administered *in vitro* to a T84 epithelial monolayer, results in the loss of tight junction integrity (Madara and Stafford, 1989). This effect is prevented by pretreatment with transforming growth factor-β1 (Planchon et al., 1994). TPN-dependent mice have been shown to have a greater than three-fold increase in IFN-γ mRNA expression, with subsequent EBF breakdown. This loss of EBF is mitigated via blockade of IFN-γ signaling using IFN-γ-knockout mice (Yang et al., 2002, 2003a).

Similarly, our work has also shown that TNF-α expression is increased in response to enteral nutrient deprivation and TPN administration (Feng and Teitelbaum, 2012). This increase is associated with mucosal atrophy and loss of EBF. Using a combination of TNF-receptor-1 (TNF-R1) and TNF-R2 knockout mice, blockade of TNF signaling prevents mucosal atrophy with TPN, but blockade of both receptors are needed to prevent the loss of EBF associated with TPN (Feng and Teitelbaum, 2012). TNF-receptor-dependent downstream mediators include nuclear factor-κB (NF-κB) and myosin light chain kinase (MLCK). TNF-receptor activation leads to up-regulation of MLCK with the activation of myosin light chain (MLC) to phospho-MLC (Feng and Teitelbaum, 2013). Intestinal epithelial cell (IEC) MLCK is known to mediate TNF-α-induced modulation of the intestinal epithelial tight junction barrier (Ye et al., 2006). The EBF is maintained by the integrity of a series of paracellular tight junctional proteins, including zonula occludens 1 (ZO-1), ZO-2, junctional adhesions, occludin as well as a large family of claudins. With the phosphorylation of MLC, an activation of actin and myosin contraction occurs on the IEC apical surface, with the dissociation of ZO-1 from the tight junction, an internalization of occludin, and a resultant deterioration of barrier function (Chen et al., 2012). This process can be progressive, as TNF-α will drive the downstream activation of NF-κB, which is responsible for initiating an inflammatory amplification cascade (Barnes and Karin, 1997). Another important regulatory role of TNF-α is the mediation of

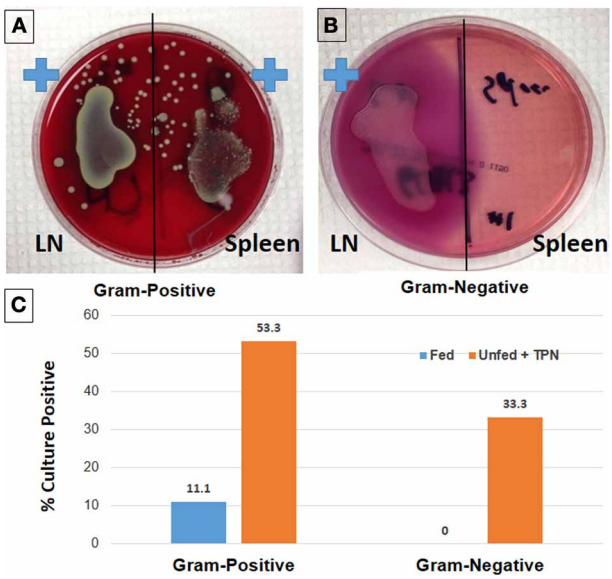

FIGURE 1 | Loss of epithelial barrier function with TPN leads to bacterial translocation and sepsis. Lymph node (LN) and spleen isolates from TPN-dependent mice were incubated on Columbia-CNA agar **(A)** to isolate Gram-positive bacteria. Similarly, culture on MacConkey agar demonstrates Gram-negative bacteria from TPN-dependent LN isolates **(B)**. Rates of bacteremia are significantly greater in TPN-dependent vs. fed mice **(C)** (Sun et al., 2006).

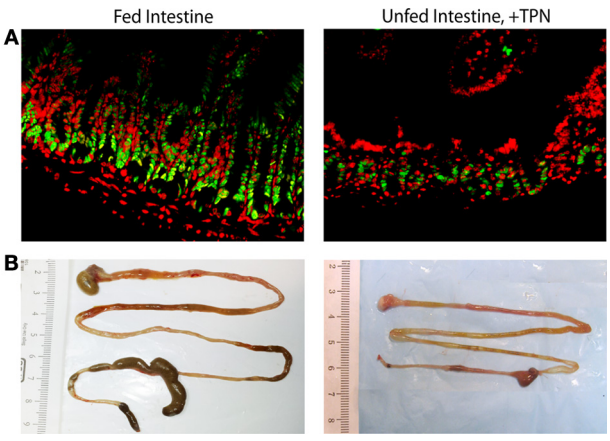

FIGURE 2 | Epithelial and whole-bowel changes with TPN. Unfed bowel demonstrates decreased epithelial cell proliferation (green = PCNA, proliferating cells; red = DAPI, all nucleated cells) compared to fed intestine **(A)**. Representative images of harvested mouse intestine demonstrate decreased length with TPN-dependence **(B)**.

IEC apoptosis. Signaling of TNF-α via the TNF-R1 and TNR-R1 receptors are principal pathways for IEC apoptosis (Feng and Teitelbaum, 2012). Interestingly, both MLCK and NF-κB are up-regulated with TPN, and these changes are prevented with TNF-α blockade (Feng and Teitelbaum, 2013).

DIMINISHED GROWTH FACTOR AND IMMUNE REGULATOR SIGNALING

In addition to mediating inflammation and apoptosis, TNF-α has recently been shown to play a critical role in enterocyte survival and regulation of IEC proliferation (Edelblum et al., 2008). A potent mediator of EC proliferation, epidermal growth factor (EGF) signaling is dependent on both ErbB-1 receptor and TNF-α signaling pathways (McElroy et al., 2008; Yamaoka et al., 2008). With TPN administration, EGF signaling is diminished secondary to both TNF-α dysregulation along with decreased ErbB-1 expression. Correction of this TPN-induced imbalance via exogenous EGF or through a blockade of TNF-R1 was shown to partially reverse the pro-apoptotic effects of excessive TNF-α signaling (Feng and Teitelbaum, 2012). Two other mucosal growth factors, keratinocyte growth factor and glucagon-like peptide 2, have also been shown to be diminished with TPN. These two growth factors, like EGF, play a role in maintenance of EBF and their diminished expression contributes to EBF loss (Feng et al., 2012).

The regulation of IEC integrity is also linked to the intraepithelial lymphocyte (IEL)-derived anti-inflammatory interleukin 10 (IL-10), which is a master regulator of the mucosal immune system (Duell et al., 2012). Knockout mice who fail to produce IL-10 demonstrate increased epithelial permeability (Madsen et al., 1999), resulting in a colitis model (Berg et al., 2002). In TPN-dependent mice, a significant decline in IEL-derived IL-10 is seen along with an associated decrease in EBF (Sun et al., 2008). By administering exogenous IL-10, the TPN-associated decline in intestinal barrier function is attenuated; suggesting that loss of IL-10 contributes to TPN-induced epithelial barrier dysfunction.

EBF BREAKDOWN IS MODULATED BY DECREASED p-Akt SIGNALING

The decrease in growth factor signaling with TPN leads to a downstream decrease in phosphatidylinositol 3-kinase (PI3K)/p-Akt signaling. PI3K/p-Akt signaling is known to play a key role in cell cycle progression and preventing apoptosis (Chang et al., 2003). With TPN, p-Akt activity diminishes in ECs, with an associated loss of EC proliferation and increased apoptosis (Feng et al., 2009). Using an Akt-activating peptide T-cell lymphoma-1 (TCL1) conjugated to a transactivator of transcription peptide sequence (TAT), a significant increase in p-Akt abundance was achieved in TPN-dependent mice, along with prevention of the loss of EC proliferation and increased apoptosis seen with TPN (Feng et al., 2010). This demonstrates the central importance of p-Akt signaling in maintaining EBF integrity.

Thus, TPN causes increased levels of inflammatory cytokines TNF-α and IFN-γ, along with decreased production of inflammatory regulator IL-10 and growth factor EGF, which together lead to altered IEC survival and proliferation. In addition, TPN-dependence appears to significantly diminish EBF. The components that make up EBF include the synthesis and release of mucus from goblet cells, transcytosis of dimeric secretory IgA [which is also lost with TPN (Fukatsu and Kudsk, 2011)], intraluminal movement of water, and the physical integrity of the epithelium itself (Clayburgh et al., 2004). Breakdown of this barrier can lead to the translocation of intestinal microbiota and/or endotoxin, which are thought to contribute to TPN-related sepsis (Kristof et al., 2011).

TPN-dependence appears to decrease the integrity of the epithelial junctional protein apparatus, which is a critical component of EBF. Junctional proteins include ZO-1, which cross-links the E-Cadherin/catenin complex and the actin cytoskeleton (Itoh et al., 1997), as well as occludin and the family of claudins, the latter of which has been found to be altered in Crohn's disease (Zeissig et al., 2007). In TPN-dependent mice, all of these junctional proteins have been found to have a significant reduction in abundance compared to enterally-fed mice (Sun et al., 2008). Expression of these proteins is linked to EBF, which can be reflected via measurement of the transepithelial potential difference and resistance using an Ussing chamber (Yang et al., 2003b). The previously described cytokine changes with TPN are also linked to junctional protein expression. For instance, exogenous IL-10 administration ameliorated TPN-induced loss of ZO-1, ZO-2, claudin-2, and occludin (Sun et al., 2008). Production of these proteins is also related to p-Akt signaling, as our work has shown that down-regulation of E-Cadherin expression in TPN-dependent mice is tightly related to a decrease in p-Akt activity (Feng et al., 2009). In addition, we demonstrated prevention of the TPN-induced loss of ZO-1 and E-cadherin expression when p-Akt activity is upregulated with TCL-1 administration. The loss in EBF is clearly multifactorial, however, as TCL-1 failed to prevent the loss of occludin expression or the decrease in transepithelial resistance with TPN (Feng et al., 2012). Interestingly, supplementation of TPN-dependent mice with intravenous glutamine, a critical amino acid, has been shown to preserve EBF and restore EC proliferation via prevention of loss of p-Akt abundance (Nose et al., 2010).

A key distinction in the mechanism of these TPN-induced changes is whether they are due to administration of the TPN solution itself or the lack of enteral feeding. To address this, we compared mice receiving TPN without feeding vs. TPN with partial (25%) oral feeding. This small amount of enteral nutrition prevented intestinal epithelial atrophy and the associated increase in proinflammatory cytokines while restoring normal tight junction function and EBF (Wildhaber et al., 2005). Thus, the lack of intraluminal nutrients appears to drive the pathophysiology of TPN-dependence.

CHANGES IN THE GUT MICROBIOME WITH TPN

While the alterations in inflammatory cytokines and growth factors thus far described have a clear role in the physiologic changes seen with TPN, the mechanisms behind these alterations are unknown. An increasing body of evidence suggests that a driving force behind these changes is a drastically altered intestinal microbiome with associated immunologic changes. Normally composed of a diverse population of bacteria numbering up to 10^{14} colony-forming units, the intestinal microbiota play an essential role in host physiology via immune stimulation and regulation, digestion of carbohydrates otherwise unavailable to enterocytes, and production of key nutrients such as short-chain fatty acids (Salzman, 2010; Sekirov et al., 2010).

The interaction between host and microbiota is a complex relationship that is not completely understood. One of the critical functions played by intestinal microbiota is modulation of the host's immune system through interaction with lamina propria (LP) cells. A principal function of LP cells is the detection and monitoring of changes in the intraluminal environment (Novak and Bieber, 2008). Microbiota interact with LP cells via the toll-like receptor (TLR) pathway (Chichlowski and Hale, 2008; Ng et al., 2010). Several bacterial components act as ligands for TLRs, such as lipopolysaccharide (LPS), which is derived from Gram-negative bacteria and binds TLR4. TLR binding leads to downstream activation of NF-κB signaling via a myeloid differentiation primary response gene 88 (MyD88) dependent pathway (Karrasch and Jobin, 2008; Abreu, 2010). Activation of NF-κB, as described above, then mediates the expression of a number of proinflammatory cytokines.

While TPN supplies adequate nutritional needs systemically to the recipient, enteral deprivation puts the intestinal microbiota in an abrupt state of nutrient withdrawal. This has been shown to dramatically alter the makeup of small intestinal microbiota from a normally benign composition of predominantly Gram-positive Firmicutes to a Gram-negative Proteobacteria-dominated population. Additional phylum-level changes include increases in Bacteroidetes and Verrucomicrobia (predominantly Akkermansia) (**Figure 3**). This shift is associated with increased TLR signaling—specifically, up-regulation of TLR-2, 4, 7, and 9. The subsequent proinflammatory state within the LP is characterized by increased TNFα, IFN-γ, downstream NF-κB activation, and a marked loss in the LP T-regulatory (T_{reg}) cell population (Miyasaka et al., 2013).

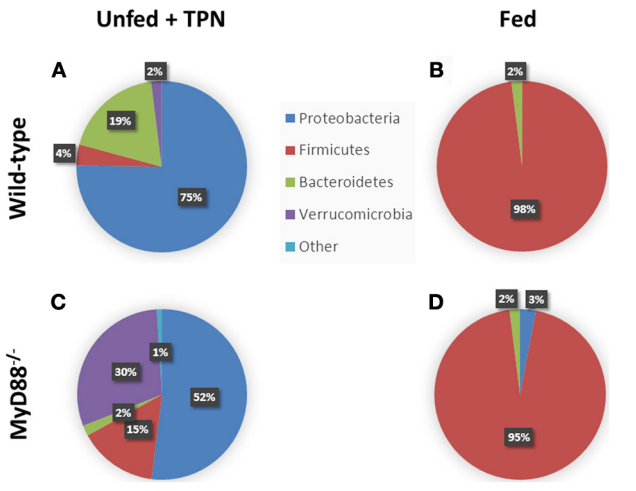

FIGURE 3 | Phylum-level changes in intestinal microbiota with TPN. Enteric bacteria from TPN-dependent mice **(A)** demonstrate a relative increase in Proteobacteria and Bacteroidetes vs. fed mice **(B)**, where Firmicutes dominates. MyD88$^{-/-}$ mice, with defective TLR signaling, demonstrate an additional expansion of Verrucomicrobia with TPN-dependence **(C)** vs. fed controls **(D)**. Akkermansia species predominate among the Verrucomicrobia. Adapted from Miyasaka et al. (2013).

The deprivation of enteral nutrients available to intraluminal bacteria alters the selection pressure determining the dominant species of microbiota. In an environment of relative starvation, Proteobacteria have been shown to demonstrate resilience (Sinclair and Alexander, 1984), while Firmicutes establish themselves in the enterally-fed state (Costello et al., 2010). While these changes are characterized by a relative increase in Gram-negatives, there remains an undefined role for Gram-positive bacteria in TPN-dependence, given these organisms activate multiple TLRs (Patten and Collett, 2013) as well as apoptosis signaling (Ulett and Adderson, 2006). Besides the lack of luminal nutrients, host factors play a role in changing the microbial environment. For instance, TPN administration has been shown to lead to an increase in goblet cell numbers (Conour et al., 2002) and a decrease in Paneth cell function. Paneth cells, located adjacent to intestinal stem cells in crypts, interact with intestinal bacteria by secreting bactericidal proteins. TPN leads to decreased expression of Paneth cell-related antimicrobial proteins REGIII-g, lysozyme, and cryptdin-4. This leads to increased susceptibility to enteroinvasion by *E. coli* (Heneghan et al., 2013). These findings demonstrate a significant change in the host-microbiome relationship with TPN. An altered bacterial population develops in an environment with diminished mucosal defenses, contributing to continued EBF breakdown and septic complications.

BLOCKADE OF MyD88 PREVENTS THE HOST RESPONSE TO AN ALTERED MICROBIOME

To investigate the mechanism by which the altered microbiome contributes to TPN-related IEC changes, we have used TPN-dependent and chow-fed MyD88 knockout mice. Microbiota interact with host LP myeloid cells via TLR-signaling, much of which is mediated via the sub-cytoplasmic membrane protein, MyD88 (Abreu, 2010). Similar changes in gut microbiota have been observed in MyD88 knockout mice given TPN, with a shift from a Firmicutes-predominant community in fed mice to a Proteobacteria-dominant bacterial community in TPN-dependent mice. In this model, it indicates that the change in bacterial composition occurs independently of MyD88, supporting the theory that the lack of enteral nutrition itself drives the microbial changes. This is in contrast to a recent study in which the targeted deletion of MyD88 within the epithelium led to distinct changes within the mucosally-associated microbial population (Frantz et al., 2012).

The TLR signaling changes normally induced by this bacterial shift, however, are abrogated in the MyD88 knockout strain. Blockade of TLR signaling in these mice prevents sensing of the altered microbiota by the host LP cells. This leaves the mucosal immune response unchanged from that of fed mice. Pro-inflammatory cytokines TNF-α and IFN-γ are not upregulated, and activation of NF-κB is prevented. In addition, MyD88 blockade led to preservation of the small intestinal T_{reg} cell population, which is almost completely lost in wild-type TPN-dependent mice. Together, prevention of these inflammatory mucosal responses allows maintenance of EC proliferation, decreased apoptosis, and preservation of EBF (Miyasaka et al., 2013).

Interestingly, MyD88 knockout mice on TPN demonstrate an increase in Akkermansia species compared to wild-type TPN-dependent mice and fed mice (**Figure 3**). This may reflect the role of mucosal responses in modulating the intraluminal environment. By preventing the dominant Proteobacteria from signaling via TLRs, the inflammatory changes in the epithelium are avoided, and this may create an altered environment that is more favorable for this strain of bacteria (Miyasaka et al., 2013). A mechanism for this change may be due to an increase in acidomucins within the small bowel mucosa during the administration of TPN, the primary substrate for Akkermansia mucinophilia (Derrien et al., 2011). The complex interaction between the various microbial species, their luminal environment, and the intestinal epithelium continues to be explored.

EVIDENCE OF BARRIER FUNCTION LOSS AND MICROBIOTA CHANGES IN HUMANS

While the outcomes of decreased EBF and subsequent septic complications associated with TPN are well-documented in humans, the mechanisms described thus far have not been thoroughly explored in human intestine. In a study of 8 healthy volunteers who received TPN as an exclusive means of nutrition for 14 days, many of the intestinal morphologic and functional changes seen in animal models were reproduced, though to a lesser extent (Buchman et al., 1995). These changes included a decrease in mucosal thickness, increased villus cell count, and an increase in the urinary lactulose-mannitol ratio (indicating an increase in intestinal permeability). Mitotic index was not significantly diminished, however. While other studies in humans have failed to replicate increased sensitivity to intravenous LPS (Santos et al., 1994) or decreased EBF (Reynolds et al., 1997), these studies are based on a limited number of patients and lack a robust evaluation of changes in mucosal physiology.

A recent study has begun to elucidate whether a similarly significant change in gut microbial diversity occurs with enteral deprivation in humans (Ralls et al., 2013). Small bowel samples from 12 patients undergoing intestinal resection were collected and mucosa-associated bacteria were analyzed. As noted in other studies of human intestinal microbiota (Costello et al., 2012), a wide variability in microbial diversity was found within all groups. While an accurate characterization of the typical makeup of intestinal microbiota with TPN in humans was not possible, an interesting finding was that the level of microbial diversity appeared to be closely related to clinical outcome. Patients with low enteric bacterial diversity were significantly more likely to develop postoperative infection or intestinal anastomotic disruption (Ralls et al., 2013).

CONCLUSION AND FUTURE DIRECTIONS

While TPN serves a life-sustaining purpose in patients who must remain deprived of enteral nutrition for a prolonged period, it is not without risk. Enteral deprivation leads to a drastically altered intestinal luminal environment, which allows for the dominance of aggressive microbiota such as Gram-negative Proteobacteria. Signaling factors such as LPS derived from these bacteria act through intestinal TLRs to stimulate an increase in pro-inflammatory mediators and a decrease in growth factors. These changes lead to a decrease in EC proliferation and increase in EC apoptosis. The final outcome is diminished EBF, characterized by a loss of epithelial tight junction proteins and increased mucosal permeability, with the subsequent septic morbidity associated with TPN (**Figure 4**).

A deeper understanding of the interaction between host and intestinal microbiota in the setting of enteric deprivation may provide novel strategies for preventing TPN-related complications. While evidence suggests that microbiota signal mucosal inflammation via TLRs using a MyD88 pathway, studies with more specific inhibition of certain TLRs may reveal that a subset of TLRs (i.e., TLR4, which binds LPS) may be primarily responsible for this process. In addition to understanding how epithelial physiology changes in this setting, it will be critical elucidate how luminal environment changes are tied to bacterial selection. Modifying this environment to select for more benign bacterial species may alleviate some of the deleterious changes seen with TPN. Finally, the altered cytokine profile produced by these changes may be alleviated pharmacologically. For example, growth factor replacement with exogenous EGF or antibody blockade of epithelial TNF-α may diminish TPN-related inflammation and loss of EBF.

Using a mouse model, significant gains have been made in the understanding of intestinal physiologic changes with fasting and TPN. Further human studies are needed to translate these

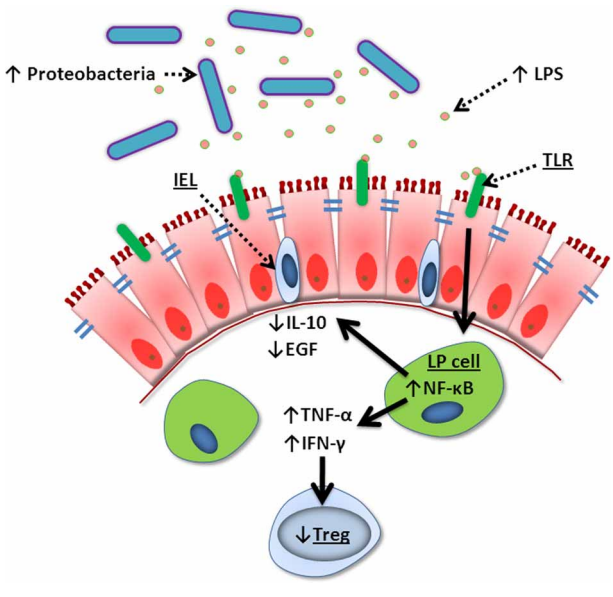

FIGURE 4 | Summary of TPN-induced epithelial signaling changes. Lack of enteral nutrition leads to a change in luminal microbiota where Gram-negative Proteobacteria dominate. Lipopolysaccharide (LPS) derived from these bacteria signal lamina propria (LP) cells via Toll-like receptors (TLR), leading to increased NF-κB transcription. This creates a pro-inflammatory state with increased TNF-α and IFN-γ, loss of T_{reg} cells, and decreased intraepithelial lymphocyte (IEL)-derived IL-10 and EGF. These changes lead to break down of tight junctions, loss of epithelial barrier function, bacterial translocation, and sepsis.

findings to the patient. With a clear understanding of these changes, novel strategies to mitigate them may make TPN a safer option for the many patients who require it.

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Inflammasome-mediated cell death in response to bacterial pathogens that access the host cell cytosol: lessons from *Legionella pneumophila*

Cierra N. Casson and Sunny Shin*

Department of Microbiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

Edited by:
Yongqun He, University of Michigan, USA

Reviewed by:
Dario S. Zamboni, Universidade de São Paulo, Brazil
Thomas Henry, Institut National de la Santé et de la recherche médicale, France

****Correspondence:***
Sunny Shin, Department of Microbiology, Perelman School of Medicine, University of Pennsylvania, 3610 Hamilton Walk, Philadelphia, PA 19104, USA
e-mail: sunshin@mail.med.upenn.edu

Cell death can be critical for host defense against intracellular pathogens because it eliminates a crucial replicative niche, and pro-inflammatory cell death can alert neighboring cells to the presence of pathogenic organisms and enhance downstream immune responses. Pyroptosis is a pro-inflammatory form of cell death triggered by the inflammasome, a multi-protein complex that assembles in the cytosol to activate caspase-1. Inflammasome activation by pathogens hinges upon violation of the host cell cytosol by activities such as the use of pore-forming toxins, the use of specialized secretion systems, or the cytosolic presence of the pathogen itself. Recently, a non-canonical inflammasome has been described that activates caspase-11 and also leads to pro-inflammatory cell death. Caspase-11 is activated rapidly and robustly in response to violation of the cytosol by bacterial pathogens as well. In this mini-review, we describe the canonical and non-canonical inflammasome pathways that are critical for host defense against a model intracellular bacterial pathogen that accesses the host cytosol—*Legionella pneumophila*.

Keywords: *Legionella pneumophila*, inflammasome, cell death, pyroptosis, caspase-11, caspase-1

INTRODUCTION

Cell death is an important innate immune effector mechanism to aid in clearance of intracellular pathogens, as it can eliminate a pathogen's replicative niche. Additionally, pro-inflammatory cell death can be critical for alerting neighboring cells to the presence of invading pathogens (Kono and Rock, 2008; Bergsbaken et al., 2009). The pro-inflammatory form of cell death known as pyroptosis is critical both for clearance of bacterial pathogens and for release of important proinflammatory cytokines *in vivo* (Fink and Cookson, 2005; Miao et al., 2010a). Pyroptosis is initiated in response to violation of the host cell cytosol by pathogenic microbes (Lamkanfi and Dixit, 2009). Violation of the cytosol can occur either by access via bacterial secretion systems, such as type IV (T4SS) or type III (T3SS) secretion systems, or by physical entry of a pathogen into the cytosol. Here, we discuss how cells induce proinflammatory cell death in response to microbes gaining cytosolic access by using *Legionella pneumophila* as a model intracellular pathogen.

NOD-LIKE RECEPTORS RESPOND TO CYTOSOLIC ACCESS BY PATHOGENS

Pattern recognition receptors (PRRs) are critical initiators of host defense against invading microorganisms (Janeway and Medzhitov, 2002; Medzhitov, 2007). Surface and endosomally-associated PRRs, such as Toll-like receptors (TLRs), recognize pathogen-associated molecular patterns found in the extracellular space (Janeway and Medzhitov, 2002). However, many pathogenic organisms have mechanisms for accessing the host cytosol. Thus, many cells encode cytosolic PRRs, such as nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (Harton et al., 2002), which act as guardians of cytosolic sanctity (Lamkanfi and Dixit, 2009). NLRs respond to "patterns of pathogenesis," such as membrane disruption, delivery of bacterial molecules into the host cytosol via specialized secretion systems, or pore-forming toxins, by activating the inflammasome (Fritz et al., 2006; Lamkanfi and Dixit, 2009; Vance et al., 2009; Davis et al., 2011; Franchi et al., 2012).

CASPASE-1-DEPENDENT INFLAMMASOMES

The canonical inflammasome is a multi-protein complex that assembles in the cytosol to activate the enzyme caspase-1, also known as interleukin-1β (IL-1β)-converting enzyme (ICE) (Martinon et al., 2002). Caspase-1 regulates secretion of IL-1 family cytokines and a pro-inflammatory form of cell death termed pyroptosis (Rathinam et al., 2012a). Caspase-1 processes IL-1β and IL-18 into their mature forms and aids in their secretion (Howard et al., 1991; Thornberry et al., 1992; Ghayur et al., 1997; Gu et al., 1997). Caspase-1 does not cleave IL-1α, though it can aid in IL-1α secretion as well (Keller et al., 2008). IL-1 family cytokines act *in vivo* to enhance immune responses against invading microorganisms (Labow et al., 1997; Bohn et al., 1998; Dinarello, 2009). Additionally, caspase-1-mediated pyroptosis enhances clearance of bacterial pathogens *in vivo* (Miao et al., 2010a).

NLRs respond to different stimuli when activating the inflammasome. Few NLRs have been shown to bind directly to their implicated substrates, and some are activated by a wide variety

of stimuli. For example, NLRP3 responds to stimuli ranging from bacterial RNA to extracellular adenosine triphosphate and uric acid crystals (Kanneganti et al., 2006; Mariathasan et al., 2006; Martinon et al., 2006). Absent in melanoma 2 (AIM2) responds to the presence of cytosolic double-stranded DNA (Hornung et al., 2009; Roberts et al., 2009). In mice, ICE-protease activating factor (IPAF)/NLR family, CARD domain containing 4 (NLRC4) mediates inflammasome activation in response to three distinct stimuli—flagellin, the conserved inner rod component of the bacterial T3SS (PrgJ), and T3SS needle proteins (Franchi et al., 2006; Miao et al., 2006, 2010b; Lightfield et al., 2011; Yang et al., 2013). Biochemical studies have shown that the NLRs neuronal apoptosis inhibitory protein 5 (NAIP5) and NAIP6 co-immunoprecipitate with flagellin, while NAIP2 interacts specifically with PrgJ and NAIP1 interacts with the needle proteins (Kofoed and Vance, 2011; Zhao et al., 2011; Yang et al., 2013). NLRC4 appears to be an important adaptor for the NAIP receptors. The adaptor protein apoptosis-associated speck-like protein containing a carboxy-terminal caspase recruitment domain (ASC) often bridges the interaction between NLRs and caspase-1, allowing for oligomerization and auto-processing of caspase-1 for activation (Srinivasula et al., 2002). Caspase-1 auto-processing is required for cytokine cleavage and secretion, though cell death can occur independently of caspase-1 proteolysis (Broz et al., 2010).

THE NON-CANONICAL INFLAMMASOME

Experiments examining inflammasome activation were first performed with macrophages from mice that lack caspase-1, and it was concluded that caspase-1 is solely responsible for inflammasome activation. However, the strain of mice used to generate the original caspase-1 knockout has a caspase-11 polymorphism that eliminates protein expression. Thus, the original mice lack both caspase-1 and caspase-11 (Kuida et al., 1995; Kayagaki et al., 2011). Though it was reported that caspase-11 mediates septic shock *in vivo*, the cell-intrinsic role of caspase-11 in response to bacterial pathogens remained unclear (Wang et al., 1996, 1998). Recently, however, a non-canonical caspase-11-dependent inflammasome has been described that contributes to IL-1α, IL-1β, and IL-18 secretion and cell death in response to many Gram-negative bacteria. Caspase-11 is activated with delayed kinetics, taking 16–24 h *in vitro*, in response to bacteria that do not typically access the host cell cytosol, such as non-pathogenic *Escherichia coli* (Kayagaki et al., 2011). For many Gram-negative bacteria, non-canonical inflammasome activation requires TIR-domain-containing adaptor-inducing interferon-β (TRIF) and type I interferon (IFN) signaling downstream of TLR4 (Broz et al., 2012; Gurung et al., 2012; Rathinam et al., 2012b). Additionally, cytosolic lipopolysaccharide (LPS) activates caspase-11 independently of TLR4 (Hagar et al., 2013; Kayagaki et al., 2013). Pathogens that access or enter the host cytosol also induce non-canonical inflammasome activation, and this activation is more rapid than for other Gram-negative bacteria. One robust activator of the non-canonical inflammasome is the intracellular pathogen *Legionella pneumophila* (Aachoui et al., 2013; Case et al., 2013; Casson et al., 2013).

Legionella pneumophila

L. pneumophila is a Gram-negative bacterium that causes the severe pneumonia Legionnaires' disease (Fraser et al., 1977; McDade et al., 1977). *L. pneumophila* uses its *dot*/*icm*-encoded T4SS to translocate effector proteins into the host cytosol to establish an endoplasmic reticulum-derived vacuole that supports bacterial replication (Marra et al., 1992; Berger and Isberg, 1993; Sadosky et al., 1993; Roy et al., 1998; Segal et al., 1998; Vogel et al., 1998). The natural host for *L. pneumophila* is amoebae in aquatic reservoirs (Rowbotham, 1980; Fliermans et al., 1981), so while it has evolved to evade amoebic host defenses, it is not thought to have evolved to evade mammalian-specific immune responses. Therefore, as a consequence of accessing the host cytosol in mammalian cells, *L. pneumophila* triggers multiple pathways that elicit cell-intrinsic immune responses and induce cell death. These robust immune responses make the bacterium valuable for studying host defense against intracellular pathogens.

L. pneumophila AND CASPASE-1-DEPENDENT INFLAMMASOME ACTIVATION

It is well-understood that *L. pneumophila* triggers inflammasome activation and pyroptosis as a consequence of flagellin expression and T4SS activity (**Figure 1**). In murine macrophages, detection of flagellin by BIRC1e/NAIP5 mediates pyroptosis and contributes to restriction of *L. pneumophila* replication both *in vitro* and *in vivo* (Growney and Dietrich, 2000; Diez et al., 2003; Wright et al., 2003; Derré and Isberg, 2004; Zamboni et al., 2006; Kofoed and Vance, 2011; Zhao et al., 2011). Flagellin-deficient *L. pneumophila* (Δ*flaA* Lp) evade NAIP5-mediated restriction and replicate in NAIP5-sufficient macrophages from C57BL/6 (B6) mice, in part because they do not induce as much caspase-1-dependent cell death as wild-type (WT) Lp (Molofsky et al., 2006; Ren et al., 2006). NLRC4 also acts upstream of caspase-1 to induce flagellin-mediated restriction of replication, pore formation in the host membrane, and IL-1β release (Zamboni et al., 2006; Silveira and Zamboni, 2010). NLRC4 co-immunoprecipitates with NAIP5, consistent with the model that NLRC4 is an adaptor for NAIP5 (Zamboni et al., 2006; Kofoed and Vance, 2011; Zhao et al., 2011). The NAIP5/NLRC4-dependent cell death induced in B6 macrophages requires cytosolic access, as T4SS-deficient mutants (Δ*dotA* Lp) do not activate the inflammasome. These data suggest that flagellin is translocated through the T4SS into the host cytosol during infection, though this has not been shown experimentally.

A/J mice express a hypomorphic allele of NAIP5 (Diez et al., 2000), and A/J macrophages still activate caspase-1 in response to WT Lp under certain infection conditions (Lamkanfi et al., 2007). However, using *Naip5*$^{-/-}$ macrophages, it was shown that NAIP5 is required for caspase-1 activation in response to WT Lp (Lightfield et al., 2008). Interestingly, NAIP6 also interacts with *L. pneumophila* flagellin (Kofoed and Vance, 2011; Zhao et al., 2011). However, NAIP6 is insufficient for the restriction of *L. pneumophila*, as *Naip5*$^{-/-}$ macrophages and mice are permissive for infection (Lightfield et al., 2008), potentially due to lower expression levels of NAIP6 relative to NAIP5 in primary macrophages (Wright et al., 2003). NAIP5 and NLRC4 also

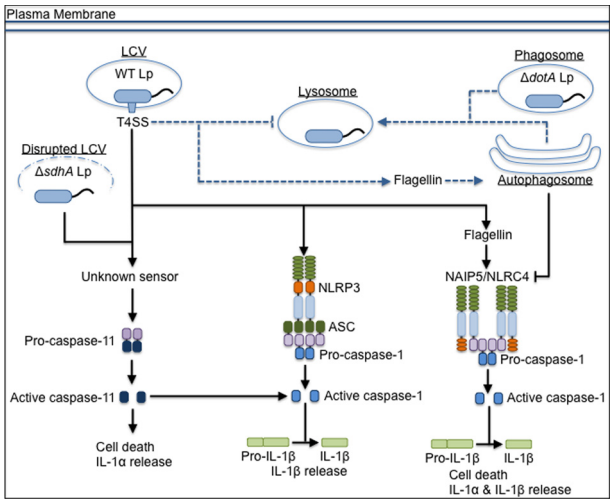

FIGURE 1 | The inflammasome-mediated response to *L. pneumophila* in murine macrophages. *L. pneumophila* that do not express a functional T4SS (Δ*dotA* Lp) traffic to the lysosome, but wild-type *L. pneumophila* (WT Lp) use the T4SS to translocate effectors into the host cytosol to establish a replicative niche, the *Legionella*-containing vacuole (LCV), and block fusion with lysosomes. WT Lp triggers canonical caspase-1-dependent inflammasome activation through detection of translocated flagellin by NAIP5/NLRC4. NLRP3 and ASC contribute to IL-1β secretion in response to WT Lp. Detection of T4SS activity through an unknown sensor leads to caspase-11 activation, which contributes to inflammasome activation. Caspase-11 also responds to bacteria that aberrantly enter the cytosol (Δ*sdhA* Lp) due to loss of LCV membrane integrity. Translocated flagellin triggers trafficking of WT Lp to the autophagosome, and induction of autophagy negatively regulates pyroptosis if there are low levels of flagellin in the host cell cytosol. Dashed lines represent vesicular trafficking patterns. Solid lines represent pathways for activation of the host response. Arrows at the end of lines represent induction, while flat bars at the end of lines represent inhibition.

contribute to the control of *L. pneumophila* replication by enhancing fusion of the *Legionella*-containing vacuole (LCV) with lysosomes during infections performed at a low multiplicity of infection (MOI) (Amer et al., 2006; Fortier et al., 2007). In addition, flagellin-dependent NLRC4 signaling leads to caspase-7-mediated restriction of *L. pneumophila* via enhanced lysosomal degradation of the bacterium (Akhter et al., 2009). NLRC4-mediated restriction *in vivo* is also partially caspase-1-independent through an unknown mechanism (Pereira et al., 2011). However, caspase-1 activation downstream of NLRC4 clearly induces pyroptosis and leads to IL-18 secretion both *in vitro* and *in vivo*, contributing to IFN-γ production and the subsequent resolution of pulmonary infection (Brieland et al., 2000; Spörri et al., 2006; Archer et al., 2009; Case et al., 2009). Thus, the NAIP5/NLRC4 inflammasome may control *L. pneumophila* replication through multiple mechanisms. Further studies are needed to determine the relative contributions of these mechanisms.

Not surprisingly, infection conditions, including MOI, can affect the detection of caspase-1 activation in response to *L. pneumophila*, as higher MOIs likely enhance the number of macrophages that harbor bacteria. At higher MOIs, infection of B6 macrophages induces both NLRC4-dependent and NLRC4-independent inflammasome activation. NLRC4-independent caspase-1 activation and IL-1β and IL-18 secretion require ASC and NLRP3, although the identity of the *L. pneumophila*-derived signal sensed via NLRP3 is unknown (Case et al., 2009, 2013; Casson et al., 2013). Caspase-1 cleavage in the absence of ASC can be detected in either the supernatant or the cytosol, depending on the MOI (Case et al., 2009; Abdelaziz et al., 2011a). ASC also drives formation of a punctate structure containing caspase-1 and NLRC4 in *L. pneumophila*-infected macrophages (Case and Roy, 2011). At early timepoints, pore formation is not observed in the absence of NLRC4, though cell death still occurs in the absence of ASC. Recruitment of NLRC4 into the ASC complex appears to dampen NLRC4 activity because pyroptosis occurs at a higher rate in the absence of ASC (Case and Roy, 2011). Further studies are needed to elucidate the temporal and spatial coordination of the ASC- and NLRC4-dependent inflammasomes and how they are triggered by *L. pneumophila*.

INFLAMMASOME ACTIVATION IN HUMAN CELLS

Unlike macrophages from most inbred mouse strains, human cells are permissive for *L. pneumophila* replication. The mechanisms underlying inflammasome-mediated control of *L. pneumophila* replication in human cells are unclear. Humans express only one homolog of the numerous murine NAIP paralogs (Scharf et al., 1996). The homolog, human NAIP (hNAIP), restricts growth of WT Lp (Vinzing et al., 2008). Additionally, the human NLRC4 homolog, human IPAF (hIPAF), also restricts *L. pneumophila* replication. Overexpression of full-length hNAIP in HEK293T cells increases cell death in response to *L. pneumophila* (Boniotto et al., 2012), and overexpression of hNAIP in the murine macrophage RAW264.7 cell line mediates flagellin-induced pyroptosis and IL-1β secretion (Katagiri et al., 2012), suggesting that it may function similarly to NAIP5. However, unlike NAIP5, hNAIP does not co-immunoprecipitate with flagellin and instead interacts with T3SS needle proteins (Zhao et al., 2011; Yang et al., 2013). Thus, it is unclear whether hNAIP senses flagellin or another *L. pneumophila*-derived ligand, how hNAIP restricts *L. pneumophila* replication, and if hNAIP contributes to cell death or IL-1β secretion in primary human cells.

The implication that the IPAF/NAIP/caspase-1 inflammasome contributes to restriction of *L. pneumophila* is pervasive, though caspase-1 activation in response to *L. pneumophila* has not been explicitly shown in primary cells from humans, a naturally susceptible host. Immortalized human alveolar epithelial cells activate caspase-1 in response to *L. pneumophila*, though primary human monocytes and monocyte-derived macrophages (MDMs) do not produce detectable levels of processed or active caspase-1 (Santic et al., 2007; Furugen et al., 2008; Abdelaziz et al., 2011b). Additionally, the expression of ASC is moderately down-regulated in infected monocytes, potentially contributing to evasion of inflammasome activation in human cells by *L. pneumophila* (Abdelaziz et al., 2011b). Future studies in primary MDMs and human alveolar macrophages are needed to clarify the role of the inflammasome in restricting *L. pneumophila* replication in human cells (**Figure 2**).

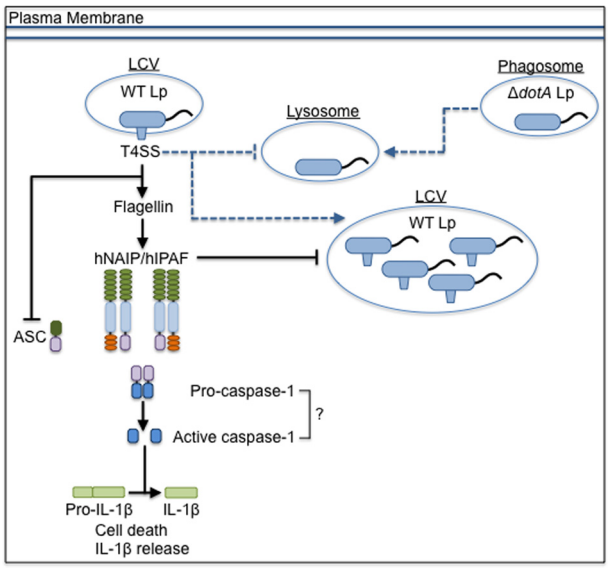

FIGURE 2 | The inflammasome-mediated response to *L. pneumophila* in human cells. *L. pneumophila* that do not express a functional T4SS (Δ*dotA* Lp) traffic to the lysosome, but wild-type *L. pneumophila* (WT Lp) use the T4SS to translocate effectors into the host cytosol to establish a replicative niche, the *Legionella*-containing vacuole (LCV), and block fusion with lysosomes. The presence of flagellin triggers signaling through hNAIP/hIPAF that blocks replication of WT Lp, though it is unclear if caspase-1 is involved in restriction. T4SS activity down-regulates the expression of ASC. Dashed lines represent vesicular trafficking patterns. Solid lines represent pathways for activation of the host response. Arrows at the end of lines represent induction, while flat bars at the end of lines represent inhibition.

INFLAMMASOME ACTIVATION AND AUTOPHAGY

In murine macrophages, autophagy is induced shortly after phagocytosis of *L. pneumophila*, as components of the autophagy pathway co-localize with the LCV (Amer and Swanson, 2005). LCVs in A/J macrophages show delayed autophagosome maturation compared to LCVs in B6 macrophages, potentially contributing to increased replication of the bacterium. When expression of the autophagy component ATG5 is silenced, *L. pneumophila* replication in A/J macrophages increases. Additionally, replication of *L. pneumophila* decreases slightly when autophagy is induced exogenously, suggesting that autophagy contributes to restriction of *L. pneumophila* replication (Matsuda et al., 2009). Under low MOI infection conditions where there is minimal induction of pyroptosis, it was revealed that the induction of autophagy dampens pyroptosis in response to *L. pneumophila*, and turnover of autophagosomes requires NAIP5, NLRC4, and caspase-1 (Byrne et al., 2013). Collectively, these data suggest that NAIP5 inflammasome activation contributes to the restriction of *L. pneumophila* replication by inducing autophagy and/or pyroptosis, depending on the MOI and amount of flagellin present. How competing host and bacterial factors influence the outcome of inflammasome activation and autophagy during infection remains unclear and may be clarified by studies examining the temporal regulation of inflammasome activation and autophagy at a single-cell level.

L. pneumophila AND NON-CANONICAL INFLAMMASOME ACTIVATION

Though Δ*flaA* Lp avoid NAIP5/NLRC4-mediated pyroptosis and can replicate in B6 macrophages, Δ*flaA* Lp trigger an additional form of cell death. Recently, caspase-11 has been shown to contribute to inflammasome activation in response to *L. pneumophila* (**Figure 1**). After MyD88 and TRIF-dependent upregulation of caspase-11, host cells undergo rapid caspase-11-mediated cell death, occurring in less than 4 h, in response to Δ*flaA* Lp (Case et al., 2013; Casson et al., 2013). Non-canonical inflammasome activation in response to Δ*flaA* Lp requires T4SS-mediated cytosolic access, as Δ*dotA* Lp do not activate caspase-11. Like caspase-1-mediated pyroptosis, caspase-11-dependent cell death leads to release of important inflammatory mediators, such as IL-1α, IL-1β, and IL-18. Caspase-11 is required for cell death and IL-1α release and additionally enhances NLRP3-dependent caspase-1 activation and IL-1β and IL-18 secretion (Case et al., 2013; Casson et al., 2013). IL-1α release *in vivo* is critical for host defense, including neutrophil recruitment to the airway space and control of bacterial burden, though there are caspase-11-independent sources of IL-1α *in vivo* as well (Barry et al., 2013; Casson et al., 2013). Caspase-11 also contributes to NAIP5/NLRC4-mediated inflammasome activation and restricts WT Lp by enhancing phago-lysosomal fusion (Akhter et al., 2012). In its non-lytic role, caspase-11 modulates actin polymerization and phosphorylation of cofilin to promote lysosomal trafficking of pathogenic, but not non-pathogenic, bacteria. Additionally, caspase-11 contributes to control of WT Lp replication *in vivo* (Akhter et al., 2012).

Caspase-11 responds not only to vacuolar bacteria that access the host cytosol through the T4SS but also to *L. pneumophila* that escape from the vacuole and aberrantly enter the cytosol (Aachoui et al., 2013). The T4SS-translocated effector SdhA is critical for bacterial growth in primary macrophages (Laguna et al., 2006; Liu et al., 2008). Macrophages infected with Δ*sdhA* Lp undergo cell death because SdhA is required to maintain LCV membrane integrity (Creasey and Isberg, 2012). Therefore, Δ*sdhA* Lp aberrantly enter the host cytosol where they become degraded, induce type I IFN, and activate caspase-1 via AIM2 (Monroe et al., 2009; Creasey and Isberg, 2012; Ge et al., 2012). In addition, Δ*sdhA* Lp induce rapid caspase-11-dependent cell death independently of bacterial flagellin (Aachoui et al., 2013). It appears that AIM2 responds to cytosolic *L. pneumophila* by producing IL-1β, whereas caspase-11 mediates cell death. However, *L. pneumophila* does not normally enter the cytosol, so the upstream mediators of caspase-11 activation may be different for Δ*sdhA*Δ*flaA* bacteria that enter the cytosol and Δ*flaA* bacteria that remain within the vacuole. Whether the bacteria physically enter the cytosol or not, these unique pathways upstream of caspase-11 are likely relevant for defense against other pathogens that lack or down-regulate flagellin during infection.

Non-canonical inflammasome activation is a recently described phenomenon, so there are many questions that remain unanswered. Currently, no NLRs have been identified that act upstream of caspase-11 to induce non-canonical inflammasome activation. As *L. pneumophila* rapidly and robustly activates caspase-11, it will be a valuable tool for future studies aiming

to identify NLRs or other host factors critical for caspase-11 activation. The only bacterial factor that has been shown to initiate non-canonical inflammasome activation is cytosolic LPS (Hagar et al., 2013; Kayagaki et al., 2013). For some Gram-negative bacteria, it is thought that bacterial RNA may access the host cytosol to activate NLRP3 and caspase-11 (Kanneganti et al., 2006; Rathinam et al., 2012b). However, translocation of *L. pneumophila* RNA to initiate inflammasome activation has not been verified experimentally. Additionally, though cytosolic LPS may trigger caspase-11 during infection with Δ*sdhA* Lp that aberrantly enter the cytosol, it is unclear if LPS is sensed by host cells to initiate non-canonical inflammasome activation in the context of infection with *L. pneumophila* that remain within the LCV. Further studies are needed to clarify what triggers the host response to Δ*flaA* Lp and to elucidate the molecular pathways that lead to caspase-11-mediated cell death.

CONCLUDING REMARKS

Studying the inflammasome pathways triggered by the pathogen *L. pneumophila* has shaped our knowledge of how host cells are poised to respond to violation by intracellular pathogens. Whether the bacterium utilizes its T4SS to access the host cytosol, additionally delivers flagellin into the cytoplasm, or physically enters the cytosol itself, the host has evolved multiple ways to restrict replication of the pathogen and trigger immunity.

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Brucella dissociation is essential for macrophage egress and bacterial dissemination

Jianwu Pei, Melissa Kahl-McDonagh and Thomas A. Ficht*

Department of Veterinary Pathobiology, Texas A&M University and Texas Agricultural Experiment Station, College Station, TX, USA

Edited by:
Yongqun "Oliver" He, University of Michigan Medical School, USA

Reviewed by:
Srinand Sreevastan, University of Minnesota, USA
Martin R. Roop II, East Carolina University, USA

***Correspondence:**
Thomas A. Ficht, Department of Veterinary Pathobiology, Texas A&M University, Veterinary Medicine Research Building, 634-676 University Drive, College Station, TX77843-4467, USA
e-mail: tficht@cvm.tamu.edu

It has long been observed that smooth *Brucella* can dissociate into rough mutants that are cytotoxic to macrophages. However, the *in vivo* biological significance and/or mechanistic details of *Brucella* dissociation and cytotoxicity remain incomplete. In the current report, a plaque assay was developed using *Brucella* strains exhibiting varying degrees of cytotoxicity. Infected monolayers were observed daily using phase contrast microscopy for plaque formation while *Brucella* uptake and replication were monitored using an immunofluorescence assay (IFA). Visible plaques were detected at 4–5 days post infection (p.i.) with cytotoxic *Brucella* 16MΔ*manBA* at an MOI of 0.1. IFA staining demonstrated that the plaques consisted of macrophages with replicating *Brucella*. Visible plaques were not detected in monolayers infected with non-cytotoxic 16MΔ*manBA*Δ*virB*2 at an MOI of 0.1. However, IFA staining did reveal small groups of macrophages (foci) with replicating *Brucella* in the monolayers infected with 16MΔ*manBA*Δ*virB*2. The size of the foci observed in macrophage monolayers infected with rough *Brucella* correlated directly with cytotoxicity measured in liquid culture, suggesting that cytotoxicity was essential for *Brucella* egress and dissemination. In monolayers infected with 16M, small and large foci were observed. Double antibody staining revealed spontaneous rough mutants within the large, but not the small foci in 16M infected monolayers. Furthermore, plaque formation was observed in the large foci derived from 16M infections. Finally, the addition of gentamicin to the culture medium inhibited plaque formation, suggesting that cell-to-cell spread occurred only following release of the organisms from the cells. Taken together, these results demonstrate that *Brucella*-induced cytotoxicity is critical for *Brucella* egress and dissemination.

Keywords: *Brucella* dissociation, macrophage cytotoxicity, egress and dissemination, plaque assay, infection foci

INTRODUCTION

Brucella is a genus of Gram-negative, facultative intracellular bacteria that cause brucellosis in a variety of animals and undulant fever in humans. Ten species have been described to date (Whatmore, 2009), and three species, *B. melitensis*, *B. abortus*, and *B. suis* provide the major threat to agriculture and public health worldwide (Boschiroli et al., 2001).

Macrophages/monocytes are the primary target cells in which *Brucella* replicate and cause persistent infection and as such are essential dissemination within the host (Baldwin and Winter, 1994; Liautard et al., 1996). Recent studies have shown that *Brucella* modulates the fate of infected macrophages and monocytes. Smooth *Brucella* infection inhibits macrophage and monocyte apoptosis by targeting the intrinsic (mitochondrial) and extrinsic (death receptor) pathways (Galdiero et al., 2000; Gross et al., 2000; Eskra et al., 2003; Fernandez-Prada et al., 2003; Tolomeo et al., 2003; He et al., 2006; Covert et al., 2009). In contrast, *Brucella* rough mutant infection results in type four-secretion system (T4SS) dependent macrophage cell death (Pei and Ficht, 2004; Pei et al., 2006, 2008b; De Jong et al., 2008; Zhong et al., 2009). It has been shown in a number of intracellular bacterial species that the regulation of host cell apoptosis is important to pathogenesis. Prevention of host cell apoptosis provides a hospitable intracellular niche for multiplication (Hacker and Fischer, 2002; Faherty and Maurelli, 2008) while induction of host cell death promotes bacterial release (Weinrauch and Zychlinsky, 1999; Gao and Kwaik, 2000b).

Although initial observations documenting rough *Brucella*-induced cell death in guinea pig macrophages occurred 50 years ago (Freeman et al., 1961), the mechanisms responsible have only been recently investigated (Pei and Ficht, 2004; Pei et al., 2006, 2008b; De Jong et al., 2008; Chen and He, 2009; Zhong et al., 2009; Chen et al., 2011), and the biological significance of the *Brucella* cytotoxicity remains undefined. In the current study, a plaque formation assay was developed to better evaluate *Brucella*-induced cytotoxicity. Comparison using *Brucella* strains with different levels of cytotoxicity provide direct evidence that cytotoxicity plays an important role in *Brucella* egress and dissemination in culture.

MATERIALS AND METHODS

BACTERIA STRAINS AND MEDIA

Bacterial strains used in these experiments include *B. melitensis* 16M, rough mutants 16MΔ*manBA* and 16MΔ*manBA*Δ*virB*2 (Pei et al., 2008b), and the rough *B. abortus* vaccine strain RB51. *Brucella* strains were routinely grown in tryptic soy agar (TSA)

or tryptic soy broth (TSB) as described previously (Pei and Ficht, 2004).

CELL CULTURE AND INFECTION

Murine macrophage-like cells J774.A1 (ATCC, TIB-67) were grown in DMEM with 10% (v/v) fetal bovine serum, 1 mM L-glutamine, and 1 mM non-essential amino acid as described previously (Pei and Ficht, 2004). For plaque assays, 1.25×10^5 cells were seeded into each well of a 24-well plate and incubated overnight at 37°C in atmosphere containing 5% CO_2 prior to inoculation with *Brucella* at various multiplicities of infection (MOI). Infections were synchronized by centrifugation at 200× g for 5 min at room temperature and the plates were incubated at 37°C for 20 min. Cell monolayers were washed with PBS (pH 7.4) three times, and complete DMEM containing 50 μg/ml of gentamicin was added to kill extracellular bacteria with incubation at 37°C for 1 h (Pei and Ficht, 2004). *Brucella* uptake was determined following a 1-h incubation by washing the monolayers with PBS and lysing the cells with 0.5% (w/v) Tween 20 in distilled water. CFUs present in the lysates were determined as described previously (Pei and Ficht, 2004).

PLAQUE FORMATION ASSAY (OAKS ET AL., 1985)

J774.A1 macrophages cultured in 24-well plates (1.25×10^5 cells/well) were infected with *Brucella* as described above. Medium was replaced with 1 ml of warm (45°C) complete DMEM without gentamicin containing 1% (w/v) ultra-pure agarose (Gibco. Gaithersburg, MD). One milliliter of complete DMEM (with or without gentamicin) was added to each well following agarose solidification. Liquid media were changed every 2 days, and cell monolayers were observed daily using phase contrast light microscopy for plaque formation. Following incubation, liquid medium was removed and replaced with sufficient formalin to fix the cells and kill *Brucella* [3.7% (v/v) formaldehyde final] with incubation overnight at room temperature.

IMMUNOFLUORESCENCE ASSAY (IFA)

Following fixation, the agarose was carefully removed and the cell monolayer washed with PBS. Infected cells were stained with goat anti *Brucella* serum and rabbit anti-rough *Brucella* monospecific serum (1:1000) in PBS-TT (PBS with 0.05% (v/v) Tween-20 and 0.05% (v/v) Triton X-100) for smooth and rough *Brucella*, respectively. Following three washes with PBS-T (PBS with 0.05% Tween-20), the cells were incubated with secondary antibodies including donkey anti goat IgG Alexa Fluor 488, chicken anti rabbit IgG Alexa Fluor 488, or chicken anti rabbit IgG Alexa Fluor 594 (Molecular Probes) (1:1000 in PBS-TT). Bacteria were revealed using IX70 fluorescence microscopy (Olympus).

DOUBLE ANTIBODY STAINING

To differentiate rough mutants from smooth *Brucella*, J774.A1 macrophages were inoculated with 16M, 16MΔ*manBA*, or a mixture of 16M and 16MΔ*manBA* (10:1). The cells were fixed at 1 h after infection and incubated with rabbit anti *Brucella* monospecific M serum (1:500 in PBS-TT) and goat anti *B. ovis* serum (1:500 in PBS-TT). Following three washes with PBS-T, the cells were incubated with chicken anti rabbit IgG Alexa Fluor 488 and donkey anti goat IgG Alexa Fluor 594 (1:1000 in PBS-TT). Bacteria were revealed using IX70 fluorescence microscopy.

PLAQUE-FORMING UNIT

To enumerate plaque-forming units (PFUs) in each well, PFUs were averaged over five randomly selected fields in 16MΔ*manBA* infected monolayers using phase contrast and IX70 fluorescence microscopy (10× objective). Since, the area covered by each field under 10× objective lens is 1.798 mm^2 and the total surface area is 200 mm^2 the number of PFU/well is equal to the average PFU/field × (200/1.798).

RESULTS

PLAQUE FORMATION ASSOCIATED WITH *BRUCELLA* CYTOTOXICITY

Previous studies have shown that *Brucella* rough mutants proliferate in murine macrophage J774.A1 causing oncotic and necrotic cell death (Pei and Ficht, 2004; Pei et al., 2006). Therefore, infection with cytotoxic *Brucella* in macrophage monolayers with low MOI was predicted to produce plaques, resulting from replication and release of bacteria that infect neighboring cells to cause localized lysis. To test this hypothesis, a plaque assay was developed using the cytotoxic mutant 16MΔ*manBA* while the non-cytotoxic *Brucella* mutant 16MΔ*manBA*Δ*virB2* was employed as control (Pei et al., 2008b). In order to detect individual plaques, J774.A1 macrophages were infected with *Brucella* at low MOI (1.0 or 0.1). All the macrophages in the wells infected with 16MΔ*manBA* at 1 MOI were lysed within 4 days, and no individual plaques were observed. Plaques were visible between 4 and 5 days in monolayers infected with 16MΔ*manBA* at an MOI of 0.1 (**Figure 1A**). Neither cell death nor plaques were observed in cell monolayers infected with 16MΔ*manBA*Δ*virB2* at MOIs of 1 or 0.1 (data not shown) (**Figure 1B**). Since the MOI used was 0.1, at most only one in 10 macrophage are expected to be infected, and this was confirmed by IFA staining of infected cells at 1 h p.i. (shown in the following section). The results suggested that rough, cytotoxic *Brucella* replicated in macrophages causing cell death, and the bacteria released re-infected neighboring cells to cause a localized cell death. The process repeated itself until an area of dead cells (plaque) was observed (**Figure 1A**). MOI of 0.1 was used in all plaque assays described in this report unless otherwise specified.

To further determine whether the plaques observed in 16MΔ*manBA* infected cells (**Figure 2A**) were caused by *Brucella* replication, the infected cell monolayer was stained using the Immunofluorescence assay (IFA) for *Brucella* at 4 days p.i. The results revealed that significant replication of 16MΔ*manBA* occurred in cells comprising the plaques (**Figure 2B**). These results indicated that plaque formation was a direct result of the intracellular replication of the cytotoxic *Brucella* mutants.

BRUCELLA CYTOTOXICITY WAS ESSENTIAL FOR BACTERIA EGRESS AND SUBSEQUENT REINFECTION

The results described in the previous section indicated that plaques were composed of dead, lysed or shedding cells containing replicating *Brucella*. However, differences in plaque size were apparent depending on the strain employed and it was hypothesized that plaque size was directly related to *Brucella* cytotoxicity,

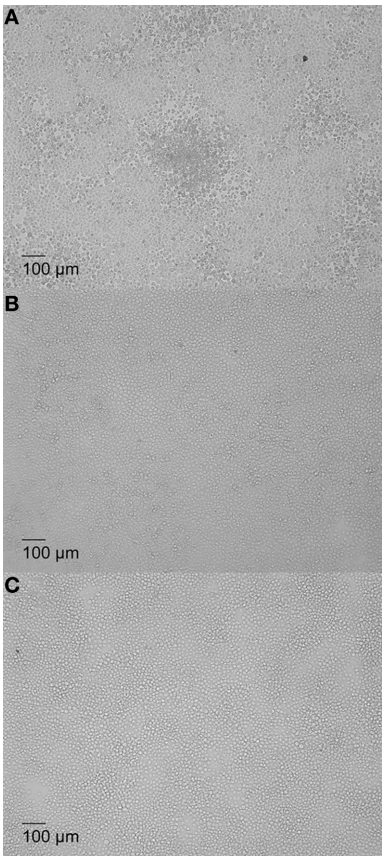

FIGURE 1 | Plaque formation in *Brucella*-infected macrophage monolayers associated with cytotoxicity. J774.A1 macrophages were infected at an MOI of 0.1 with *B. melitensis* mutant 16MΔ*manBA* **(A)**, 16MΔ*manBA*Δ*virB*2 0.1 **(B)**, or left uninfected, as control **(C)**. The agarose (1% w/v) overlay and DMEM were added at 1 h p.i. as described in Materials and Methods, and incubated an additional 5 days. The cells were fixed overnight in 3.7% (w/v) formaldehyde and observed using IX70 microscopy. Scale bar, 100 µm.

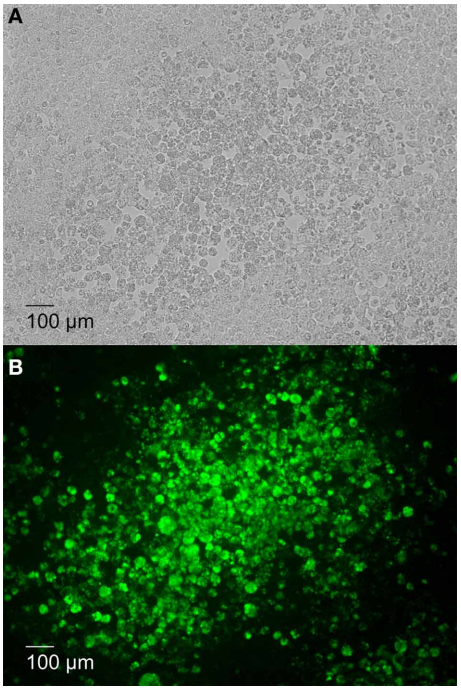

FIGURE 2 | Plaque formation corresponds with increased intracellular *Brucella* replication. J774.A1 macrophages were infected with 16MΔ*manBA* at an MOI of 0.1. An agarose over lay and DMEM were added at 1 h p.i. and incubated an additional 4 days. Following fixation in 3.7% (w/v) formaldehyde, rough *Brucella* were visualized via IFA and IX70 microscopy under phase contrast field **(A)** and fluorescent field **(B)** (showing *Brucella* replication in green) microscopy. Scale bar, 100 µm.

as measured by LDH release (Pei and Ficht, 2004). To test this hypothesis, J774.A1 macrophages were infected with *Brucella* strains exhibiting different levels of cytotoxicity: 16MΔ*manBA* (high cytotoxicity), 16MΔ*manBA*Δ*virB*2 (no cytotoxicity), and *B. abortus* rough vaccine strain RB51 (low cytotoxicity) (Pei and Ficht, 2004; Pei et al., 2008b). *Brucella* replication and spread (designated foci) were detected by 4 days p.i. using IFA. As predicted, foci of infected macrophage monolayers containing RB51 were smaller than those in 16MΔ*manBA*-infected cells, but larger than the foci in monolayers infected with non-cytotoxic mutant 16MΔ*manBA*Δ*virB*2 (**Figure 3**). These results suggested that cytotoxicity was directly related to *Brucella* dissemination and subsequent re-infection presumably as a result of enhanced egress, suggesting a possible role for cytotoxicity in the spread of infection.

To further determine whether plaques can develop from an individual bacterium, uptake and replication of cytotoxic and non-cytotoxic *Brucella* were examined in J774.A1 monolayers infected with 16MΔ*manBA* or 16MΔ*manBA*Δ*virB*2. The infected monolayers were fixed at 1 h p.i. and visualized via rough-specific *Brucella* IFA staining to evaluate bacterial uptake. The cell monolayers were fixed at 2, 3, and 4 days p.i. and stained to monitor plaque (cytotoxicity) and focus (replication) formation. Staining of the infected cells following 1 h incubation confirmed a low-level of bacterial uptake, per infected cell (**Figures 4A,E**) and no demonstrable bacterial aggregation. By day 2, individual cells containing replicating *Brucella* were detected in the monolayers (**Figures 4B,F**), indicating that the bacteria replicated within the macrophages. By day 3, groups of cells containing *Brucella* were observed in the infected monolayers (**Figures 4C,G**). By day 4, large plaques consisting of dead cells were observed in the 16MΔ*manBA* infected cells using light microscopy (as shown in **Figure 2A**), and the cells within the foci were full of *Brucella* as revealed by IFA (**Figure 4D**). Although no plaques were observed in 16MΔ*manBA*Δ*virB*2 infected monolayers at day 4 using light microscopy, foci containing large numbers of 16MΔ*manBA*Δ*virB*2 were observed following IFA staining (**Figure 4H**). However, the foci detected by IFA were much smaller than those in the monolayers infected with 16MΔ*manBA* (**Figure 4D**). These results suggest that individual plaques or foci are formed by replication of a single bacterium, and further confirmed that the cytotoxicity was important for re-infection via extracellular spread to neighboring cells via egress from the host macrophage.

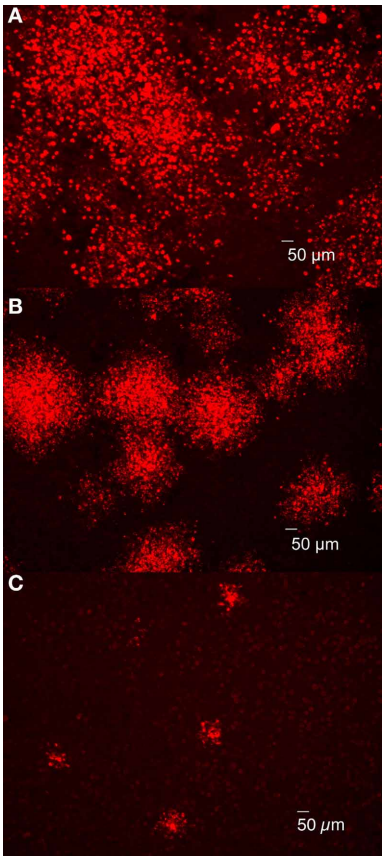

FIGURE 3 | Brucella cytotoxicity was essential for bacterial egress and subsequent re-infection. J774.A1 macrophages were infected with 16MΔ*manBA* **(A)**, RB51 **(B)** or 16MΔ*manBA*Δ*virB2* **(C)** at an MOI of 0.1. Agarose overlay and DMEM were added at 1 h p.i. and incubated for 4 days. Following formaldehyde fixation, the cells were stained via IFA to detect rough *Brucella* observed using IX70 microscopy. Scale bar, 50 μm.

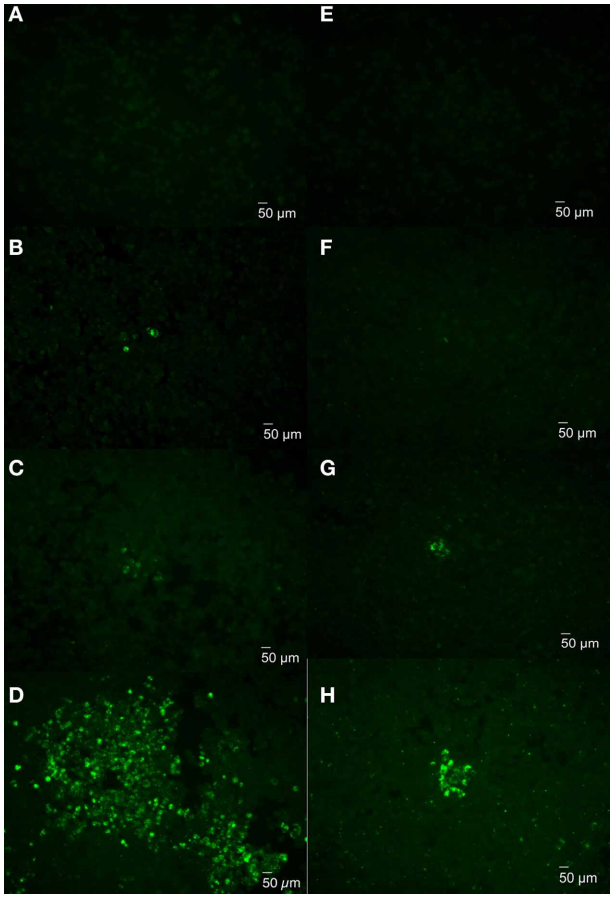

FIGURE 4 | Monitoring plaque formation during *Brucella* infection. J774.A1 macrophages were infected with 16MΔ*manBA* **(A–D)** or 16MΔ*manBA*Δ*virB2* **(E–H)** at an MOI of 0.1. Agarose overlay and DMEM were added at 1 h p.i. and incubated an additional 4 days. The cells were fixed at 1 h **(A,E)**, 2 days **(B,F)**, 3 days **(C,G)**, and 4 days **(D,H)** p.i., and stained to visualize intracellular *Brucella* via IFA. Scale bar, 50 μm.

BRUCELLA DISSOCIATION ASSISTS ORGANISM DISSEMINATION

Previous studies in our lab revealed that *Brucella* dissociation occurs *in vitro* and *in vivo* at elevated frequency and can result in an accumulation of rough variants in infected macrophages (Turse et al., 2011). However, previous studies have also revealed low levels of cytotoxicity associated with infection by wild type smooth *Brucella* at elevated MOI (Pei et al., 2008b). In order to determine whether ongoing dissociation, rather than low-level toxicity, is necessary for bacterial egress and dissemination, infection foci were evaluated for dissociation using a double antibody staining method described in the Materials and Methods. The results confirm the binding specificities of the rabbit anti-*Brucella* mono-specific M serum for wild type 16M (**Figures 5A,C**), and the goat anti-*B. ovis* serum for rough variants (**Figures 5B,D**). Antibody specificity and the capacity to distinguish smooth vs. rough *Brucella* during a mixed infection were confirmed using cells infected with a 10:1 mixture (smooth to rough) of *Brucella*, in which smooth *Brucella* were revealed in green (**Figure 5E**) and rough *Brucella* were revealed in red (**Figure 5F**).

To detect rough dissociation in infected cells, J774.A1 macrophage monolayers were infected with 16M and fixed at 1 h and 4 days p.i. Because no antibiotic was added during experimentation, overgrowth of 16M in the media prevented further analysis beyond 4 days p.i. Double antibody staining of the cells fixed at 1 h p.i. confirmed a low-level bacterial uptake without bacterial aggregation or detectable dissociation (data not shown). Staining of the cells fixed at 4 days p.i. revealed two types of foci: small foci consisting of a few cells with replicating *Brucella* (**Figure 6A**) and large foci in which the cells that had not sloughed-off the surface of the plate were retained (**Figures 6B,C**). Double antibody staining confirmed the presence of significant numbers of rough *Brucella* (**Figures 6D,F**) mixed with smooth *Brucella* (**Figures 6E,F**) in the large foci that were not detected in the small foci (data not shown). These results are consistent with the hypothesis that rough dissociation enhances *Brucella* dissemination.

To determine whether each *Brucella*-infected macrophage formed a plaque or focus of infection, PFUs in each well were

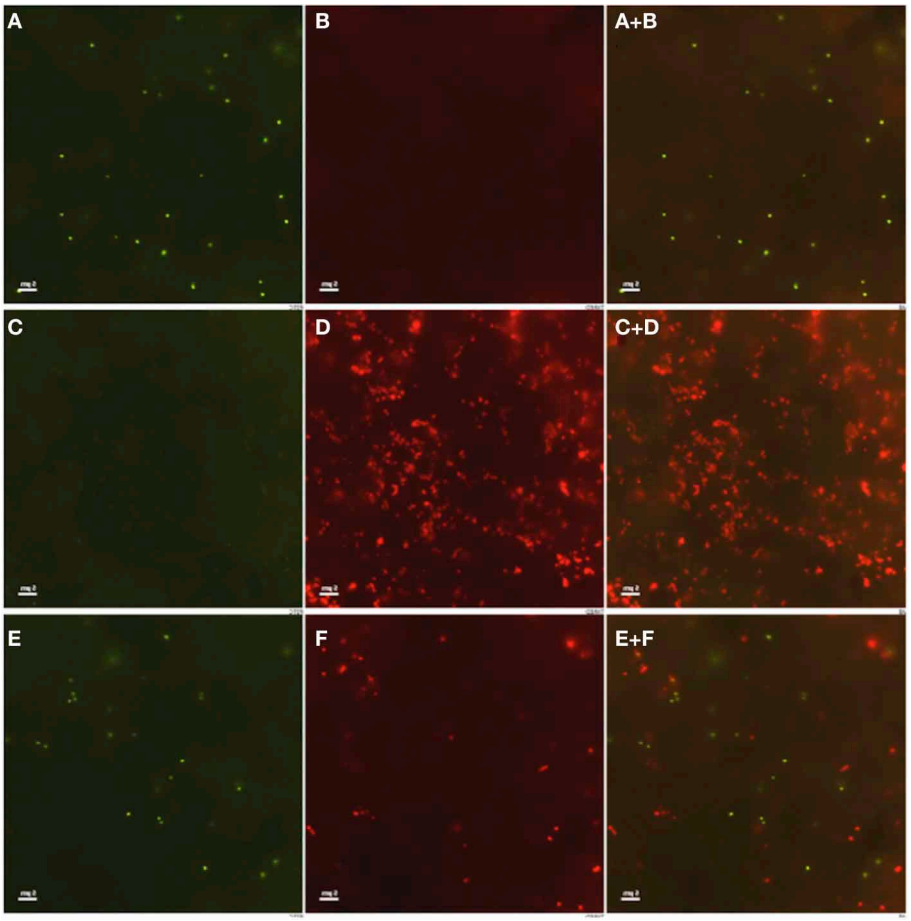

FIGURE 5 | Double antibody staining differentiates rough variants from smooth *Brucella*. J774.A1 macrophages were inoculated at an MOI of 50 with 16M **(A,B)**, 16MΔ*manBA* **(C,D)** or a 10:1 mixture of 16M and 16MΔ*manBA* **(E,F)**. The cells were fixed at 1 h p.i. and incubated with rabbit mono-specific anti-M serum (1:500 in PBS-TT) and goat anti-*B. ovis* serum (1:500 in PBS-TT). Intracellular smooth **(A,C,E)** and rough **(B,D,F)** *Brucella* were revealed following incubation with conjugated secondary antibodies chicken anti rabbit IgG Alexa Fluor 488 and donkey anti goat IgG Alexa Fluor 594 (1:1000 in PBS-TT). Panels **A**+**B, C**+**D, E**+**F** are derived by merging the appropriate channels. Scale bar, 5 μm.

evaluated via IFA staining at 4 days p.i. and compared with the uptake colony forming unit (CFUs) determined by gentamicin protection assay at 1 h p.i. The results indicated that 26.5% of the invading 16MΔ*manBA* formed plaques; only 4.6% of the invading 16M and 0.25% of the invading 16MΔ*manBA*Δ*viB2* formed foci.

INHIBITION OF *BRUCELLA* DISSEMINATION WITH THE ADDITION OF GENTAMICIN TO GROWTH MEDIUM

Bacterial spread from cell to cell can be accomplished by invasion of neighboring cells without release into the medium, such as *Listeria monocytogenes* (Mounier et al., 1990), or following release and re-infection of neighboring cells. To determine whether *Brucella* spread from cell to cell directly or via medium, experiments were performed in which gentamicin (50 μg/ml) was added to the overlay and DMEM so as to inactivate bacteria on the cell surface or released into the media and, as such, are not protected by intracellular uptake. Comparison of rough *B. melitensis* 16MΔ*manBA* and smooth 16M via IFA staining revealed small foci consisting of cells with replicating *Brucella* formed in monolayers infected with 16MΔ*manBA* (**Figure 7A**) and 16M (**Figure 7B**). But, neither plaques nor cell death were detected via phase contrast microscopy by 4 days p.i. (data not shown). In addition, the size of the foci was much smaller compared with those present in the infected monolayers without gentamicin treatment (**Figures 7C,D**). These results are consistent with the hypothesis that *Brucella* dissemination occurs via release into the medium with subsequent cell reinfection, and does not occur via cell-to-cell contact.

DISCUSSION

Pathogens have developed various strategies to evade host innate and adaptive immune systems (Finlay and McFadden, 2006), one of which is to manipulate host cell viability (Guiney, 2005). Similarly, both necrotic/apoptotic and anti-apoptotic cell death have been reported in macrophage during *Brucella* infection (Freeman et al., 1961; Gross et al., 2000; Fernandez-Prada et al., 2003; Pei and Ficht, 2004). Although several mechanisms have been thoroughly studied (Eskra et al., 2003; He et al., 2006; Pei et al., 2006, 2008b; De Jong et al., 2008; Chen and He, 2009; Zhong

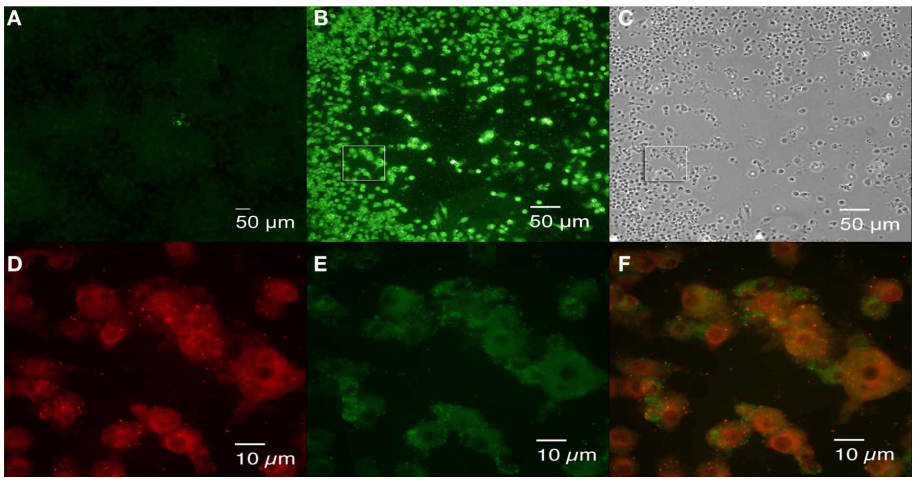

FIGURE 6 | Brucella dissociation detected in large foci following 16M infection. J774.A1 macrophages were infected with 16M at an MOI of 0.1. Agarose overlay and fresh DMEM were added at 1 h p.i. and incubation was continued for an additional 4 days. Following fixation in 3.7 % (w/v) formaldehyde, double antibody staining (rabbit mono-specific anti-M serum and goat anti *B. ovis* serum) was performed as described in the legend to **Figure 5**. Small **(A)** and large **(B)** foci were observed. Cell death was observed in the large foci (panel **C**, a bright field image of panel **B**). Rough variants (red, **D**) arising during growth of the parental organism (green, **E**) were detected in the large foci. Panel **(F)** is the merged image of panels **(D,E)**, an enlarged square shown in **(B)**. Scale bars, 50 μm in **(A–C)**; 10 μm in **(D–F)**.

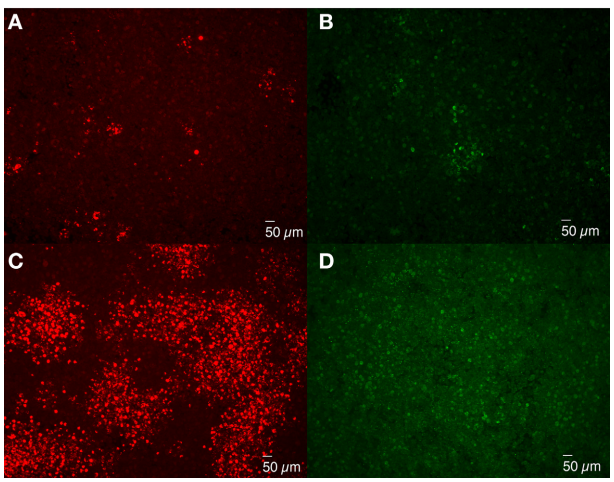

FIGURE 7 | Extracellular cell-to-cell dissemination of *Brucella*. J774.A1 macrophages were infected with 16MΔ*manBA* **(A,C)** or 16M **(B,D)** at an MOI of 0.1. An agarose overlay was added at 1 h p.i. along with fresh DMEM supplemented with **(A,B)** or without **(C,D)** 50 μg/ml of gentamicin. Following an additional 4 days of incubation, the cells were fixed and stained to visualize intracellular *Brucella* via IFA. Scale bar, 50 μm.

et al., 2009; Chen et al., 2011), their biological significance has not been established. The current study demonstrates that *Brucella* cytotoxicity enhances bacterial egress and dissemination.

An extensive amount of work has been performed regarding *Brucella* invasion and subversion of host cell functions thought to be critical to establishment of a hospitable replicative niche. However, another critical aspect of *Brucella* pathogenesis is organism egress from the host cell and continued dissemination. Seven potential exiting strategies or mechanisms used by different pathogens have been described in the literature to date (Hybiske and Stephens, 2008). *Legionella* generates pores in phagosomal or cellular membranes during different stages of infection inducing host cell cytotoxicity that is required for bacterial egress (Kirby et al., 1998; Alli et al., 2000; Gao and Kwaik, 2000a; Molmeret et al., 2002; Zink et al., 2002; Chen et al., 2004). *Salmonella* replication induces macrophage oncosis resulting in bacterial release from the cell (Sano et al., 2007). Similarly, our previous studies have revealed that rough *Brucella* infection induced pore formation on macrophage cell membranes to cause cell necrosis/oncosis (Pei et al., 2006). We provide support for this idea in the current study by demonstrating that *Brucella* cytotoxicity determines focus size, consistent with the idea that *Brucella* cytotoxicity is important for bacterial egress and dissemination.

Plaque formation was not apparent in the presence of gentamicin indicating that *Brucella* infection of neighboring cells occurs via extracellular dissemination of the organism. The reduced size of the foci formed under these conditions confirms the importance of extracellular spread of the organism to infection. Although the results obtained do not rule out the capacity of *Brucella* to disseminate using actin-based protrusion (used by *Listeria monocytogenes*, *Shigella flexneri, Rickettsia rickettsi, Rickettsia conorii, Burkholderia pseudomallei,* and *Mycobacterium marinum*), budding (by *Orientia tsutsugamushi*), or extrusion (*Chlamydia* spp.) (Hybiske and Stephens, 2008); the difference in the size of the foci observed suggests that *Brucella* dissemination is primarily the result of cell lysis and extracellular dissemination through the medium.

It has been proposed that two steps are required for *Legionella* release from infected cells. First, replicating *Legionella* form pores in the phagosomal membranes causing phagosome disruption. Second, bacteria released into the cytoplasm form pores in cell membrane resulting in cell lysis (Molmeret and

Abu Kwaik, 2002). Our previous study has shown that rough *Brucella* are retained within intact vacuoles identified within dead macrophages (Pei et al., 2006), suggesting that phagosomal membranes are not disrupted, and suggesting that only a single step is involved in the release of *Brucella* from macrophages. A complete or well-defined description of *Brucella*-containing vacuoles (BCV) is not available, but the result described suggests that a putative *Brucella* "cytotoxin" might only form pores on the cell membrane, not on the BCV membrane. Inhibition of plaque formation by gentamicin treatment suggested that these BCVs do not protect the organisms from gentamicin. Since rough *Brucella* are sensitive to complement- and cationic peptide-mediated lysis, organisms released in intact BCV derived from lysed macrophages may be protected by the vacuoles and phagocytosed by surviving macrophages to start a new round of infection. Therefore, the one-step release may facilitate *Brucella* dissemination.

At the MOI used in the plaque assays, i.e., $\leq$0.1, bacterial uptake by infected cells is not expected to exceed more than one bacterium per infected cell. Yet, massive bacterial replication can be detected as soon as 4 days p.i. (**Figures 2**, **4**). This is consistent with previous reports documenting rough *Brucella* mutant replication in HeLa cells and macrophages (Godfroid et al., 1998; Ugalde et al., 2000; Porte et al., 2003; Pei and Ficht, 2004; Pei et al., 2008b). These results demonstrate that *Brucella* cytotoxicity is not simply due to an uptake of excessively high numbers of *Brucella*, but derive from the replication of cytotoxic *Brucella* within the macrophages. The results also reveal that individual plaques are formed from cells infected with a single bacterium. The small foci associated with non-cytotoxic 16MΔ*manBA*Δ*virB*2 result from their poor release from infected cells, despite significant levels of intracellular replication. It is not clear why the foci in non-cytotoxic strain infected monolayers was lower than that in cytotoxic strain infected monolayers. However, one possibility is that the foci formed by the non-cytotoxic strain are so small that they are easily missed during enumeration.

The T4SS is the predominant virulence factor identified in *Brucella* to date. It is widely accepted that the T4SS is essential for *Brucella* survival within host cells, and is consistent with the attenuated survival of *Brucella* Δ*virB* mutants in the mouse model (Hong et al., 2000). Yet, despite the well-defined role of the T4SS in *Brucella* intracellular trafficking (Comerci et al., 2001; Delrue et al., 2001; Celli et al., 2003), recent reports show that *virB* mutants survive in the host as well as wild type organisms for the first 3 days *in vivo* (Roux et al., 2007). Since the T4SS is essential for *Brucella* cytotoxicity (De Jong et al., 2008; Pei et al., 2008b; Zhong et al., 2009) and subsequent bacterial dissemination, *in vivo* attenuation of *virB* mutants could be explained by a failure to disseminate within the host just as well as failing to obtain a replication niche. This hypothesis is supported by our recent studies showing that the 16MΔ*manBA*Δ*virB*2 mutant was cleared from infected mice within 1 week, while Δ*manBA* and Δ*virB*2 single mutants persist in mice beyond 4 weeks (Pei and Ficht, unpublished data).

To evade the immune system for survival in the host, many pathogenic Gram-negative bacteria have the ability to alter their LPS structure, including smooth-rough variation (Lukacova et al., 2008). The current results suggest that the spontaneous appearance of rough variants from smooth *Brucella* may function in the dissemination of infection. *Brucella* dissociation, shown to be enhanced in acidic environments (Braun, 1946a), may induce dissociation and subsequently assist *Brucella* dissemination from within acidic phagosomes (Porte et al., 1999; Boschiroli et al., 2002). It should be pointed out that dissociated *Brucella* can still revert to smooth phenotype (Braun, 1947). Therefore, it is possible that some smooth *Brucella* dissociated into a rough phenotype when needed to disseminate, and can still revert to smooth phenotype following egress to resist intracellular killing.

It has been demonstrated that *Brucella* dissociation is genetically based (Braun, 1946a; Mancilla et al., 2010, 2012, 2013; Turse et al., 2011). Each individual bacterium may have a different dissociation rate. In the current study, the dissociation rate was not determined, but a study conducted by Braun showed that the percentage of rough organisms in individual cultures was different (Braun, 1946a). This could be the reason why different size foci were observed following smooth *Brucella* infection. Mancilla et al. demonstrated that phage integrase-mediated excision of genomic island 2 (GI-2) and ISBm1-mediated excision of *wbk*A glycosyltransferase gene were partially responsible for *Brucella* rough dissociation (Mancilla et al., 2010). Although knockout of these genes could not eliminate the dissociation, it would be interesting

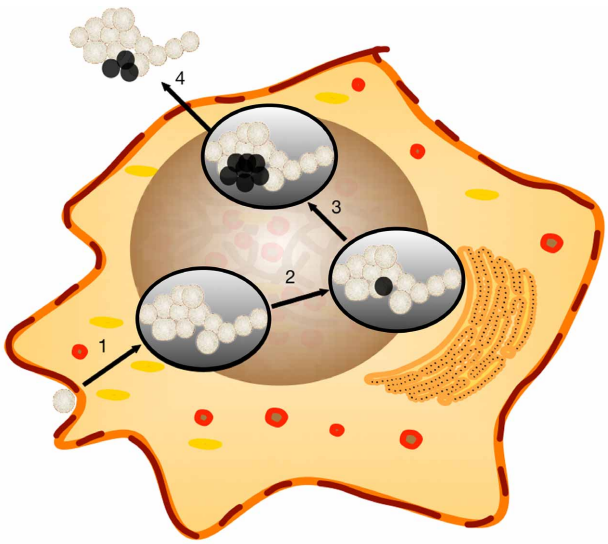

FIGURE 8 | Working model of *Brucella* host cell egress. *Brucella* dissociation has been previously reported to continue unabated within the host cell. The properties of rough derivatives would lend themselves to bacterial dissemination and the induction of an inflammatory response sufficient to attract new target cells. Step 1, Smooth *Brucella,* that are resistant to extracellular killing mechanisms, successfully invades target macrophages where replication occurs within a *Brucella*-containing vacuole (BCV). Step 2, dissociation occurs in association with replication within the BCV as observed *in vitro*. Step 3, cytotoxic activity, enhanced in rough mutants, begins to break down the the cellular membrane. Step 4, *Brucella* are released from the cell and successive rounds of replication and dissociation continue. Rough *Brucella* infection and macrophage necrotic cell death induce inflammatory responses, which recruit more macrophages to the infection sites.

to determine the disseminating abilities of these mutants in comparison with the wild type. Starr et al. reported recently that forming autophagic *Brucella*-containing vacuoles (aBCV) promoted *Brucella* egress in HeLa cells. However, the phenomenon was not tested in macrophages in this report (Starr et al., 2012). Since autophagy is involved in *Brucella* survival and replication in macrophages (Qin et al., 2008; Guo et al., 2012; Starr et al., 2012), its role in *Brucella* egress from macrophage needs to be investigated.

Brucella dissociation was observed more than 60 years ago (Stearns and Roepke, 1941; Braun, 1945, 1946a,b). However, the biological significance was not identified. Our current study revealed that *Brucella* dissociation enhanced bacterial dissemination, which may enhance *Brucella* virulence. A working model was proposed based on the results from this report and published studies (**Figure 8**). During smooth *Brucella* infection, the organism traffics to ER-like compartments and replicates (Celli et al., 2003). During replication, some of the organisms dissociate into a rough phenotype and accumulate in the host cells (Turse et al., 2011). The rough mutants with enhanced T4SS produce more cytotoxic factors (Pei et al., 2008b). Once rough mutant accumulation reaches a threshold level, the host cell will die from necrosis and apoptosis (Pei and Ficht, 2004; Pei et al., 2006; De Jong et al., 2008; Chen and He, 2009). The cell contents including the organisms will be released. Smooth *Brucella* will subsequently infect more macrophages and start a new round of replication and dissociation. Rough mutants may be killed by complement or other cationic peptide-mediated lysis (Allen et al., 1998). Rough *Brucella* induced cytokine and chemokine release (Rittig et al., 2003; Pei et al., 2008a) and macrophage necrotic cell death result in inflammatory responses, which in turn recruits more macrophages to the infection sites to help *Brucella* dissemination (**Figure 8**). This working model is strongly supported by the undulant fever presentation of human brucellosis.

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Cell death paradigms in the pathogenesis of *Mycobacterium tuberculosis* infection

Dinesh Kumar Parandhaman[1,2] and Sujatha Narayanan[1]*

[1] Department of Immunology, National Institute for Research in Tuberculosis, Chennai, India
[2] Department of Immunology, International Centre for Genetic Engineering and Biotechnology, New Delhi, India

Edited by:
Yongqun "Oliver" He, University of Michigan Medical School, USA

Reviewed by:
Dario S. Zamboni, Universidade de São Paulo, Brazil
Subramanian Dhandayuthapani, Texas Tech Health Sciences Center, USA

***Correspondence:**
Sujatha Narayanan, Department of Immunology, National Institute for Research in Tuberculosis (Formerly Tuberculosis Research Centre), Indian Council of Medical Research, Mayor Sathayamoorthy Road, Chetpet, Chennai 600031, Tamilnadu, India
e-mail: sujatha.sujatha36@gmail.com

Cell death or senescence is a fundamental event that helps maintain cellular homeostasis, shapes the growth of organism, and provides protective immunity against invading pathogens. Decreased or increased cell death is detrimental both in infectious and non-infectious diseases. Cell death is executed both by regulated enzymic reactions and non-enzymic sudden collapse. In this brief review we have tried to summarize various cell death modalities and their impact on the pathogenesis of *Mycobacterium tuberculosis*.

Keywords: apoptosis, pyroptosis, intrinsic pathway, extrinsic pathway, autophagy, *Mycobacterium tuberculosis*

INTRODUCTION

Cell death is a primordial event in embryogenesis, metamorphosis, and in innate immune response against the invading pathogens. Cell death as a defense mechanism is also documented in the plant kingdom (Kabbage et al., 2013). Cell death is executed in a series of ordered biochemical cascades and is referred as programmed cell death or PCD.

Till early 2000, cell death was discussed as dichotomy in terms of either apoptosis or necrosis. However, with the growth of science many distinct modes of cell death with well-organized signaling cascades were unraveled. Currently, there exists nine different forms of cell death namely apoptosis (Fink and Cookson, 2005), autophagy (Fink and Cookson, 2005), mitoptosis (Chaabane et al., 2012), necrosis (Fink and Cookson, 2005), necroptosis (Galluzzi and Kroemer, 2008), netosis (Remijsen et al., 2011), oncosis (Fink and Cookson, 2005), pyroptosis (Fink and Cookson, 2005), and pyronecrosis (Willingham et al., 2007). It is still a puzzle whether these pathways are different features of the same response or physiologically distinct responses. Apoptosis as an defense mechanism initiates both innate and adaptive immunity (Behar et al., 2010). However, pathogenic organisms have developed mechanisms to modulate apoptosis for their survival. Apoptosis of the infected cells have been reported to be a favorable outcome for the dissemination of infections like Yersinia, Francisella, etc. (Ruckdeschel et al., 1997; Wickstrum et al., 2009). On the contrary, impairment of apoptosis provides a survival niche to many intracellular pathogens including *Mycobacterium tuberculosis* (Behar et al., 2010), leads to auto immunity, cancer and degenerative disorders (Elmore, 2007). Studies in *M. tuberculosis* have identified a causal relationship between virulence of the strain and induction of apoptosis. Inhibition of apoptosis favors *M. tuberculosis* survival in many ways like preventing bactericidal effects, T-cell priming, etc. (Velmurugan et al., 2007). In contrast, a recent report states that apoptosis inducing strains could disseminate *M. tuberculosis* infection (Aguilo et al., 2013). Necrotic cell death of burdened *M. tuberculosis* infected cells was shown to pave way for re-infection (Butler et al., 2012). In here, we summarize various apoptotic modalities and their role in the pathogenesis of *M. tuberculosis*. Furthermore, we share our experience in analyzing these responses in *M. tuberculosis* infection.

MODELS OF CELL DEATH

APOPTOSIS

First represented in the article by Kerr, Wyllie, and Currie in 1972 (Elmore, 2007). Apoptosis is an energy dependent regulatory process that disintegrates the dying cell by enclosing the cytoplasmic contents inside membrane bound vesicles called apoptotic bodies. These apoptotic bodies are engulfed by the phagocytic cells by a process called efferocytosis thereby efficiently clearing the dying cell without any inflammatory responses (Lee et al., 2009). Three pathways namely extrinsic/ligand-mediated pathway, intrinsic/mitochondrial pathway, and the granzyme B-mediated pathway regulate the process of apoptosis upon activation by physiological or pathological conditions (Elmore, 2007). The major players in apoptosis are caspases, adaptor proteins, tumor necrosis factor (TNF) receptor (TNF-R) super family, and Bcl-2 family of proteins (Strasser et al., 2000). There are

three categories of caspases; initiators (caspase-2,-8,-9,-10), effectors or executioners (caspase-3,-6,-7), and inflammatory caspases (caspase-1,-4,-5) (Elmore, 2007). Caspase-activated DNases activate endonuclease that produce the typical internucleosomal DNA cleavage during apoptosis (Strasser et al., 2000). Adapter proteins play a major role in apoptosis as a link between caspases and the TNF-R by mediating homotypic interactions between the domains death domain, the death effector domain, and the caspase recruitment domains (Strasser et al., 2000).

Bcl-2 family of proteins are classified into three types that fall into pro-survival and pro-apoptotic categories based on the amino acid sequence homology to Bcl-2 homology regions BH1–BH4. Pro -survival Bcl-xL, Bcl-w, A1/Bfl-1, Mcl-1, and Boo/Diva have three or four bcl-2 homology regions while the pro-apoptotic members called Bax-like death factors Bax, Bcl-xS, Bak, and Bok/Mtd contain two or three homology regions (Pecina-Slaus, 2010). The third group of proteins Bad, Bik/Nbk, Bid, Hrk/DP5, Bim/Bod, and Blk, etc. that possess only a BH3 region are potent inducers of apoptosis (Strasser et al., 2000).

Apoptotic pathways

- **Extrinsic pathway** is initiated by binding of the ligands like TNF-α, FasL, CD95L, TRAIL, etc. to their respective receptors TNFR, Fas/CD95, and DR3 on the cell surface. This activates the initiator caspases such as caspases 8 and 10 that results in the formation and activation of death inducing signaling complex (DISC) that activates caspase 3 (Pecina-Slaus, 2010; Kalimuthu and Se-Kwon, 2013). Caspase 3 activation leads to cleavage of various death substrates that results in the characteristic hallmarks of apoptosis like DNA fragmentation, membrane blebbing, etc. (Kalimuthu and Se-Kwon, 2013).
- **Intrinsic pathway** of apoptosis is trigged due to the intracellular death signals. Mitochondrial enzyme endonuclease G, Bcl-2 family of proteins like Bax, Bid, and other mitochondrial proteins AIF, DIABLO [SMAC (second mitochondria-derived activator of caspases)], and cytochrome C plays a major role in this response (Kalimuthu and Se-Kwon, 2013). Upon the stimulus, the BH3-only protein Bid activates Bax and Bak that results in conformational change and oligomerization, forming an oligomeric pore in the outer mitochondrial membrane called permeability transition pores (Ferri and Kroemer, 2001; Kalimuthu and Se-Kwon, 2013). This results in the release of cytochrome C and other pro-apoptotic factors from the mitochondria into the cytosol. Cytochrome C interacts with Apaf and activates caspase-9 forming a multi-protein subunit complex called casposome (apoptosome) comprising cytochrome C, Apaf-1, procaspase-9, and ATP. In the absence of death stimulus, inhibitor of apoptosis family proteins (IAP) inactivates the caspase activity by direct binding. However, upon apoptotic stimuli IAPs are negatively regulated by SMAC and that leads to the activation of caspase-3 (Pecina-Slaus, 2010; Kalimuthu and Se-Kwon, 2013). Furthermore, extrinsic pathway was found to influence the intrinsic pathway of apoptosis by truncation of Bid (Cillessen et al., 2007).
- **Granzyme B**-mediated pathway utilizing the extrinsic mode of apoptosis is used by cytotoxic T lymphocytes as a mechanism to kill its target. Besides this, the secretion of pore forming granules containing serine proteases granzyme A and granzyme B also execute apoptosis that is both dependent and independent of caspase activation (Elmore, 2007).

AUTOPHAGY

It is a regulated homeostatic response conserved in all living cells degrading their own cytoplasm. Autophagy is a predominant cell survival response that is involved either in nutrient turnover or energy production during stress or removal of long lived cells or to protect against invading intracellular pathogens (Chaabane et al., 2012). Three forms of autophagy namely macroautophagy, microautophagy, and chaperone-mediated autophagy exist. During the autophagy, damaged organelle is lined with an isolation membrane called the phagophore that enlarges forming the double membrane structure called autophagosome. The autophagosome fuses either with late endosomes or lysosomes causing cell death (Levine and Deretic, 2007; Remijsen et al., 2011). Autophagy is regulated by autophagy-related proteins, serine/threonine kinase, mammalian target of rapamycin (mTOR), class I and class III phosphoinositide 3-kinases (PI3Ks) (Levine and Deretic, 2007; Su et al., 2013).

MITOPTOSIS

Apoptotic changes inside the mitochondria are called mitoptosis. Mitoptosis is still in infancy and no specific factors have been identified. The identification is based on morphological changes like disintegrating cristae, swollen mitochondria, etc. (Chaabane et al., 2012).

NECROSIS

Accidental cell death induced due to pathological or physiological conditions are called necrosis. During necrosis, swelling of organelles like endoplasmic reticulum, mitochondria occurs thereby rupturing the plasma membrane. This leaks the intracellular contents of the necrotic cell into the intercellular space causing inflammatory responses (Fink and Cookson, 2005; Chaabane et al., 2012).

NECROPTOSIS

In the year 2008, Hitomi et al. reported that necrosis could be a regulated process of cell death. The activation of serine/threonine kinase RIP1, BH3 only protein Bmf, and mitochondrial dysfunction executes necroptosis (Galluzzi and Kroemer, 2008).

NETosis

In 2004, the findings of Brinkman group unveiled another cell death program named by Steinberg in 2007 called NETosis (Mesa and Vasquez, 2013). One among the defense mechanisms used by neutrophils is the extrusion of intracellular material in the form of extracellular traps (ETs) to the surrounding extracellular medium. This concentrates the microbicidal substances to trap and kill pathogens (Mesa and Vasquez, 2013). Release of ETs by neutrophils is called NETs and mast cells as MCETs. NETs are composed of DNA and histones, and they are resistant to degradation by proteases, insensitive to caspase inhibition and necrostatins (cytoprotective agents) (Mesa and Vasquez, 2013). During NETosis both the nuclear and granular membranes disintegrate

leaving the plasma membrane intact (Remijsen et al., 2011). NETosis is activated by pathogens, platelets activated with LPS and in eosinophils (Remijsen et al., 2011). Formation of NET is both nuclear and mitochondrial in origin.

ONCOSIS

It is the swelling of cells that involves rapid plasma membrane breakdown, and swollen nuclei without internucleosomal DNA fragmentation. Oncosis depletes cellular energy and leads to failure of the ionic pumps in the plasma membrane. It is elicited by agents that disrupt the ATP production of the cell (Fink and Cookson, 2005).

PYROPTOSIS

Apoptosis in general does not induce an inflammatory response. However, apoptosis in Shigella, Salmonella, Francisella, and Legionella infections produce inflammatory responses that are called as pyroptosis (Carneiro et al., 2009; Lee et al., 2011). Pyroptosis is executed by the formation of inflammasomes by bacterial products involving NLRC 4 (Nod-like receptor—NLR), that activates caspase-1 and the processing of IL-1β and IL-18 cytokines promoting cell death (Fink and Cookson, 2005; Carneiro et al., 2009).

PYRONECROSIS

Cathepsin B-dependent apoptosis that is independent of caspase-1 activation and inflammasome formation is called pyronecrosis. This mode of apoptosis is observed in shigellosis (Willingham et al., 2007; Carneiro et al., 2009).

OTHER APOPTOTIC MODELS

- **Tumor suppressor protein 53** (TP53) induced apoptosis involves the transcriptional induction of redox proteins, generation of reactive oxygen species, and oxidative degradation of mitochondrial components that results in cell death. TP53 was shown to transcriptionally regulate proapoptotic proteins like Bax and NOXA (Yamada et al., 2002).
- **NF-kB** expression is implicated in the survival of living cells. NF-kB family contains five proteins namely c-Rel, RelA, RelB, p50/p105, and p52/p100. NF-kB as a homo or hetero dimers bind to the kB sites on their target DNA and regulate their expression (Barkett and Gilmore, 1999). NF-kB is activated by various stimuli like pathogens, mitogens, proinflammatory cytokines, etc. It plays a major role in immune responses and affects the expression of genes c-IAP-1 and c-IAP-2, Fas ligand, c-myc, p53, etc. involved in apoptosis (Zhang and Ghosh, 2001). Two TNF receptors TNFRSF8 and TNFRSF9 were shown to promote apoptosis, former activating, and latter inactivating NF-kB expression (Wang et al., 2008).

APOPTOSIS AND *MYCOBACTERIUM TUBERCULOSIS*

M. tuberculosis infections with virulent strains have been reported to inhibit macrophage apoptosis (Behar et al., 2010). Varied mechanisms of apoptotic suppression have been reported in *M. tuberculosis* infections (**Table 1**) unraveling the tactics of this pathogen to generate a protective niche inside the host. Among the various cell death modalities described above, only three apoptotic responses were documented in *M. tuberculosis* infection

Table 1 | Apoptotic mechanisms in the pathogenesis of *M. tuberculosis*.

S.no	Mechanisms of apoptosis	Year	References
1	Treatment of macrophages post-infection with exogenous ATP reduces viability	1994	Molloy et al., 1994
2	Extrinsic apoptosis	1997	Keane et al., 1997
3	Virulent strains induce IL-10-dependent sTNFR2 forming inactive TNF-α-TNFR2 complex	1998	Fratazzi et al., 1999
4	Granulysin and perforin reduce the viability of *M. tuberculosis*	1998	Stenger et al., 1998
5	Treatment of Fas ligand post-infection reduces the viability	1998	Oddo et al., 1998
6	Degree of apoptosis is strain-dependent	2000	Keane et al., 2000
7	ManLam prevents apoptosis by altering Ca^{2+} levels	2000	Rojas et al., 2000
8	*M. tuberculosis* apoptosis down regulates CD14	2000	Santucci et al., 2000
9	Apoptosis of avirulent strains dependent on group IV cytosolic phospholipase A_2 and TNF-α	2001	Duan et al., 2001
10	Reduced viability using exogenous ATP is executed using P2X7 receptor	2001	Fairbairn et al., 2001
11	Anti-apoptotic Mcl-1expression by virulent strains decreases apoptosis	2003	Sly et al., 2003
12	Detour pathway of antigen presentation	2003	Schaible et al., 2003
13	19 kDa lipoprotein induces apoptosis by TLR2 signaling	2003	Lopez et al., 2003
14	Virulent strains induce necrosis	2006	Park et al., 2006
15	Methyl glyoxal plays role in apoptosis	2006	Rachman et al., 2006
16	TLR-2-mediated activation of NF-kB and c-FLIP protects infected cells from FasL-induced apoptosis	2006	Loeuillet et al., 2006
17	PE_PGRS33 induces TNF-α secretion using TLR-2 signaling and genetic alterations in PE_PGRS33 decreases TNF-α secretion	2006	Basu et al., 2007
18	High MOI induces TNF-α independent apoptosis leading to mycobacterial spread	2007	Lee et al., 2006

(Continued)

Table 1 | Continued

S.no	Mechanisms of apoptosis	Year	References
19	Higher MOI leads to caspase independent apoptosis involving both mitochondria and lysosymes	2007	O'Sullivan et al., 2007
20	ESAT-6 induces apoptosis	2007	Derrick and Morris, 2007
21	Bystander apoptosis elicited by avirulent strains are independent of TNF-α,Fas,TRAIL, TGF-β, TLR2, and MyD88	2008	Kelly et al., 2008
22	Virulent strains prevents apoptotic envelope formation leading to necrosis	2008	Gan et al., 2008
23	Virulent strains produce more lipoxinA_4 promoting necrosis and avirulent strain induces PGE_2 that prevents necrosis	2008	Chen et al., 2008
24	Formation of NETs unable to kill *M. tuberculosis*	2008	Ramos-Kichik et al., 2009
25	Prevents pyroptosis using *zmp*1 by inhibiting inflammasome formation required for IL-1β secretion	2008	Master et al., 2008
26	pstS1 induces TNF-α, FasL,Fas TNFR1, TNFR2, and TLR-2 mediated apoptosis	2008	Sanchez et al., 2009
27	TNF-α-mediated caspase-8 apoptosis by p38MAPK, ASK-1, and $FLIP_S$ degradation	2009	Kundu et al., 2009
28	Virulent strains inhibit plasma membrane repair promoting necrosis	2009	Divangahi et al., 2009
29	Neutrophil activation leads to ectosome release	2010	Gonzalez-Cano et al., 2010
30	*nuoG* neutralize NOX2 derived ROS inhibiting extrinsic apoptosis	2010	Miller et al., 2010
31	Rv3654c and Rv3655c genes prevent extrinsic apoptosis	2010	Danelishvili et al., 2010
32	*eis* is involved in suppressing autophagy in a redox dependent JNK activation	2010	Shin et al., 2010
33	Higher MOI induces host cell lipolysis and PHOPR kinase plays a role in this response	2011	Divangahi et al., 2009
34	PE_PGRS33 interacts with host mitochondria and probably involved in primary necrosis	2011	Cadieux et al., 2011
35	Dendritic cells undergo caspase independent apoptosis	2011	Ryan et al., 2011
36	ROS mediated necrosis as a survival strategy in neutrophils	2012	Corleis et al., 2012
37	ESAT-6 induced apoptosis is regulated by BAT3	2012	Grover and Izzo, 2012
38	Rv3364c prevents pyroptosis by inhibiting cathepsinG	2012	Danelishvili et al., 2012
39	*pknE* inhibits various modes of apoptosis in response to nitric oxide stress of the macrophages	2012	Kumar and Narayanan, 2012
40	nuoG mutant reveals decreased neutrophil apoptosis reduces CD4 T cell activation	2012	Blomgran et al., 2012
41	Virulence determines cytotoxicity whereas strain characteristics determine the mode of cell death	2012	Butler et al., 2012
42	ESAT-6 is involved in inhibiting autophagy	2012	Romagnoli et al., 2012
43	sigH or its regulated genes suppresses apoptosis, modulates innate immune responses, and reduces chemotaxis	2012	Dutta et al., 2012
44	Infection with avirulent mycobacteria induces mitochondrial exhaustion while virulent promotes mitochondrial function thereby increasing ATP synthesis	2012	Jamwal et al., 2013
45	LpqH induces both extrinsic and intrinsic apoptosis	2012	Sanchez et al., 2012
46	Virulent Mycobacterial strains induce apoptosis by ESX-1 system and colonize new cells	2013	Aguilo et al., 2013
47	Validation of burst size hypothesis in *in vivo* model	2013	Repasy et al., 2013
48	*pknE* involved in the copathogenesis of HIV/TB coinfection	2014	Parandhaman et al., 2014

This table illustrates varied apoptotic mechanisms identified in the pathogenesis of M. tuberculosis. The abbreviations MOI denote multiplicity of infection, ManLam, mannosylated lipoarabinomannan; PGE_2, prostaglandinE2; ROS, reactive oxygen species; ATP, adenosine tri phosphate.

namely apoptosis (*nuoG*, *SecA2*, *pknE*, *lpqH*, *esxA* (ESAT-6), PE_PGRS33, *pstS-1*, Rv3654c, and Rv3655c), pyroptosis (*zmp1*, Rv3364c), and autophagy (*eis*) (Hinchey et al., 2007; Velmurugan et al., 2007; Jayakumar et al., 2008; Master et al., 2008; Sanchez et al., 2009, 2012; Danelishvili et al., 2010, 2012; Shin et al., 2010).

SERINE/THREONINE PROTEIN KINASES (STPK)

Two component signaling systems were considered as the standalone mechanism of signaling in prokaryotes in response to environmental cues. However with the availability of various molecular techniques serine, threonine, and tyrosine mediated phosphorylation events unique to eukaryotes were documented in pathogenic prokaryotes like *M. tuberculosis*, *Streptococcus* species, Staphylococcus spp, *Pseudomonas* spp, etc. (Chao et al., 2009; Chakraborti et al., 2011). Among the 11 STPKs that *M. tuberculosis* encodes, only five of them *pknE*, *pknG*, *pknH*, *pknI,* and *pknK* were reported to support intracellular survival (Walburger et al., 2004; Papavinasasundaram et al., 2005; Jayakumar et al., 2008; Gopalaswamy et al., 2009; Malhotra et al., 2010). Our data for the first time proved that PknE was the only STPK to inhibit apoptosis (Jayakumar et al., 2008).

PknE IN INNATE IMMUNITY

The function of *pknE* was established from our studies using the deletion mutant Δ*pknE* generated using specialized transduction. Deletion of *pknE* had reduced intracellular survival, increased apoptosis, and reduced proinflammatory responses (Jayakumar et al., 2008). Subsequent molecular pathogenesis studies revealed that the deletion of *pknE* promotes macrophage cell death dependent on intrinsic pathway of apoptosis, TP53, and *Arg2*. This apoptosis was independent of TNF-α, iNOS, Akt, *Arg1,* and pro-inflammatory cytokines (Kumar and Narayanan, 2012). *M. tuberculosis* encounters reactive nitrogen and oxygen intermediates inside the macrophages as one among the host defenses. Characterization of the promoter of the *pknE* gene showed its elevated expression during nitric oxide (NO) stress (Jayakumar et al., 2008). Macrophage experiments performed using NO donor sodium nitroprusside to mimic the host microbicidal activity confirmed that, *pknE* in response to NO stress suppresses innate immune responses (Kumar and Narayanan, 2012). *In vitro* studies carried with the deletion mutant showed defective growth in pH 7.0 and lysozyme (a cell wall-damaging agent) with better survival in pH 5.5, SDS (surfactant stress), and kanamycin (a second-line anti-tuberculosis drug). Δ*pknE* was reduced in cell size during growth in liquid media and exhibited hypervirulence in a guinea pig model of infection (Kumar et al., 2012). The data from the *in vitro* studies highlighted the role of *pknE* in adaptive responses of *M. tuberculosis*. Recently we reported that, deletion of *pknE* results in defective phosphorylation kinetics of MAPKs (p38MAPK, Erk½, and SAPK/JNK) and their transcription factors ATF-2 and c-JUN. Deletion of *pknE* also revealed crosstalks in the host macrophages where Erk½ signaling was found to be influenced by SAPK/JNK and p38 pathways independently. Modulations in intra cellular signaling altered the expression of coreceptors CCR5 and CXCR4 in macrophages infected with the deletion mutant of *pknE* that were authenticated using HIV tropic strains (Parandhaman et al., 2014). For the first time, our data showed that difference in apoptosis and intracellular signaling events, and the virulence capacity of the *M. tuberculosis* strain could influence the copathogenesis of HIV infection (Parandhaman et al., 2014). Collectively the reports show that *pknE* has a role suppression of innate immunity and help *M. tuberculosis* to adapt to the different environmental condition that it encounters.

CONCLUSION

Molecular techniques have revolutionized our understanding of pathogenic organisms and their interactions with the immune system. Pathogenic organisms have evolved host mimicking properties and utilize the host responses for their own survival and propagation. This review has addressed the various mechanisms of cell death that is vital for initiating an innate and adaptive immunity against the invading pathogen. As novel cell death paradigms evolve, it adds to the complexity of how temporally and spatially the immune system coordinates these responses. Most of the cell death models described here disrupt the energy source of the cell, mitochondria indicating whether these paradigms are interconnected response of a single biochemical event and this still remains a puzzle. Adding complexity to this conundrum is that, pathogenic organisms like *M. tuberculosis* is able to inhibit the various apoptotic models that were discovered so far. This arise the question whether *M. tuberculosis* by educating itself avoids cell death or has antigens that are poor inducers of cell death and that await further studies.

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