Francisella Inflammasomes: Integrated Responses to a Cytosolic Stealth Bacterium

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Abstract *Francisella tularensis* is a facultative intracellular bacterium causing tularemia, a zoonotic disease. *Francisella* replicates in the macrophage cytosol and eventually triggers cytosolic immune responses. In murine macrophages, *Francisella novicida* and *Francisella tularensis* live vaccine strain lyse in the host cytosol and activate the cytosolic DNA receptor Aim2. Here, we review the mechanisms leading or contributing to Aim2 inflammasome activation, including the role of TLRs and of IFN signaling and the implication of the guanylate-binding proteins 2 and 5 in triggering cytosolic bacteriolysis. Furthermore, we present how this cytosolic Gram-negative bacterium escapes recognition by caspase-11 but can trigger a non-canonical caspase-8 inflammasome. In addition, we highlight the differences in inflammasome activation in murine and human cells with pyrin, NLRP3, and AIM2 involved in sensing *Francisella* in human phagocytes. From a bacterial prospective, we describe the hiding strategy of *Francisella* to escape recognition by innate sensors and to resist to bacteriolysis in the host cytosol. Finally, we discuss the inability of the inflammasome sensors to detect *F. tularensis* subspecies *tularensis* strains, making them highly pathogenic stealth microbes.

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Contents

1 Introduction

1.1 The Francisella *Genus*

Francisella is the only genus within the *Francisellaceae* family (McLendon et al. 2006). These gamma-proteobacteria from the *Thiotrichales* order are small and pleiomorphic Gram-negative bacteria. The *Francisella* genus is divided into several species including *Francisella tularensis* and *Francisella novicida* (Table 1). *Francisella tularensis* is the most pathogenic species for humans. Yet, the virulence of *F. tularensis* strains is highly dependent on the subspecies they belong to. *F. tularensis* subspecies *tularensis* (also named type A strains) and its representative strain SCHU S4 are highly infectious and highly virulent with a lethal dose estimated to be lower than 10 bacteria for humans. *F. tularensis* subspecies *holarctica* (type B strains, hereinafter referred to as *F. holarctica*), although responsible for numerous infections of immunocompetent humans, is slightly less pathogenic. The live vaccine strain (LVS) is a strain derived from a virulent *F. holarctica* strain through sequential, in vitro passages. Although not licensed for vaccination, *F. holarctica* LVS is commonly used to study *Francisella* virulence factors and host immune responses. *F. novicida* is a species closely related to *F. tularensis* with greater than 97 % nucleotide identities between the two species (Larsson et al. 2009). *F. novicida* strains have been isolated from a few human

Table 1 Summary of the nomenclature, the virulence in mice and men, and the ability to activate the inflammasome complex of the most studied *Francisella* strains

Name	Representative strain	Alternative strain name	Virulence in mice	Virulence in humans	Inflammasome activation
F. novicida	U112	–	++	–	++
F. tularensis subsp. *holarctica*	LVS	Type B	+[a]	–[a]	+[a]
F. tularensis subsp. *tularensis*	SCHU S4	Type A	+++	+++	–

[a]Data are shown for *F. holarctica* LVS. Clinical strains from *F. holarctica* are virulent in mice and humans but, to our knowledge, have not been characterized in regard to inflammasome activation

patients, most of them with immune deficits or underlying medical conditions (Kingry and Petersen 2014). Due to its high virulence in mouse models of tularemia and an intracellular life cycle very similar to the one of virulent *F. tularensis* strains, *F. novicida* strain U112 is used to study tularemia.

In this review, we will present the modalities of the inflammasome activation upon *F. novicida* infections and we will discuss the similarities and differences observed between *F. novicida*, *F. holarctica* LVS, and the highly virulent *F. tularensis* type A strains.

1.2 Francisella tularensis*, the Agent of Tularemia*

Tularemia is a zoonotic disease with no transmission from human to human. Several forms of tularemia are observed depending on *F. tularensis* route of entry into the human host. Tularemia is associated with flu-like symptoms with high fever and lymphadenopathies. The most common form is the ulceroglandular tularemia following entry of the bacterium through the skin either through the bite of an infected arthropod or after direct contact with an infected animal (Sjostedt 2007). Ulceroglandular tularemia is characterized by a necrotic ulcer at the site of infection and a swollen draining lymph node. Consumption of contaminated food or freshwater leads to oropharyngeal tularemia (Gurcan 2014) with the rapid development of cervical lymphadenopathy. Finally, the most life-threatening disease is respiratory tularemia, which is triggered upon inhalation of *F. tularensis*. The severity of this infection coupled to high infectivity of *F. tularensis* has led the Center for Diseases Control to classify *F. tularensis* as a bioterrorism class A agent.

1.3 Intracellular Life Cycle

F. tularensis is a facultative intracellular pathogen found in vivo mostly in phagocytic cells including macrophages, neutrophils, dendritic cells, and monocytes (Hall et al. 2008). In contrast to other intracellular bacteria such as *Salmonella*

or *Shigella*, *F. tularensis* is unable to direct its entry into non-phagocytic cells. *F. tularensis* thus relies on host phagocytic receptors to invade host cells. *F. tularensis* uptake by macrophages proceeds through an atypical phagocytosis termed "looping phagocytosis." Bacterial opsonization and several host receptors are involved in facilitating *F. tularensis* entry (Clemens et al. 2005; Clemens and Horwitz 2007). The engagement of a specific entry receptor modulates the subsequent pro-inflammatory response including IL-1β release (Dai et al. 2013).

Following uptake, *F. tularensis* rapidly lyses the phagosome (Golovliov et al. 2003; Clemens et al. 2004; Checroun et al. 2006) to reach the host cytosol. Escape into the host cytosol is dependent on an atypical type VI secretion system (T6SS) encoded in the *Francisella Pathogenicity Island* (FPI) (Nano et al. 2004; Barker et al. 2009; Broms et al. 2010). While the T6SS is thought to secrete lytic effectors into the host cytosol, the mechanisms underlying vacuolar escape remain to be identified (Broms et al. 2010). Upon reaching the host cytosol, *F. tularensis* replicates rapidly thanks to a metabolic adaptation allowing bacterial growth in this cellular compartment (Barel and Charbit 2013). Due to the cytosolic localization of *Francisella* strains, these bacteria are used as a model to study the cytosolic innate immune responses including the inflammasome complex. The vacuolar mutants deleted of the *FPI* locus, of key *FPI* genes, or mutated in the master regulator gene *mglA* (Baron and Nano 1998) are often used in comparison with wild-type strains to discriminate the immune responses elicited in the vacuole/phagosome from the cytosol-specific responses.

2 Overview of the Innate Immune Responses to *Francisella* Infection

The inflammasome is highly connected with other innate immune pathways (Henry et al. 2007; Cole et al. 2008; Jones and Weiss 2011; Man et al. 2015; Meunier et al. 2015). We will thus briefly review the roles of the Toll-like receptors (TLRs) and of the pathways leading to type I IFN secretion during *Francisella* infection.

2.1 TLRs and NF-κB Activation

Bacteria from the *Francisellaceae* family are Gram-negative bacteria harboring lipopolysaccharide (LPS) on the external leaflet of their outer membrane. It was noted early on that, in contrast to the typical *Enterobacteriaceae* LPS, *Francisella* LPS does not have any endotoxin properties and is unable to induce IL-1 in human monocytes (Sandstrom et al. 1992). Indeed, *Francisella* LPS is not recognized by TLR4 (Hajjar et al. 2006). This lack of recognition is due to three peculiar features of *Francisella* lipid A. First, while most Gram-negative bacteria have hexa-acylated

LPS, *Francisella* lipid A is tetra-acylated. Second, the four fatty acyl chains are made of a chain of 16–18 carbons in length as opposed to 12–14 for *Escherichia coli* lipid A (Gunn and Ernst 2007; Kanistanon et al. 2008). Third, the carbon in position 4′ of the glucosamine is lacking the typical phosphate group observed in most Gram-negative lipid A (Wang et al. 2007).

TLR2 is thus the main pattern recognition receptor (PRR) responsible for transcription factor NF-κB activation during *Francisella* infection (Katz et al. 2006; Li et al. 2006). TLR2 activation occurs through the recognition of bacterial lipoproteins (Thakran et al. 2008) and is observed both at the plasma membrane and in the phagosome before *Francisella* escapes into the host cytosol (Cole et al. 2010).

2.2 Signaling Pathways Leading to Type I IFN Production

In addition to the pro-inflammatory response triggered at the plasma membrane or in the phagosome, escape of *Francisella* into the host cytosol triggers the "cytosolic innate immune responses." Henry et al. studied these responses by comparing the transcriptional responses of bone marrow-derived macrophages (BMDMs) infected with either the cytosolic wild-type *F. novicida* strain (U112) or a vacuolar mutant (ΔFPI). They demonstrated that macrophages detecting *Francisella* specifically in the cytosol secrete type I interferon (IFN-α and IFN-β) (Henry et al. 2007). Cole et al. confirmed this finding using *F. holarctica* LVS (Cole et al. 2008). In agreement with the cytosolic localization required to trigger IFN-β secretion, the type I IFN response upon *F. novicida* infection is independent of TLR2 and of the TLR adaptors MyD88 and TRIF (Henry et al. 2007; Jones and Weiss 2011). Type I IFN induction during *Francisella* infection is dependent on STING (Jones et al. 2010; Jin et al. 2011) and could be recapitulated by transfecting into the host cytosol of a proteinase K-resistant, DNase1-sensitive factor contained in a *Francisella* extract (Jones et al. 2010). The cytosolic DNA sensor cyclic GMP-AMP synthase (cGAS) is required for type I IFN production in response to *F. novicida* infection (Storek et al. 2015; Man et al. 2015). In addition to cGAS, Storek et al. demonstrated that in infected BMDMs, the DNA sensor Ifi204 (the murine homologue of human IFI16) is required to induce IFN-β production. The respective roles of the cGAS and Ifi204 remain unknown (Storek et al. 2015). Altogether, the results demonstrate that detection of *F. novicida* DNA in the host cytosol by the synergistic action of two DNA sensors triggers type I IFN secretion. Downstream of the DNA sensors, STING contributes to activate the transcription factors IRF1 and 3 (Henry et al. 2007; Jin et al. 2011; Man et al. 2015) to induce IFN-β and expression of numerous interferon-stimulated genes (ISGs) (Fig. 1).

The pathway leading to type I IFN secretion upon *F. holarctica* LVS infection might be slightly different. Indeed, type I IFN secretion is neither observed in *Sting*$^{-/-}$ BMDMs (Jin et al. 2011) nor in *Tlr2*$^{-/-}$ BMDMs (Cole et al. 2007). At the

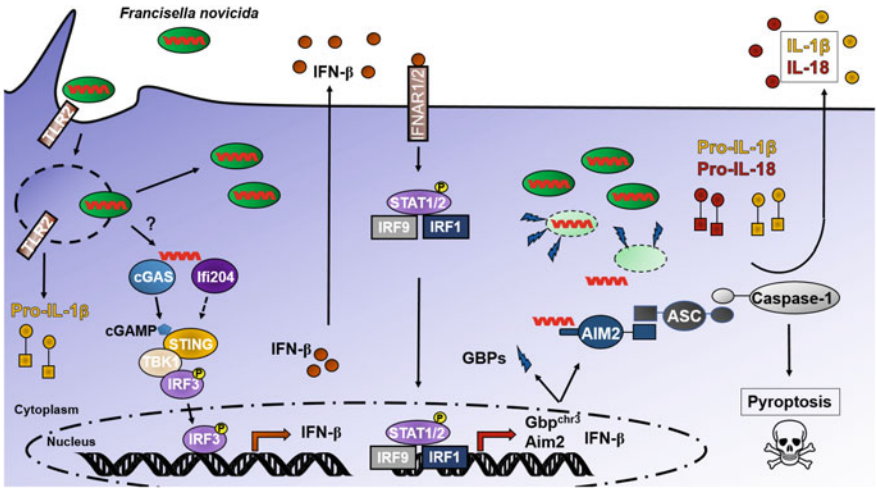

Fig. 1 Innate immune pathways leading to the Aim2 inflammasome activation during *F. novicida* infection of BMDMs (see text for details)

present time, it remains unclear whether TLR2 is directly involved in type I IFN response upon *F. holarctica* LVS infection, possibly through the MyD88-IRF1-IRF7 pathways elicited in the endosomal compartment (Dietrich et al. 2010) or whether TLR2 is required upstream of the release of LVS DNA into the host cytosol to elicit the cGAS/Ifi204-STING pathway.

3 *Francisella* Activates the Aim2 Inflammasome in Murine Phagocytes

Most of the published work on the modalities of inflammasome activation during *Francisella* infection has been performed in murine macrophages using *F. novicida* or *F. holarctica* LVS. First, we will describe activation of the inflammasome in mice and in murine phagocytes upon *F. novicida* and *F. holarctica* LVS infections (Fig. 1). In a second time, we will highlight the similarities and differences between human and murine cells and between *F. novicida*, *F. holarctica* LVS, and the highly pathogenic *F. tularensis* subsp. *tularensis* strains.

3.1 Aim2 Inflammasome and the IFN Requirement

3.1.1 In Vitro and In Vivo Role of the Inflammasome

The role of the inflammasome was demonstrated early on by Monack and colleagues (Mariathasan et al. 2005). In 2005, Mariathasan et al. described a cell death

response that was dependent on both ASC and caspase-1 as well as on the cytosolic localization of *F. novicida* in BMDMs. This cell death is associated with caspase-1 processing, IL-1β, and IL-18 release. As expected, TNF-α release (which levels are regulated by NF-κB) is independent of both ASC and caspase-1. The in vitro results were validated in a mouse model of tularemia. Mariathasan et al. demonstrated that ASC and caspase-1 are required for the survival of the mice to *F. novicida* infection while Nlrc4 (known at the time as IPAF) is not. Indeed, when 1.5×10^5 *F. novicida* colony-forming units (CFU) were injected subcutaneously, 100 % of *Asc*$^{-/-}$ and *Casp1*$^{-/-}$ mice died within 3–5 days. In contrast, with the same inoculum, 25 % of the wild-type mice survived the infection (Mariathasan et al. 2005). Cells expressing active caspase-1 are detected in the liver, in the spleen of LVS-infected mice (Wickstrum et al. 2009), and in the lung of *F. novicida*-infected mice (Mares et al. 2008). In agreement with the key role of the inflammasome pathway in these organs, *F. novicida* burden at 2 days post-inoculation is 100- to 1000-fold higher in the spleen, liver, and lung of inflammasome-deficient mice than in the corresponding organs of wild-type mice (Mariathasan et al. 2005).

Caspase-1-dependent cytokines orchestrate the innate immune response against *Francisella*. Indeed, IL-18 is key to induce IFN-γ production by NK cells in *F. novicida*-infected mice (Pierini et al. 2013; del Barrio et al. 2015). Interestingly, the survival of *Il-18*$^{-/-}$ mice to LVS infection can be rescued by IFN-γ administration. In contrast, *Il-1r1*$^{-/-}$ mice remained highly susceptible to LVS infection even upon IFN-γ administration (del Barrio et al. 2015). Re and colleagues identified that IL-1β is required early during infection to produce anti-LPS IgM (del Barrio et al. 2015). Anti LVS-IglM was observed in the serum of infected wild-type mice 7 days post-infection but were greatly reduced in *Asc*$^{-/-}$ and *Il-1b*$^{-/-}$ mice. B1a B cells are responsible for this specific IgM response, and their numbers were reduced in the spleen of infected *Il-1b*^{KO} mice. This IglM response was demonstrated to be protective likely through opsonization and increased phagocytosis (del Barrio et al. 2015). Finally, caspase-1 inhibition reduces IL-23p19 level (an IL-1β-regulated cytokine controlling IL-17 levels) in LVS-infected macrophages (Skyberg et al. 2013).

Using IL-1β- and IL-18-neutralizing antibodies injections in wild-type mice, Monack and colleagues demonstrated that mature IL-1β and IL-18 are required to fight *Francisella* infection. However, the susceptibility of caspase-1-deficient mice is due only in part to the lack of these two active cytokines. These results were confirmed by comparing mice doubly deficient for IL-1β and IL-18 (*Il-1β/Il-18*^{DKO}) to wild-type and *Casp1*$^{-/-}$ mice. *Il-1β/Il-18*^{DKO} mice display an intermediate phenotype in terms of bacterial burden in the spleen, liver, and lung between wild-type and *Casp1*$^{-/-}$ mice (Henry and Monack 2007). These results led the authors to conclude that the cell death defect in caspase-1-deficient mice was also participating in the strong susceptibility of caspase-1-deficient mice (Mariathasan et al. 2005; Henry and Monack 2007). Similarly to what have been reported for *Salmonella typhimurium*, *Legionella pneumophila,* and *Burkholderia thailandensis* (Miao et al. 2010), pyroptosis is thus a potent innate immune mechanism against

Francisella by removing its replicative niche and contributing to the clearance of this pathogen.

Of note, a Fas-mediated apoptotic pathway has been described for macrophages infected with *F. novicida* (Rajaram et al. 2009) but this pathway was characterized in RAW 264.7 cells, which are deficient for the inflammasome adaptor ASC (Pelegrin et al. 2008). While both apoptotic and pyroptotic pathways may occur during LVS or *F. tularensis* infections, in primary macrophages infected with *F. novicida*, the cell death is clearly inflammasome dependent.

3.1.2 Type I IFN Signaling Is Required for *Francisella*-mediated Inflammasome Activation

This inflammasome response in infected BMDMs was rapidly characterized as Nlrp3 independent (known at the time as cryopyrin) (Mariathasan et al. 2006). The first clue on the potential receptor-sensing *Francisella* in the cytosol came in 2007 with the demonstration that macrophages deficient in the type I IFN receptor (IFNAR1) were specifically deficient for inflammasome activation in response to *Francisella* infection (Henry et al. 2007). While the induction of pro-IL-1β protein is similar in wild-type and in *Ifnar1*$^{-/-}$ macrophages, secretion of mature IL-1β, IL-18, and caspase-1 processing and cell death are fully deficient in the latter cells. The same phenotype is also observed in *Irf3*$^{-/-}$ macrophages, which are deficient for IFN-β production during *F. novicida* infection. However, addition of rIFN-β to *Irf3*$^{-/-}$ macrophages restores inflammasome activation in these cells (Henry et al. 2007). These results were strengthened by the study of cGAS- and STING-deficient BMDMs, which are deficient both for type I IFN induction and inflammasome activation in response to *F. novicida* infection (Jones et al. 2010; Storek et al. 2015). Altogether, these studies indicate that type I IFN production and signaling are required to trigger inflammasome activation in response to *F. novicida* infection. In 2007, this finding led Henry et al. (2007) to suggest that the inflammasome receptor-sensing *Francisella* in the cytosol could be a member of the interferon-stimulated genes (ISGs) family, which includes proteins with pyrin domain.

3.1.3 Aim2 Is the Inflammasome Receptor Detecting *Francisella* in the Host Cytosol

Absent in melanoma 2 (AIM2) was identified in 2009 as an inflammasome receptor-sensing cytoplasmic DNA (Roberts et al. 2009; Burckstummer et al. 2009; Fernandes-Alnemri et al. 2009; Hornung et al. 2009). *Aim2* is an ISG coding a protein with a N-terminal pyrin domain and a C-terminal HIN domain. Through its HIN domain, AIM2, a cytosolic protein, binds double-stranded (ds)DNA. dsDNA is recognized by AIM2 in a sequence-independent manner as the HIN domain binds the dsDNA sugar-phosphate backbone (Jin et al. 2012). A DNA strand of at least

80 bp in length is required upon transfection to trigger IL-1β release. dsDNA in the cytosol may thus act as a scaffold to oligomerize AIM2. The resulting complex in turn recruits ASC oligomers through homotypic interactions of AIM2 and ASC pyrin domains leading to the formation of the active inflammasome complex (Jin et al. 2012).

The role of Aim2 as the *Francisella* inflammasome receptor was demonstrated independently in 2010 by three groups (the Fitzgerald, Alnemri, and Monack teams) who had generated *Aim2*$^{-/-}$ mice (Fernandes-Alnemri et al. 2010; Rathinam et al. 2010; Jones et al. 2010). Among several Aim2-activating pathogens, Fitzgerald and colleagues demonstrated that *Aim2*$^{-/-}$ macrophages were deficient for caspase-1 processing and IL-1β release in response to LVS infection (Rathinam et al. 2010). The Alnemri and Monack groups used *F. novicida* to demonstrate that, even at high multiplicity of infection, Aim2 was required for ASC oligomerization, caspase-1 processing, pro-IL-1β processing, IL-1β release, and macrophage death (Fernandes-Alnemri et al. 2010; Jones et al. 2010). The requirement for Aim2 is highly specific since neither *Nlrp3*$^{-/-}$ nor *Mefv*$^{-/-}$ BMDMs (the latter being deficient in the pyrin receptor) display any deficiency in response to *F. novicida* infection (Fernandes-Alnemri et al. 2010). In agreement, with the cytosolic localization of AIM2 and with previous studies (Mariathasan et al. 2005; Gavrilin et al. 2006), the vacuole-restricted mutant (Δ*FPI*) is unable to trigger Aim2 activation. The direct recognition of *Francisella* DNA by Aim2 was demonstrated by immunofluorescence. Macrophages were infected with *F. Novicida* pre-labeled with Hoechst, a DNA intercalating dye. Following infection, Aim2 specks or clusters were observed co-localizing with Hoechst-labeled material in close proximity to bacteria. In addition, Aim2 specks co-localize with ASC specks likely corresponding to the mature inflammasome complex. Importantly, Aim2 specks are also observed in ASC-deficient macrophages further demonstrating the role of Aim2 as a receptor upstream of ASC (Fernandes-Alnemri et al. 2010; Jones et al. 2010). Of note, Aim2 is also required in bone marrow-derived dendritic cells (BMDC) to trigger inflammasome activation in response to *F. novicida* infection (Belhocine and Monack 2012).

Aim2 is also critical for the immune response in vivo in a mouse model of tularemia. As previously reported for *Casp1*$^{-/-}$ and *Asc*$^{-/-}$, *Aim2*$^{-/-}$ mice are highly susceptible to *F. novicida* infection with 100 % of the mice dying in 5 days as opposed to 30 % of wild-type mice (Fernandes-Alnemri et al. 2010). The fast death is associated with a 100- to 1000-fold increase in the bacterial burden in the spleen, the lung, and the liver and a much lower concentration of IL-18 in the serum of *Aim2*$^{-/-}$ mice compared to wild-type mice (Fernandes-Alnemri et al. 2010; Jones et al. 2010). The Aim2 inflammasome is thus a key receptor for the innate immune defense against *Francisella.*

The discovery of Aim2 as the receptor-sensing *Francisella* in the host cytosol gave an opportunity to question the role of type I IFN signaling in Aim2 induction and Aim2 inflammasome activation. While Aim2 expression was strongly induced upon *Francisella* infection in a STING- and type I IFN receptor-dependent manner in experiments performed by Jones and colleagues, Aim2 was not induced during

similar experiments performed by Fernandes-Alnemri et al. Interestingly, while several groups confirmed a role for the type I IFN pathway [IRF3/IRF7 (Rathinam et al. 2010); IRF3, IFNAR1 (Fernandes-Alnemri et al. 2010); STING, IFNAR1 (Jones et al. 2010)] in potentiating the Aim2 inflammasome, the type I IFN pathway is not required for Aim2 activation following transfection with synthetic DNA (p(dA:dT)) or following transfection with *F. novicida* genomic DNA (Fernandes-Alnemri et al. 2010; Rathinam et al. 2010; Meunier et al. 2015). This result indicates that the basal level of Aim2 is sufficient to detect cytosolic DNA and suggests that type I IFN and ISGs are required specifically during *Francisella* infection upstream of the Aim2 inflammasome.

3.2 *Gbp-mediated Bacteriolysis Is Required to Trigger Aim2 Inflammasome Activation*

One of the key questions regarding the activation of the cGAS/Ifi204 DNA sensor or of the Aim2 inflammasome during *F. novicida* infection is the source of cytosolic DNA. Indeed, bacterial DNA is normally enclosed within the bacterium and not accessible to cytosolic DNA sensors. During immunostaining experiments, Jones and colleagues had observed that Aim2 specks were localized in proximity to irregular-shaped bacterial remnants (Jones et al. 2010) suggesting that bacteriolysis may be a prerequisite for the release of genomic bacterial DNA into the host cytosol. This observation coupled to the lack of requirement for type I IFN signaling to activate the Aim2 inflammasome in response to synthetic DNA led Meunier et al. (2015) to hypothesize that type I IFN signaling may be involved in the bacteriolysis mechanism. As type I IFN signaling induces hundreds of ISGs in *F. novicida*-infected macrophages (Henry et al. 2007), they performed a RNA interference screening on 483 ISGs in *F. novicida*-infected BMDMs. They identified that the knockdown of the genes encoding two guanylate-binding proteins (Gbps): Gbp2 and Gbp5 strongly reduced Aim2 inflammasome activation upon *F. novicida* infection (Meunier et al. 2015). At the meantime, Kanneganti and colleagues demonstrated that in addition to IFNAR1, IFNAR2, Stat1, and IRF9, the IFN-inducible transcription factor IRF1 was required for *F. novicida*-mediated Aim2 inflammasome activation (Man et al. 2015). IRF1 regulates IFN-β induction during *F. novicida* infection. However, Man et al. demonstrated that the addition of IFN-β in *Irf1*$^{-/-}$ BMDM does not rescue Aim2 inflammasome activation during *F. novicida* infection. This result suggested that IRF1 action is required downstream of the IFNAR receptor to induce one of several specific ISGs. Using a gene expression profiling approach, Man and colleagues identified Gbps as a family of protein strongly up-regulated by IRF1 and IFNAR1 in *F. novicida*-infected macrophages. A subsequent siRNA screen on the Gbp family confirmed Gbp2 and Gbp5 as two specific Gbps required for Aim2 inflammasome activation in response to *F. novicida* infection (Man et al. 2015).

Gbps are interferon-inducible, dynamin-like GTPases involved in cell-autonomous immunity. They may act as scaffolding proteins to target antimicrobial proteins such as the NADPH oxidase, autophagic proteins, and the IFN-γ-inducible GTPase p47 to membrane-bound intracellular pathogens (Meunier and Broz 2016). In addition, Gbp5 directly binds NLRP3 and may promote NLRP3 inflammasome assembly in response to pathogenic bacteria and to ATP and Nigericin (Shenoy et al. 2012) although the latter observation is controversial (Meunier et al. 2014). In addition, Gbps contribute to caspase-11 activation following infection with *Salmonella typhimurium* (Meunier et al. 2014) and *Legionella pneumophila* (Pilla et al. 2014). The mechanisms controlling the enhanced activation of this non-canonical inflammasome are still unclear with three mechanisms proposed. (1) Gbps could favor the disruption of pathogen-containing vacuole, thus enhancing the release of the bacteria into the host cytosol (Meunier et al. 2014). (2) Gbps could act downstream of the release of the bacteria into the host cytosol (Pilla et al. 2014) by targeting cytosolic bacteria and triggering the shedding of LPS into the cytosol. (3) Gbps could directly act onto the non-canonical caspase-11 complex to promote its multimerization (Finethy et al. 2015). Eleven *Gbp* genes (and two *Gbp* pseudogenes) are present in two clusters on mouse chromosomes 3 and 5. Several of the chromosome 3-encoded Gbps control bacterial (Kim et al. 2011) and parasitic replication (Yamamoto et al. 2012; Degrandi et al. 2013). Using BMDMs from *Gbp5*$^{-/-}$ *and Gbp2*$^{-/-}$ mice and from mice deficient for the cluster of *Gbps* (*Gbp1, Gbp2, Gbp3, Gbp5, and Gbp7*) present on the chromosome 3 (*Gbp*^{chr3}), Meunier et al. and Man et al. confirmed that Gbps and particularly Gbp2 and Gbp5 are required for Aim2 inflammasome activation during *F. novicida* infection. Indeed, *Gbp*^{chr3} macrophages secrete lower levels of IL-1β and IL-18 than wild-type macrophages during the infection. Similarly, *Gbp*^{chr3} macrophages are largely resistant to *Francisella*-induced pyroptosis. Since Gbps are known to target pathogen-containing phagosomes/vacuoles and destabilize the integrity of these compartments (Yamamoto et al. 2012; Meunier et al. 2014), Meunier et al. and Man et al. hypothesized that Gbps might attack *Francisella*-containing phagosomes leading to the release of bacteria into the host cytosol. Yet, there was no difference in the *F. novicida* escape rate between wild-type and *Gbp*^{chr3}-deficient macrophages. This result indicates that Gbps act after *Francisella* escape into the host cytosol strengthening a role of Gbps in triggering cytosolic bacteriolysis.

By confocal microscopy, both Gbp2 and Gbp5 were observed surrounding the bacteria. Interestingly, using GFP-expressing bacteria, Man et al. noticed that bacteria decorated with Gbp5 tend to lose their GFP expression suggesting a loss of viability. Accordingly, a greater number of cytosolic bacteria are permeable to propidium iodide in wild-type macrophages compared to *Gbp*^{chr3}-deficient macrophages indicating a Gbps-dependent loss of bacterial membrane integrity (Meunier et al. 2015). In addition, *Francisella* replication is impaired in wild-type macrophages compared to Gbp^{chr3}-deficient and *Irf1*$^{-/-}$ BMDMs as shown by CFU assay and by quantification of bacterial loads in single cells using confocal microscopy or high-resolution microscopy in flow. How Gbps trigger bacteriolysis following recruitment onto/on close proximity to cytosolic *Francisella* is still unclear. Bosio and colleagues have shown that the anti-oxidant N-acetyl cysteine

and the mitochondrial-specific reactive oxygen species (ROS) scavenger, mitoTEMPO inhibit *F. novicida*-mediated inflammasome activation (Crane et al. 2014). ROS can target bacterial lipids and outer membrane proteins (Fang 2004). Mitochondrial ROS may thus participate in the killing of *F. novicida* inside the host cytosol leading to the release of DNA and Aim2 activation. Yet, the precise role of mitochondrial ROS and their connection with Gbps remain to be deciphered.

The roles of IRF1 and Gbps in inflammasome activation were validated in vivo in a mouse model of tularemia (Man et al. 2015; Meunier et al. 2015). Indeed, after *F. novicida* intradermal inoculation, both $Irf1^{-/-}$ and Gbp^{chr3}-deficient mice showed reduced IL-18 concentration in the serum. Furthermore, $Irf1^{-/-}$, $Gbp2^{-/-}$, and Gbp^{chr3}-deficient mice had a higher bacterial burden in the liver and the spleen than wild-type mice. Finally, in a survival experiment, all $Irf1^{-/-}$, $Gbp2^{-/-}$, and Gbp^{chr3}-deficient mice died within 4 days of infection, while most wild-type mice survived until the end of the experiment (day 8–10).

Importantly, while Gbps expression is induced by type I IFN, Gbps are also strongly induced by IFN-γ in a manner requiring the transcription factors Stat1 and IRF1 (Briken et al. 1995). In vitro, BMDMs do not produce IFN-γ and the IFN signaling is only due to autocrine and paracrine type I IFN signaling following engagement of the cytosolic DNA sensors. However, in vivo, IFN-γ produced early on by NK cells, T cells, and several different phagocyte populations (Bokhari et al. 2008; De Pascalis et al. 2008; Pierini et al. 2013) is likely to control Gbps induction. Indeed, while IFN-γ signaling is critical in vivo to fight *Francisella* infection (Anthony et al. 1989; Elkins et al. 1996), type I IFN signaling is deleterious in vivo during infections with several intracellular bacteria including *Francisella* (Auerbuch et al. 2004; Stanley et al. 2007; Henry et al. 2010).

3.3 *TLR2 Controls pro-IL-1β Level and the Kinetics of AIM2 Inflammasome Activation*

As previously mentioned, TLR2 is the main TLR responsible for NF-κB activation during *Francisella* infection (Katz et al. 2006). As such, induction of pro-IL-1β is fully abolished in *F. holarctica* LVS-infected $Tlr2^{-/-}$ BMDMs and BMDCs (Li et al. 2006; Cole et al. 2007; Dotson et al. 2013). Upon *F. holarctica* LVS infection, IFN-β induction is also fully dependent on TLR2 (Cole et al. 2007). During *F. novicida* infection, TLR2 controls pro-IL-1β level but does not play a significant role in IFN-β induction (Jones and Weiss 2011). In addition, during *F. novicida* infection, TLR2 signaling modulates inflammasome activation kinetics. Indeed, wild-type macrophages display more ASC specks, secrete more mature caspase-1 and IL-18, and die faster than $Tlr2^{-/-}$ macrophages (Jones and Weiss 2011). Importantly, the requirement for TLR2 signaling is also observed in vivo since (i) immunostaining with an antibody against active caspase-1 p20 subunit reveals more caspase-1 specks in the spleen of infected wild-type mice than in the spleen of

Tlr2$^{-/-}$ mice; (ii) TLR2-deficient mice present less IL-18 in their serum than wild-type mice do (Jones and Weiss 2011). While differences exist between LVS- and *F. novicida*-infected macrophages, these studies clearly demonstrate that TLR2 is required for full inflammasome activation during *Francisella* infection. Besides the role of TLR2 on IFN-β induction observed during LVS infection, the contributions of TLR2 to the induction of mitochondrial ROS (West et al. 2011), to Gbps-induced bacteriolysis, or to a possible priming of the Aim2 inflammasome remain to be evaluated.

4 *Francisella* and the Non-canonical Inflammasomes

4.1 Francisella *LPS Escapes Caspase-11 Recognition*

The presence of LPS inside the cytosol is detected by caspase-11 (Kayagaki et al. 2013; Hagar et al. 2013; Shi et al. 2014) leading to activation of this inflammatory caspase in a complex termed a non-canonical inflammasome (Kayagaki et al. 2013). The caspase-11 inflammasome is thus a molecular sentinel monitoring the cytosol for the presence of Gram-negative bacteria (Aachoui et al. 2013). As a Gram-negative bacterium replicating in the cytosol, *Francisella* was thus a likely pathogen to activate caspase-11. Although the expression of caspase-11, an IFN-inducible gene, is induced upon *F. novicida* infection (Akhter et al. 2012), *F. novicida* does not activate caspase-11 (Hagar et al. 2013). Miao and colleagues identified that *F. novicida*-mediated evasion from caspase-11 recognition is associated with its specific lipid A structure (Hagar et al. 2013). Indeed, as previously described, *Francisella* LPS is tetra-acylated while LPS from most Gram-negative bacteria are hexa-acylated (Okan and Kasper 2013). LpxF is a periplasmic phosphatase, which specifically removes the 4′ monophosphate group in the lipid A. Raetz and collaborators have observed that the presence of the 4′ monophosphate group in the lipid A of the *lpxF* mutant impairs the cleavage of the 3′ acyl chain. The lack of the LpxF enzyme thus results in the generation of a penta-acylated lipid A (Wang et al. 2007). Strikingly, while purified tetra-acylated LPS from *F. novicida* does not activate caspase-11, purified penta-acylated LPS from *lpxF* mutant does so (Hagar et al. 2013). This result highlights LpxF as a key enzyme to escape caspase-11 recognition. However, the *lpxF* mutant is also severely impaired for growth in axenic medium and for resistance to antimicrobial peptides indicating that this enzyme is also required for membrane integrity and the general fitness of the bacterium (Wang et al. 2007). Of note, LPS from the *lpxF* mutant does not activate TLR4 (Wang et al. 2007) while it does activate caspase-11 in the cytosol indicating that TLR4 and caspase-11 pathways have different structural requirements (Hagar et al. 2013).

4.2 ASC-dependent Caspase-1-independent Pathways

While the canonical inflammasome is defined by caspase-1 activation, Mariathasan et al. (2005) observed early on that, upon *F. novicida* infection, the kinetics of cell death differed between ASC- and caspase-1-deficient macrophages. This observation suggested the presence of an ASC-dependent caspase-1-independent cell death pathway. This non-canonical pathway was characterized by Pierini et al. (2012). They identified that while wild-type macrophages die rapidly by pyroptosis, caspase-1-deficient macrophages die 4–6 h later with features typical of apoptosis such as condensed nuclei and a ladder-like DNA pattern indicative of internucleosomal fragmentation. Importantly, neither Aim2-deficient nor ASC-deficient macrophages display any apoptotic nor necrotic features at the same time point. While in wild-type macrophages, the Aim2/ASC speck co-localizes with active caspase-1 (Jones et al. 2010), in caspase-1-deficient macrophages, the Aim2/ASC complex recruits and activates caspase-8 (Pierini et al. 2012). The Aim2/ASC-dependent, caspase-1-independent apoptosis is inhibited by caspase-8 or caspase-9 inhibitors or by ectopic expression of Bcl-2 or Bcl-X_L, two anti-apoptotic proteins blocking the mitochondrial intrinsic pathway. Altogether, these data led to the conclusion that upon *F. novicida* infection, in the absence of caspase-1, Aim2/ASC complex recruits and activates caspase-8, which in turn activates the mitochondrial intrinsic pathway and caspase-9. The combined action of both caspase-8 and caspase-9 then triggers activation of the executioner caspase-3, leading to apoptosis of the infected macrophages (Pierini et al. 2012). Importantly, the ability of ASC platforms to activate caspase-8 is not restricted to *Francisella* infection or to Aim2 activation since Nlrp3, Aim2 and Nlrc4 complexes can recruit and activate caspase-8 to trigger apoptosis in response to a variety of stimuli (Sagulenko et al. 2013; Man et al. 2014). In addition to promoting apoptotic cell death, the ASC-dependent caspase-1-independent pathway also regulates the IL-18/IFN-γ cascade in a mouse model of tularemia (Pierini et al. 2013). While the level of IL-18 and IFN-γ in the serum of *F. novicida*-infected mice are much lower in caspase-1-deficient mice than in wild-type mice, the levels of the two cytokines are much higher in *Casp1*$^{-/-}$ mice than in *Asc*$^{-/-}$ mice. Neutralization of IL-18 in infected *Casp1*$^{-/-}$ mice reduces IFN-γ levels in the serum demonstrating that bioactive IL-18 can be generated in a caspase-1-independent manner. In vitro, caspase-8, expressed in the presence of Aim2, ASC, and pro-IL-18, triggers specifically the release of IL-18. Altogether, these results demonstrate that, as presented above for the apoptotic cell death, in vivo, in the absence of caspase-1, caspase-8 can be activated in the Aim2/ASC complex leading to pro-IL-18 processing, release of bioactive IL-18, and activation of NK cells to trigger IFN-γ production (Pierini et al. 2013). In a caspase-1-proficient context, the relevance of this caspase-8 pathway remains to be understood. Indeed, caspase-8 is inducing an apoptotic cell death outcompeted by the rapid pyroptosis and caspase-8 is triggering the IL-18/IFN-γ cascade in a manner less efficient than the canonical caspase-1-inflammasome. Such a fail-safe mechanism could be important in humans bearing

defective caspase-1 variants (Luksch et al. 2012) or during infections with pathogens blocking specifically caspase-1 activity.

5 Inflammasome Activation in Human Cells

BMDMs have been the main cellular model to study inflammasome activation upon *Francisella* infection. Yet, there is clear evidence that the inflammasome complexes assembled in human phagocytes contain receptors that differ from the ones sensing *Francisella* in murine macrophages (Gavrilin et al. 2009; Atianand et al. 2011).

Inflammasome activation in human cells was first studied in monocytes (Fig. 2). Gavrilin et al. demonstrated that the cytosolic localization of *F. novicida* is required in human monocytes to trigger IL-1β release (Gavrilin et al. 2006). Indeed, various compounds blocking *F. novicida* escape into the cytosol abolish IL-1β secretion while pro-IL-1β level in the cell lysates is unaffected. Similarly, a *mglA* mutant, which remains in the vacuole, is unable to trigger IL-1β release in human monocytes (Gavrilin et al. 2006). Finally, while heat-killed *Francisella* readily induces pro-IL-1β expression, live bacteria are required to activate caspase-1 and IL-1β release in primary human dendritic cells (Li et al. 2006).

Inflammasome activation by *Francisella* in human cells (Fig. 2) is associated with three intracellular protein sensors, AIM2, NLRP3, and pyrin (encoded by the *Mefv* gene) (Gavrilin et al. 2009; Atianand et al. 2011). As described above, several groups have observed that murine *Nlrp3*$^{-/-}$ and *Mefv*$^{-/-}$ BMDMs have a normal inflammasome response upon *F. novicida* infection indicating the key requirement for Aim2 in this experimental system (Mariathasan et al. 2006; Fernandes-Alnemri et al. 2010; Ulland et al. 2010; Atianand et al. 2011). Surprisingly, human macrophages rely on both NLRP3 and AIM2 to activate the inflammasome upon

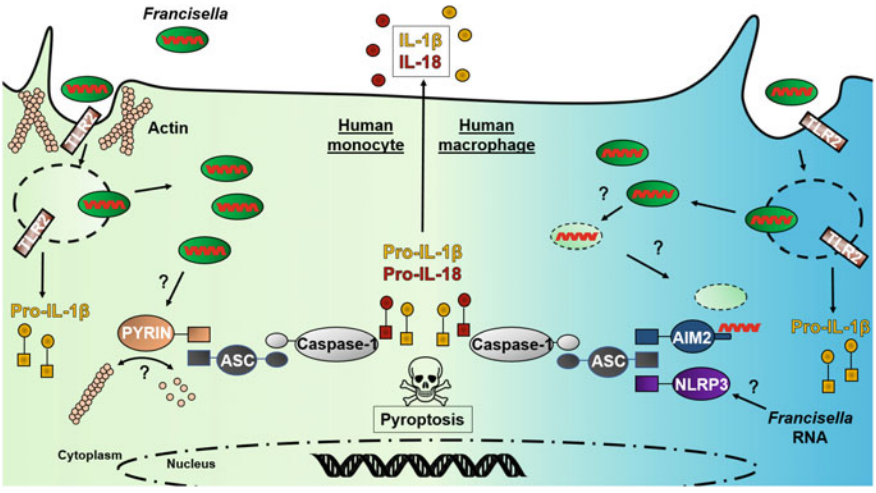

Fig. 2 Models for the different inflammasomes activated upon *Francisella* infection of human monocytes (*left panel*) and human macrophages (*right panel*) (see text for details)

F. novicida infection (Atianand et al. 2011). Indeed, knockdown of either *Nlrp3* or *Aim2* reduced IL-1β release in PMA-differentiated THP-1 macrophages. A slightly higher IL-1β inhibition was observed upon combined knockdown of both *Nlrp3* and *Aim2* suggesting that the two PRRs are involved in sensing *F. novicida* in the human macrophage cytosol (Atianand et al. 2011). The role of NLRP3 was further confirmed in an inflammasome reconstitution system in 293T cells. In this system, NLRP3 but not NLRP12 co-localizes with ASC specks upon *F. novicida* infection and expression of NLRP3 strongly increases IL-1β release upon LVS or *F. novicida* infections. While this study by Harton and colleagues (Bedoya et al. 2007) clearly demonstrates the role of NLRP3 in human cells, the reasons explaining the species specificity of the NLRP3 response are unknown. The nucleotide-binding domain, leucine-rich repeat region of human NLRP3 differs from murine Nlrp3 in the distribution of potential serine/threonine phosphorylation sites, oxidant-sensitive cysteine residues, and lysines that may serve as ubiquitination sites to regulate NLRP3 activation (Atianand et al. 2011; Py et al. 2013). Furthermore, human NLRP3 may recognize a broader panel of ligands than its murine counterpart. Indeed, human NLRP3 senses multiple types of RNAs (bacterial mRNA, rRNA, tRNA; synthetic ssRNA) whereas murine Nlrp3 preferentially recognizes only bacterial mRNA (Sha et al. 2014). The exact nature of the PAMPs or of the danger signal leading to NLRP3 activation in human cells during *Francisella* infection is still elusive.

Pyrin is expressed in primary monocytes and in THP-1 monocytes but its expression is greatly reduced in primary macrophages cultured in the absence of M-CSF or in PMA-differentiated THP-1 cells. In primary human monocytes and in THP-1 monocytes, *Mefv* knockdown reduces IL-1β release upon *F. novicida* infection. Conversely, ectopic expression of pyrin in PMA-differentiated THP-1 cells increases IL-1β release upon *F. novicida* infection (Gavrilin et al. 2009). These results indicate that Pyrin is required in human monocytes to activate the inflammasome complex in response to *F. novicida*. Recently, pyrin was identified as a sensor of the modification of Rho GTPases activity. It is still unclear whether pyrin directly senses Rho GTPases activity or the consequences of Rho dysfunction on the actin cytoskeleton (Xu et al. 2014). Numerous bacteria target Rho GTPases and the actin cytoskeleton (Lemichez and Aktories 2013; Welch and Way 2013). Yet, in contrast to other cytosolic bacteria, *F. tularensis* does not use actin-based motility and has no Rho GTPases-targeting toxins. The molecular mechanisms governing pyrin activation during *F. novicida* infection of human monocytes thus remain mysterious. Inflammasome activation has not been investigated in murine monocytes. However, pyrin is expressed at functional level in murine macrophages (Xu et al. 2014) and $Mefv^{-/-}$ BMDMs, in contrast to $Aim2^{-/-}$ macrophages, do not display any defect in inflammasome activation. The discrepancy observed between human and murine cells might be linked to the differences between the pyrin proteins in the two species. Murine pyrin lacks the C-terminal B30.2 domain, a domain known in certain immune proteins (e.g., butyrophilin 3A1, TRIM5α) to bind PAMPs (bacterial phospho-antigen, HIV-1 capsid, respectively) (Yang et al. 2012; Sandstrom et al. 2014). While the identity of the PAMP (cytosolic gDNA)

and the inflammasome receptor (Aim2) is well established in murine macrophages, further work is needed in human cells to fully understand the nature of the detected PAMP/DAMP and the interaction of the different inflammasome receptors.

6 Lessons from the Bacterial Side: Study of Hypercytotoxic Mutants

Numerous *F. novicida* and *F. holarctica* LVS mutants with a hypercytotoxic phenotypes have been identified either through genetic screens (Jones 2010; Lai et al. 2010) or through phenotypic characterization of defined mutants of interest (Weiss et al. 2007; Platz et al. 2010; Ulland et al. 2010; Huang et al. 2010; Jayakar et al. 2011; Lindemann et al. 2011; Peng et al. 2011; Mahawar et al. 2012; Dotson et al. 2013; Doyle et al. 2014). As both *F. novicida* and *F. holarctica* LVS have the ability to replicate extensively in the macrophage cytosol before being detected by Aim2, the hypercytotoxic mutants were thought to be mutated in specific virulence factors inhibiting either the recognition by innate immune receptors or directly innate immune pathways. Deletion of the gene encoding an OmpA-like protein (FTL_0325/FTT_0831c) results in an *F. holarctica* LVS mutant hyperactivating the inflammasome pathway (Mahawar et al. 2012). Upon ectopic expression in HEK293T cells, FTL_0325/FTT_0831c inhibited NF-κB activation suggesting that the hyperinflammasome phenotype of the corresponding mutant is due to an inability of the mutant to specifically block this pathway (Mahawar et al. 2012). However, Norgard and colleagues identified that the OmpA-like protein FTL_0325 contributes to cell division, maintenance of cell shape, and structural integrity suggesting that the hyperinflammasome phenotype was more likely to result from enhanced bacteriolysis in the host cytosol (Robertson et al. 2014). TolC is an outer membrane protein involved in type I secretion pathway. The hypercytotoxic phenotype of the *tolC* mutant led Platz et al. to suggest that TolC was implicated in the secretion of a toxin inhibiting host cell death (Platz et al. 2010). While this hypothesis cannot be formerly excluded, TolC is well known in *E. coli* as a member of the Tol-Pal system required for membrane integrity (Lloubes et al. 2001) suggesting that this mutant might be more sensitive to bacteriolysis. Infection with a LVS mutant in *ripA*, a gene coding an inner membrane protein, leads to a stronger MAPK response and a faster inflammasome response than upon wild-type LVS infection (Huang et al. 2010). RipA was later on identified as a protein-modulating lipid A synthesis, suggesting that the ability of *F. tularensis* strains to resist to host antimicrobial factors and/or to remain undetected may be associated with membrane remodeling (Miller et al. 2014). This idea was strengthened by work from Weiss and colleagues, who identified that *F. novicida* used the CRISPR-cas9 endonuclease (FTN_0757) to down-regulate the expression level of a bacterial lipoprotein (FTN_1103) during infection. This down-regulation enhances envelope integrity, decreases TLR2 recognition, and reduces inflammasome activation (Jones

et al. 2012; Sampson et al. 2014). In addition to the *ripA* mutant, several mutants in LPS/capsule biosynthesis genes are hypercytotoxic (Lai et al. 2010; Jayakar et al. 2011). Δ*kdtA*, Δ*lpcC*, Δ*manB*, and Δ*manC* mutants are hypercytotoxic and their LPS is lacking the O-antigen and has a defect in the lipid A core. The respective roles of the defect in the lipid A core and of the lack of O-antigen are unclear since *wbtA* mutants (which have a normal lipid A core but lack the O-antigen) have been reported as hypercytotoxic by Peng et al. (2011) but not hypercytotoxic by Lai et al. (2010). Conversely, a *htrB* mutant (deleted in a gene encoding a lipid A acylase) displays a LPS with a normal O-Antigen but is hypercytotoxic (Lai et al. 2010). Altogether, these data indicate that the lipid A structure is a key element to limit bacterial detection by the inflammasome pathway possibly through the maintenance of membrane integrity, resistance to cytosolic antimicrobial factors and spontaneous or host-induced bacteriolysis. MviN, a lipid II flippase, is an enzyme involved in peptidoglycan maturation. In contrast to *mviN* mutants in *E. coli*, an *mviN* mutant in LVS grows normally in axenic medium but presents an aberrant morphology suggesting it might be more susceptible to bacteriolysis than wild-type LVS (Ulland et al. 2010). While it is clear that envelope integrity and its regulation during infection are key factors to avoid recognition by TLR2 and the Aim2 inflammasome, most of the above-cited mutants have been shown by Peng et al. to display aberrant morphologies during growth in minimal medium and to lyse more in the host cell cytosol. This result suggests that under stressful conditions, numerous membrane proteins cooperate with peptidoglycan and LPS-biosynthetic proteins to ensure proper membrane integrity, a lack of PAMP exposure at the surface and a resistance to spontaneous and host-induced bacteriolysis inside the host cytosol (Peng et al. 2011). Altogether, the data from all these studies suggest that *Francisella* does not actively inhibit the inflammasome pathway but rather use a hiding strategy associated with tight envelope integrity and modification of the structure (e.g., LPS) or the level of expression of its surface exposed PAMP (e.g., TLR2-recognized bacterial lipoprotein). This stealth strategy is particularly striking for highly virulent *F. tularensis* subsp. *tularensis* strains.

7 Hypervirulent Strains Escape Inflammasome Detection

Considering the different *Francisella* species and the different *F. tularensis* subspecies, there is a good correlation between the ability of the various strains to escape recognition by the inflammasome and their ability to cause disease in humans (Table 1). As previously mentioned, *F. novicida* infection leads to a strong inflammasome activation although this activation occurs with a delay allowing significant replication in macrophages and in vivo (Mares et al. 2008). This delay in inflammasome activation is even stronger upon infection with *F. holarctica* LVS and is associated with a lower IL-1β release than upon *F. novicida* infection (Carlson et al. 2007; Ghonime et al. 2015). Finally, infection with *F. tularensis* type

A strains elicits no or a very weak inflammasome activation (Wickstrum et al. 2009; Bauler et al. 2011; Crane et al. 2014; Ghonime et al. 2015).

While numerous groups have observed caspase-1 activation, IL-1β release, and pyroptosis in macrophages infected with LVS (Mariathasan et al. 2005; Gavrilin et al. 2006; Li et al. 2006; Wickstrum et al. 2009; Rathinam et al. 2010), apoptosis is also observed in *F. holarctica* LVS-infected macrophages with an involvement of the intrinsic pathway proceeding through caspase-9 and caspase-3 activation and PARP cleavage (Lai and Sjostedt 2003). This apoptotic pathway has been observed in the J774 macrophage cell line, which is deficient for *F. novicida*-mediated inflammasome cell death (TH unpublished observation) but also in BMDMs (Doyle et al. 2014). These observations suggest that *F. holarctica* LVS may be less detected by the inflammasome and that another cell-intrinsic mechanism may trigger mitochondrial apoptosis to synergize with pyroptosis to fully remove the bacterial replicative niche. The ability of *F. holarctica* LVS to dampen inflammasome activation correlates with a general ability of this strain to dampen pro-inflammatory signaling (Telepnev et al. 2003). Indeed, infection with *F. holarctica* LVS inhibits LPS-induced TNF-α and IL-1β release. This inhibition is dependent on FPI-encoded genes, and it is still unclear whether it is a direct consequence of phagosomal escape or whether a specific T6SS-effector modulates this pro-inflammatory response (Telepnev et al. 2003; Broms et al. 2010, 2011).

As mentioned above, infection with *F. tularensis* subs. *tularensis* strains, leads to almost no IL-1β (undetectable to less than 20 ng/ml depending on the studies) and no IL-18 (undetectable to less than 50 ng/ml depending on the studies) release (Wickstrum et al. 2009; Crane et al. 2014; Ghonime et al. 2015). Death of SCHU S4-infected macrophages occurs very late, suggesting that pyroptosis does not play a major role in restricting SCHU S4 replication. The low inflammasome response is not due to an inability of the NLRP3 inflammasome to be primed upon infection. Indeed, when the NLRP3 priming event was evaluated using ATP as a second signal, *F. novicida*, LVS, and *F. tularensis* strains had a similar NLRP3 priming activity (Ghonime et al. 2015). This result suggests that infection with SCHU S4 does not provide the signal 2 required for inflammasome activation. Bosio and co-workers (Crane et al. 2014) demonstrated that *F. novicida* is more sensitive to H_2O_2-mediated killing than SCHU S4. As previously mentioned, ROS are required for *F. novicida*-mediated inflammasome activation. The resistance of SCHU S4 to ROS could thus limit ROS-mediated bacteriolysis of SCHU S4 in the cytosol, DNA release, and AIM2 inflammasome activation (Crane et al. 2014). While this model is appealing, further evidence remains to be provided to understand the role of mitochondrial ROS and the inability of *F. tularensis* strain to be detected by the inflammasome. In addition to the resistance to ROS-mediated killing, the ability of *F. tularensis* SCHU S4 to escape recognition by the inflammasome pathway might be related to its LPS and capsule structure. Indeed, mutants lacking genes in a locus required for O-antigen and capsule biosynthesis are hypercytotoxic upon macrophage infection (Lindemann et al. 2011).

The ability of SCHU S4 strain to modulate IL-1β response is also dependent on its entry pathway in human macrophages. Indeed, the efficient uptake of SCHU S4

strain by human macrophages requires C3 opsonization and the presence of the CR3 receptor at the macrophage surface. Despite an increase entry into host cells, infection with C3-opsonized *F. tularensis* leads to a decreased IL-1β release compared to an infection with non-opsonized *F. tularensis*. This CR3-mediated immune suppression is not specific for the inflammasome pathway but may act through inhibition of TLR2 signaling highlighting the connections between these pathways (Dai et al. 2013).

In vivo, in a mouse model of tularemia with a type A strain, an immunostaining approach to detect active caspases revealed a predominant activation of caspase-3 suggesting that the absence of inflammasome activation is also true in vivo. Although the role of caspase-1 has not been deeply investigated, the formation of granuloma and the frequency of $TUNEL^+$ cells were similar in the wild-type and $Casp1^{-/-}$ mice infected with type A strain (Wickstrum et al. 2009). Only a few cells displayed active caspase-1 staining both in the liver and in the spleen. Interestingly, active Caspase-1 was restricted to the splenic marginal zones (Wickstrum et al. 2009), which contain macrophage subsets with great ability to phagocyte pathogens (Borges da Silva et al. 2015). Altogether, while the results suggest that the inflammasome does not play a major role to fight highly virulent *F. tularensis* subsp. *tularensis* strains, inflammasome activation may be important in dedicated macrophage subsets localized in strategic places in the lymphoid organs to orchestrate downstream immune responses.

8 Concluding Remarks

Inflammasome-deficient mice have demonstrated the key role of the inflammasome to fight *F. novicida* infection. The mechanisms leading to inflammasome activation are now well established with the action of the interferon-inducible proteins Gbp2 and Gbp5 required to lyse *F. novicida* and to release its genomic DNA into the host cytosol. Aim2 is then the key sensor to initiate inflammasome complex formation and trigger the ensuing inflammatory cascade. Interestingly, the ability of *Francisella* strains to activate the inflammasome pathway is inversely correlated with their pathogenicity toward human. In addition to having a non-toxic lipid A escaping recognition by both TLR4 and caspase-11, *Francisella* has evolved a tight membrane associated with resistance to bacteriolysis and down-regulation of potential PAMPs. This strategy allows *F. novicida* and *F. holarctica* LVS to replicate in the cytosol before being eventually detected by the inflammasome sensors. This furtiveness is even higher in the highly virulent *F. tularensis* subsp. *tularensis* strains since macrophages infected with these strains do not demonstrate any inflammasome activation markers.

One striking feature of *F. novicida* and *F. holarctica* LVS inflammasome in murine macrophages is their exclusive dependence on the Aim2 receptor. This is unique since several inflammasome sensors are involved in the detection of most other intracellular bacteria, including *Listeria monocytogenes*, which is detected by

Nlrp3, Nlrc4, and Aim2 (Wu et al. 2010). This strict dependence is even more surprising considering that in human phagocytes, three different receptors (PYRIN, NLRP3, and AIM2) have been implicated in the detection of *Francisella* infection.

The inflammasome complex is highly interconnected with other immune pathways acting both upstream of Aim2 and downstream of caspase-1-dependent cytokines. Indeed, the TLR2 and the cGas/Ifi204-STING pathways potentiate or are required for Aim2 inflammasome activation in *Francisella*-infected cells. Surprisingly, for a cytosolic bacterium, the contribution of NOD1/2, the cytosolic sensors of peptidoglycan fragments, seems minimal. Downstream of caspase-1, there are clear evidences that IL-18 regulates IFN-γ level and that IL-1β regulates the IL-23/IL-17 cascade and a protective IgM response mediated by B1a B cells (del Barrio et al. 2015). IFN-γ (Anthony et al. 1989) and to a lesser extent IL-17 (Lin et al. 2009; Khader and Gopal 2010; Skyberg et al. 2013), are two very important cytokines to fight *Francisella* and other intracellular pathogens highlighting the key role of the inflammasome pathway in the orchestration of the immune response.

Of note, while this review focuses on mouse and human cells, *F. noatunensis*, a species responsible for the systemic granulomatous inflammatory disease, francisellosis in cod (Furevik et al. 2011) triggers IL-1β release in zebrafish leukocytes in a caspase-1-like dependent manner (Vojtech et al. 2012). The inflammasome pathway is thus likely to be very important in other *Vertebrates* species in response to other *Francisella* species.

Finally, while the understanding of the mechanisms controlling the inflammasome activation during *Francisella* infection has greatly progressed since the first report in 2005 (Mariathasan et al. 2005), there are still a number of questions to be solved, five of which are listed below.

First, while cytosolic DNA is detected early on during infection by cGAS and Ifi204, Aim2 activation by cytosolic DNA occurs much later during infection and requires the bacteriolytic action of Gbp2 and Gbp5. This observation suggests a hierarchy of sensing between the different DNA sensors, which is not understood.

Second, the mechanisms leading to the recruitment of Gbps onto cytosolic *Francisella* and to their bacteriolytic actions remain unknown. The presence of a CAAX-motif (prenylation motif) in the C-terminus of Gbp2 and Gbp5 suggests the involvement of an undefined membrane compartment. Furthermore, other interferon-inducible proteins might participate in Gbp2/5 action although such actors remain to be identified.

Third, the role of the autophagy pathway in counterbalancing inflammasome activation during *Francisella* remains to be assessed. Indeed, autophagy targets AIM2 inflammasome components during transfection of synthetic DNA (Shi et al. 2012). Furthermore, autophagy clears the cytosol from O-antigen mutants (Case et al. 2014) and from replication-deficient *Francisella* dying in the cytosol (Chong et al. 2012) possibly decreasing the source of cytosolic DNA to activate the Aim2 inflammasome.

Fourth, the specificity of the human inflammasomes remains to be understood. The activation of pyrin in monocytes remains mysterious in absence of any known

modification of the actin cytoskeleton upon *Francisella* infections. Similarly, the danger signals or the PAMP leading to activation of the human NLRP3 receptor but not of the murine Nlrp3 remains to be identified.

Fifth, while in vivo experiments demonstrate that the inflammasome is an important mechanism to fight *F. novicida* and *F. holarctica* LVS infection, the repertoire of cells sustaining inflammasome activation in vivo during infection remains to be determined. Particularly, the role of the inflammasome pathway in neutrophils or in macrophages from the splenic marginal zones would be interesting to investigate to understand why despite efficient in vitro inflammasome activation, *F. novicida* kills wild-type mice at a very low infectious dose.

No doubt that the next decade will provide us with exiting findings regarding the activation of the inflammasome and how *Francisella* escapes this recognition to remain one of the most infectious pathogen.

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