



Asc-dependent and independent mechanisms contribute to restriction of *Legionella pneumophila* infection in murine macrophages

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The apoptosis-associated speck-like protein containing a caspase recruitment domain (Asc) is an adaptor molecule that mediates inflammatory and apoptotic signals. *Legionella pneumophila* is an intracellular bacterium and the causative agent of Legionnaire's pneumonia. *L. pneumophila* is able to cause pneumonia in immuno-compromised humans but not in most inbred mice. Murine macrophages that lack the ability to activate caspase-1, such as caspase-1^{-/-} and Nlrp4^{-/-} allow *L. pneumophila* infection. This permissiveness is attributed mainly to the lack of active caspase-1 and the absence of its down stream substrates such as caspase-7. However, the role of Asc in control of *L. pneumophila* infection in mice is unclear. Here we show that caspase-1 is moderately activated in Asc^{-/-} macrophages and that this limited activation is required and sufficient to restrict *L. pneumophila* growth. Moreover, Asc-independent activation of caspase-1 requires bacterial flagellin and is mainly detected in cellular extracts but not in culture supernatants. We also demonstrate that the depletion of Asc from permissive macrophages enhances bacterial growth by promoting *L. pneumophila*-mediated activation of the NF-κB pathway and decreasing caspase-3 activation. Taken together, our data demonstrate that *L. pneumophila* infection in murine macrophages is controlled by several mechanisms: Asc-independent activation of caspase-1 and Asc-dependent regulation of NF-κB and caspase-3 activation.

Keywords: inflammasome, caspase-1, *Legionella pneumophila*, Asc

INTRODUCTION

The apoptosis-associated speck-like protein containing a caspase recruitment domain (Asc encoded by the *Pycard* gene) is an adaptor molecule that mediates inflammatory and apoptotic signals and is predominantly expressed in monocytes and mucosal epithelial cells (Taniguchi and Sagara, 2007; Hasegawa et al., 2009). Asc contains an N-terminal pyrin/PAAD (PYD) death domain and a C-terminal CARD protein-protein interaction domain (CARD; Masumoto et al., 1999, 2001; Liepinsh et al., 2003; Stehlik et al., 2003). Both domains enable Asc to recruit other PYD and CARD-containing proteins through homotypic protein-protein interactions (Fernandes-Alnemri et al., 2007; Mariathasan, 2007). Proteins with pyrin and/or caspase recruitment domains have roles in inflammation, apoptosis, and innate immunity. Many pyrin domain proteins, such as Asc, modulate NF-κB activity. Asc also participates in the assembly of multi-protein complexes called "inflammasomes" (Srinivasula et al., 2002; Fernandes-Alnemri et al., 2007; Fernandes-Alnemri and Alnemri, 2008). Within the inflammasome, Asc is able to link caspase-1 to NOD-like receptors (NLRs) via its CARD domain, leading to the

activation of caspase-1 (Srinivasula et al., 2002; Fernandes-Alnemri et al., 2007; Fernandes-Alnemri and Alnemri, 2008). NLRs act as intracellular sensors to stress, and foreign molecules like microbial components (Kanneganti et al., 2007; Martinon and Tschopp, 2007). Once activated, caspase-1 subsequently cleaves pro-IL-1β/IL-18 and accompanies the active cytokines when secreted out of the cell (Stehlik et al., 2002, 2003; Liepinsh et al., 2003; Hasegawa et al., 2005, 2007, 2009; Sarkar et al., 2006; Bedoya et al., 2007). Caspase-1 also activates caspase-7 in response to flagellin or LPS (Franchi et al., 2008; Lamkanfi et al., 2008, 2009; Akhter et al., 2009; Lamkanfi and Kanneganti, 2010; Shaw et al., 2010). Many Gram-negative bacteria, such as *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Shigella flexneri*, and *Legionella pneumophila*, are recognized in murine macrophages by the NLR Nlrp4/Ipaf leading to caspase-1 activation through the inflammasome (Abdelaziz et al., 2010; Amer, 2010).

Furthermore, when Asc is over expressed, it cooperates with Nlrp3 (cryopyrin) and Nlrp12 (Pypaf7) in order to promote NF-κB activity in an over expression system (Masumoto et al., 2003; Hasegawa et al., 2005). However, other reports show that Asc is

a suppressor of NF- κ B activity and that it uses its CARD interaction not only to induce caspase-1 activation, but also to down regulate NF- κ B signaling (Sarkar et al., 2006; Bedoya et al., 2007). Additionally, other studies demonstrate that Asc can be either an inducer or an inhibitor of NF- κ B depending on expression level and location (Stehlik et al., 2002; Yu et al., 2006). Thus, Asc regulates the inflammasome and other signaling complexes depending on the stoichiometry of its expression and also whether certain other PYD family proteins are expressed upon activation. On the other hand, Asc mediates apoptosis by serving as an adaptor molecule for Bax and regulates a p53-Bax mitochondrial pathway of apoptosis resulting in the activation of caspase-2 and -3 (Ohtsuka et al., 2004; Hasegawa et al., 2007; Fernandes-Alnemri and Alnemri, 2008; Motani et al., 2010).

Legionella pneumophila is an intracellular bacterium and the causative agent of Legionnaire's pneumonia (Horwitz and Silverstein, 1981, 1983; Nash et al., 1984). The ability of *L. pneumophila* to cause pneumonia in humans is dependent on its capability to evade the immune system and multiply within human monocytes and derived macrophages (Horwitz and Silverstein, 1981, 1983; Nash et al., 1984). In murine macrophages, *L. pneumophila* activates the Nlr4 inflammasome leading to the production of active caspase-1 and IL-1 β (Amer et al., 2006; Ren et al., 2006; Zamboni et al., 2006; Coers et al., 2007; Lightfield et al., 2008; Akhter et al., 2009; Abdelaziz et al., 2010; Amer, 2010; Vance, 2010). Then, Naip5 mediates caspase-7 activation downstream of caspase-1 which restricts the intracellular survival of the organism (Akhter et al., 2009; Abdelaziz et al., 2010). Therefore, mice and their derived macrophages lacking Nlr4, caspase-1, or caspase-7 allow *L. pneumophila* growth and are ideal models to study *L. pneumophila* pathogenesis (Amer et al., 2006; Ren et al., 2006; Zamboni et al., 2006; Coers et al., 2007; Lightfield et al., 2008; Akhter et al., 2009; Abdelaziz et al., 2010; Amer, 2010; Vance, 2010).

Here we demonstrate that murine macrophages control *L. pneumophila* infection through an Asc-dependent mechanism by decreasing NF- κ B activation and an Asc-independent mechanism by modestly activating caspase-1 in the cytosol. Together, our data show that Asc controls *L. pneumophila* infection in the absence of caspase-1.

RESULTS

Asc CONTROLS *L. PNEUMOPHILA* INFECTION IN MURINE MACROPHAGES LACKING CASPASE-1

WT mouse macrophages effectively activate and release caspase-1 in response to *L. pneumophila* infection. This is accompanied by the fusion of the *L. pneumophila*-containing phagosome with the lysosome, bacterial degradation, and elimination (Amer et al., 2006; Akhter et al., 2009). Consistent with that, caspase-1 knockout ($^{-/-}$) macrophages are permissive to *L. pneumophila* replication. Asc is an adaptor molecule involved in caspase-1 activation in response to a variety of agents. However, its role during *L. pneumophila* infection is not well established. To characterize the role of Asc during *L. pneumophila* infection, Asc was down regulated in both caspase-1 $^{-/-}$ (Figure 1A) and WT macrophages (Figure 1B) using Asc specific siRNA. After transfection, Asc protein level declined in both sets of cells, while other components of the inflammasome, such as Nlr4 and pro-caspase-1, were not affected (Figures 1A,B). After depletion

of Asc, cells were infected with *L. pneumophila* and the bacterial replication was assessed by counting colony-forming units (CFU). Depletion of Asc in caspase-1 $^{-/-}$ cells supported significantly more *L. pneumophila* replication compared to caspase-1 $^{-/-}$ cells treated with control siRNA (Figure 1C). In WT mouse macrophages, depletion of Asc did not have an effect on the bacterial growth compared to that of cells treated with control siRNA or untreated cells (Figure 1D). Therefore, Asc controls *L. pneumophila* replication in murine macrophages in the absence of caspase-1.

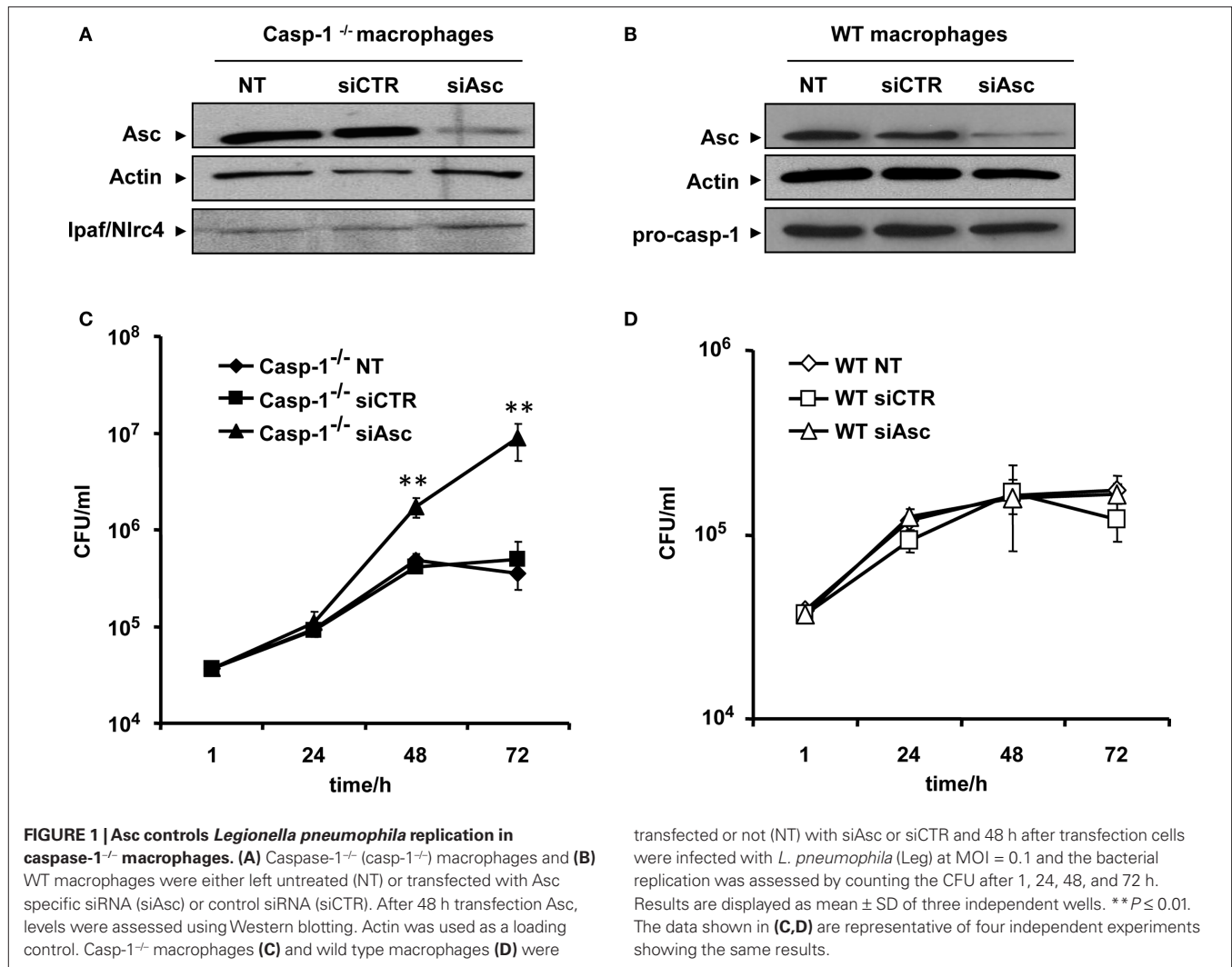
Asc REDUCES NF- κ B ACTIVATION INDUCED BY *L. PNEUMOPHILA* INFECTION

It has been shown by several reports that *L. pneumophila* triggers NF- κ B activation in TLR5 dependent and independent manner depending on the stage of the infection (Bartfeld et al., 2009; Losick et al., 2010). Nevertheless, the role of Asc in NF- κ B modulation depends on its protein levels and its location within the cells (Stehlik et al., 2002; Sarkar et al., 2006; Yu et al., 2006; Bedoya et al., 2007). Our results showed that Asc controls *L. pneumophila* replication in a caspase-1-independent manner. To determine if the mechanism by which Asc restricts *L. pneumophila* (independently of caspase-1) involves the NF- κ B pathway, Asc was depleted in caspase-1 $^{-/-}$ macrophages by siRNA against Asc. Then, cells were infected with *L. pneumophila* and NF- κ B activation was assessed in nuclear extracts by electrophoretic mobility shift assay (EMSA). We found that caspase-1 $^{-/-}$ macrophages depleted for Asc allowed more NF- κ B activation at 1, 4, and 8 h of infection compared with cells treated with control siRNA (Figure 2A). These results suggest that Asc decreased NF- κ B during *L. pneumophila* infection. To confirm these findings, Asc $^{-/-}$ and WT macrophages were infected with *L. pneumophila* and EMSA assay was performed. Initially, WT and Asc $^{-/-}$ macrophages showed NF- κ B activation within 1 h after infection. This activation declined in WT macrophages by 8 h infection whereas NF- κ B pathway remained activated in Asc $^{-/-}$ 8 h after *L. pneumophila* infection (Figure 2B). Thus, in the context of *L. pneumophila* infection, Asc hinders NF- κ B activation, and decreases cell survival.

CASPASE-1 IS ACTIVATED IN THE CYTOSOL OF MURINE MACROPHAGES LACKING Asc

The involvement of Asc in Nlr4 inflammasome is still unclear (Zamboni et al., 2006). To discern the role of Asc in *L. pneumophila*-mediated caspase-1 activation, Asc $^{-/-}$ macrophages were left untreated or infected with *L. pneumophila* at a low multiplicity of infection (MOI). Then, cleaved caspase-1 was examined in cellular extracts and culture supernatants of Asc $^{-/-}$ macrophages and of their WT counterparts. Notably, cells lacking Asc still allowed the cleavage of caspase-1 within their cytosols when infected with *L. pneumophila* (Figure 3A). The amount of cleaved caspase-1 in Asc $^{-/-}$ cell lysates was less than that of WT macrophages (Figure 3A). Cleaved caspase-1 was detected in culture supernatants of infected WT macrophages but not that of infected Asc $^{-/-}$ macrophages (Figure 3B). This data indicates that in the absence of Asc, a fraction of caspase-1 is cleaved by a yet unknown mechanism.

To further investigate the role of Asc in caspase-1 activation during *L. pneumophila* of WT macrophages, Asc was depleted in WT murine macrophages, as in Figure 1B and then infected with *L. pneumophila*. The amount of cleaved caspase-1 detected



in extracts of cells treated with Asc specific siRNA was less than that in cells treated with control siRNA (Figure 4A). Accordingly, the release of active caspase-1 from siAsc treated cells was diminished (Figure 4B). In support of this data, total cleaved caspase-1 in combined cell extracts and culture supernatants was less in Asc heterozygote macrophages than that of WT ones (Figure A1 of Appendix). Furthermore, IL-1 β release from macrophages was impeded when Asc was depleted (Figure 4C). Therefore, Asc contributes to the activation of a portion of pro-caspase-1, while the rest of caspase-1 pro-form is cleaved independently of Asc.

Asc-INDEPENDENT ACTIVATION OF CASPASE-1 WITHIN MACROPHAGE CYTOSOLS REQUIRES FLAGELLIN AND CONTRIBUTES TO THE RESTRICTION OF *L. PNEUMOPHILA* INFECTION

The activation of caspase-1 requires flagellin, thereby promoting the restriction of *L. pneumophila* infection (Amer et al., 2006; Ren et al., 2006; Zamboni et al., 2006; Lamkanfi et al., 2007; Akhter et al., 2009; Case et al., 2009; Amer, 2010; Kang et al., 2010). Thus, we next examined the growth of *L. pneumophila* in WT, caspase-1^{-/-}, and Asc^{-/-} macrophages. As previously reported by our group and by others, caspase-1^{-/-} macrophages were permissive, whereas WT and

Asc^{-/-} macrophages restricted *L. pneumophila* replication (Amer et al., 2006; Ren et al., 2006; Zamboni et al., 2006; Lamkanfi et al., 2007; Akhter et al., 2009; Case et al., 2009; Amer, 2010; Kang et al., 2010). However, our data demonstrate that caspase-1 is cleaved in the cytosol of Asc^{-/-} macrophages, therefore, we examined if this cytosolic cleavage required flagellin and if it is sufficient for restriction of *L. pneumophila* infection. WT macrophages were infected with the *L. pneumophila* mutant lacking flagellin (Fla), then, caspase-1 activation in cell extracts was examined by Western blots. Figure 5 shows that the Fla mutant did not activate caspase-1 within the cytosols of macrophages (Figure 5A). Because caspase-1 activation requires flagellin and is accompanied with restriction to *L. pneumophila* infection, we examined the growth of *L. pneumophila* mutants lacking flagellin (Fla) in macrophages lacking Asc in comparison to WT cells, since both cells lack caspase-1 activation in response to Fla (data not shown). Specifically, Figure 5B demonstrates that the growth of Fla mutants in Asc^{-/-} macrophages exceeds that exhibited in WT cells. Yet, NF- κ B activation during Fla was similar in WT and Asc^{-/-} macrophages (Figure 5C). Therefore, there must be another pathway mediated by Asc that maintains the replication of Fla mutant under control in WT macrophages and

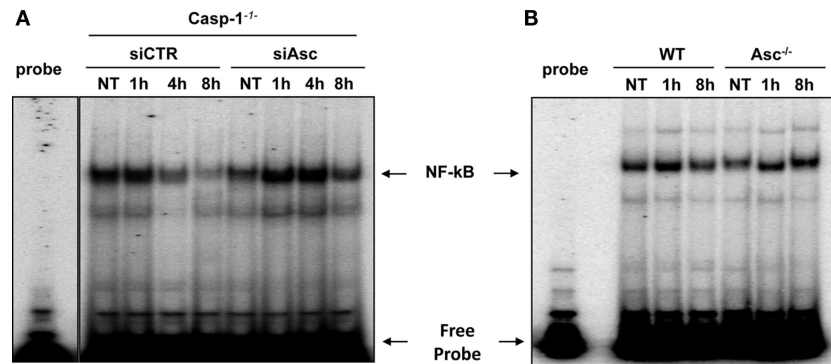


FIGURE 2 | Asc hinders the activation of NF-κB induced by *L. pneumophila*.

(A) Caspase-1^{-/-} (casp-1^{-/-}) macrophages were transfected with Asc specific siRNA (siAsc) or control siRNA (siCTR) and 48 h after transfection cells were infected or not (NT) with *L. pneumophila* (Leg) at MOI = 0.5 for 1, 4, and 8 h.

Afterward, NF-κB activation was examined using electrophoretic mobility shift assay (EMSA). **(B)** WT and Asc^{-/-} mouse macrophages were infected or not (NT) with *L. pneumophila* (Leg) at MOI = 0.5 for 1 and 8 h. Subsequently, NF-κB activation was examined using EMSA.

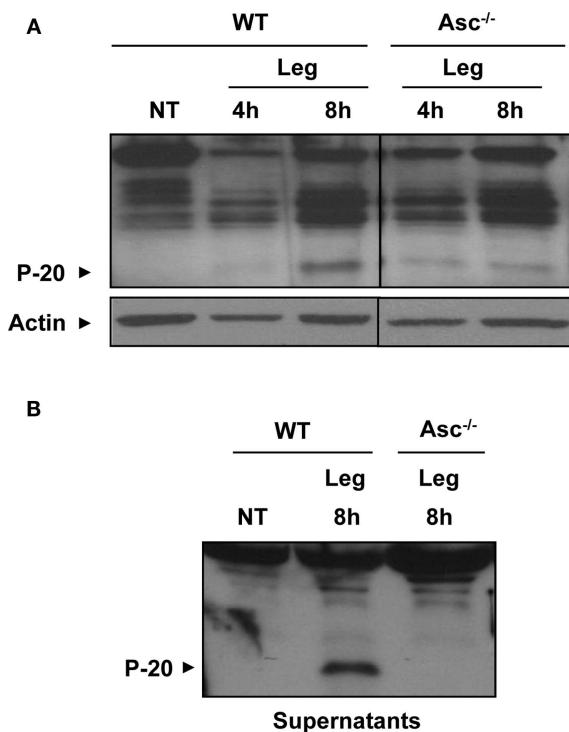


FIGURE 3 | A fraction of caspase-1 is activated in the cytosol of murine macrophages lacking Asc.

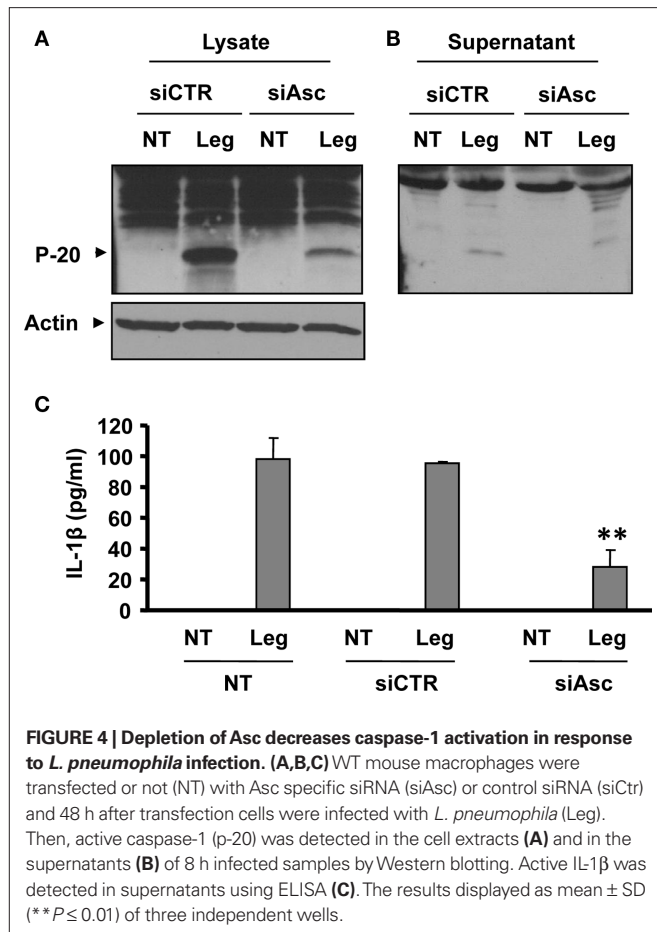
(A) WT and Asc^{-/-} mouse macrophages were either not treated (NT) or infected with *L. pneumophila* (Leg) at MOI = 0.5 for 4 and 8 h. Then active caspase-1 (p-20) was detected in the cell extracts **(A)** and the supernatants **(B)** by Western blotting. Actin was used as a loading control.

activated during *L. pneumophila* infection (Molmeret et al., 2004). First, we tested if the Fla mutant activates caspase-3 in wild type macrophages. Until 4 h post infection only WT *L. pneumophila* activated caspase-3 (**Figure 6A**). However, at 5 h post infection, the Fla mutant activated caspase-3 in WT macrophages but not in Asc^{-/-} macrophages (**Figure 6B**). Taken together, these data suggest that Asc is involved in caspase-3 activation which mediates cell death in WT macrophages at later stages of Fla infection.

LEGIONELLA PNEUMOPHILA MODULATES THE EXPRESSION OF Nlr4 INFLAMMASOME COMPONENTS IN WILD TYPE MOUSE MACROPHAGES

In WT murine macrophages *L. pneumophila* flagellin is detected by Nlr4 with subsequent activation of caspase-1 (Amer et al., 2006; Ren et al., 2006; Zamboni et al., 2006; Lamkanfi et al., 2007; Akhter et al., 2009; Case et al., 2009; Amer, 2010; Kang et al., 2010). This response is completely lacking in human phagocytes. In contrast to murine phagocytes, human phagocytes which are permissive to *L. pneumophila* do not activate caspase-1 in response to the pathogen (Abdelaziz et al., 2011). This lack of activation is due to the down regulation of ASC in human cells upon infection with *L. pneumophila* (Abdelaziz et al., 2011). To characterize the effect of *L. pneumophila* on the expression of the Nlr4 inflammasome components in the mouse, murine WT macrophages were infected with *L. pneumophila* and the expression of caspase-1, IL-1β, Nlr4, and Asc was assessed on both mRNA and protein levels. The expression of both caspase-1 and IL-1β proteins were induced significantly within 4 h after infection and remained up regulated for 24 h of infection (**Figures A2A,B** of Appendix). Subsequently, both caspase-1 and IL-1β were activated early upon infection (**Figures A2A,B** lower panel of Appendix) and their active forms were released into the supernatant (Data not shown). Remarkably, in contrast to mouse macrophages, the expression of Asc was enhanced 24 h after infection (**Figure A2C**). As for Nlr4, mRNA, and protein levels were decreased later in infection (**Figure A2D** lower panel of Appendix). We found that this decrease in Nlr4 protein levels is due to its release with the rest of the inflammasome components into the media (data not shown). Therefore, *L. pneumophila* differentially modulates the expression of Asc in murine and in human phagocytes.

is missing in Asc^{-/-} macrophages. To answer this question, we next determined the levels of LDH release in culture supernatants of WT and Asc^{-/-} macrophages infected with *L. pneumophila* or the Fla mutant. We found that the absence of Asc allows more host cell survival during Fla infection. To further understand the mechanism by which Asc modulates cell survival, we examined the activation of caspase-3, a caspase involved in apoptosis and know to be



DISCUSSION

Asc is an adaptor molecule necessary for the assembly and activation of several inflammasome complexes in response to stress signals and microbial molecules (Taniguchi and Sagara, 2007; Hasegawa et al., 2009). Asc is required for caspase-1 activation downstream of Nlr4/IpaF during *Salmonella* or *Shigella* infections, yet its role during *L. pneumophila* infection has been controversial (Zamboni et al., 2006; Akhter et al., 2009; Case et al., 2009). In this study, we demonstrate that a fraction of caspase-1 is activated within the cytosol of infected macrophages independently of Asc. Data from our group and others indicate that restriction of *L. pneumophila* infection is mediated by several mechanisms. This can occur either via caspase-1-dependent or independent mechanisms. Moreover, caspase-1 dependent control of *L. pneumophila* infection can be either Asc-dependent or independent.

First, caspase-1-dependent restriction of *L. pneumophila* is by modulation of pyroptosis and phagosome–lysosome fusion. Several studies have demonstrated that at high MOIs with *L. pneumophila*, the activation of caspase-1 leads to pyroptotic cell death, which contributes to resistance to infection (Zamboni et al., 2006; Case et al., 2009). Other reports have shown that during extreme MOIs of *L. pneumophila*, murine caspase-1 is activated independently of Nlr4 (Case et al., 2009). This is at odds with other data that clearly demonstrates that the activation of caspase-1 during physiological levels of infection requires Nlr4 (Amer et al., 2006; Ren et al., 2006; Zamboni et al., 2006; Coers et al., 2007; Lightfield et al., 2008;

Akhter et al., 2009; Abdelaziz et al., 2010; Amer, 2010; Vance, 2010). It seems that these discrepancies are largely due to different infection doses. Consequently, it is plausible to suggest that the activation of caspase-1 by high bacterial burdens overrides the signaling pathways controlling their activation at physiological levels of infection. Yet, our data suggest that Asc is required for the recruitment and cleavage of at least a part of pro-caspase-1 protein into its active form since the amount of cleaved p20 detected in the cell extracts of Asc^{-/-} macrophages was much less than that of WT macrophages.

Secondly, macrophages deploy an Asc-dependent restriction mechanism to control *L. pneumophila* infection by controlling NF- κ B pathway. Dixit and colleagues demonstrated that macrophages from Asc^{-/-} mice are markedly resistant to *Salmonella typhimurium* induced cell death (Mariathasan et al., 2004). However, in their study, cell death was mainly attributed to caspase-1 mediated pyroptosis while other pathways such as NF- κ B activation were not examined. Several reports by Isberg and Abu Kwaik showed that *L. pneumophila* activates NF- κ B in two phases to extend the host cell survival to permit intracellular growth (Losick and Isberg, 2006; Abu-Zant et al., 2007; Bartfeld et al., 2009). They clearly demonstrated that NF- κ B activation is absolutely required for *L. pneumophila* growth within macrophages. *L. pneumophila* promotes two phases of NF- κ B activation, a TLR5-dependent and a TLR5-independent activation at an early and later stages of infection respectively (Losick and Isberg, 2006; Bartfeld et al., 2009). The role of Asc in *L. pneumophila*-induced NF- κ B activation was not explored previously. A number of studies suggest that Asc promotes NF- κ B activation (Chamaillard et al., 2003; Masumoto et al., 2003; Hasegawa et al., 2005). Conversely, several studies by Wewers and Reed showed that THP-1 cells treated with small interfering RNA for human ASC decreased their caspase-1 activity while enhancing their NF- κ B signal (Stehlik et al., 2002; Sarkar et al., 2006; Yu et al., 2006). The later studies showed that the interaction of caspase-1 and RIP2 mediates NF- κ B activation which is prevented by human ASC as it hinders their interaction. Here, we report that mouse Asc deters the activation of NF- κ B during *L. pneumophila* infection since the depletion of Asc allowed more NF- κ B activation and additional *L. pneumophila* growth.

Importantly, murine Asc is induced in response to *L. pneumophila* infection (Figure A2). This is in stark contrast to human ASC, which is down regulated in human monocytes upon infection with *L. pneumophila* (Abdelaziz et al., 2011). The down regulation of human ASC contributed to the permissiveness to *L. pneumophila* growth. These findings are among the first reports clarifying the mechanism of permissiveness of human monocytes to *L. pneumophila* (Abdelaziz et al., 2011). Thus, regulating Asc availability in human phagocytes could be a mechanism employed by *L. pneumophila* to modulate caspase-1, NF- κ B, and pyroptosis.

The detection of cleaved caspase-1 in supernatants of cultured cells has been widely used as the hallmark for caspase-1 activation. Few studies have shown that Nlr4/IpaF^{-/-} and Asc^{-/-} macrophages fail to produce cleaved caspase-1 in response to *L. pneumophila*. The absence of active caspase-1 promoted *L. pneumophila* growth. Yet, it is unclear as to why Asc^{-/-} macrophages do not allow *L. pneumophila* replication. Here we show that in Asc^{-/-} murine macrophages, a small portion of caspase-1 is cleaved during *L. pneumophila* infection but is not detected in culture supernatants. However, we cannot exclude that cleaved caspase-1 was released in amounts below our detection

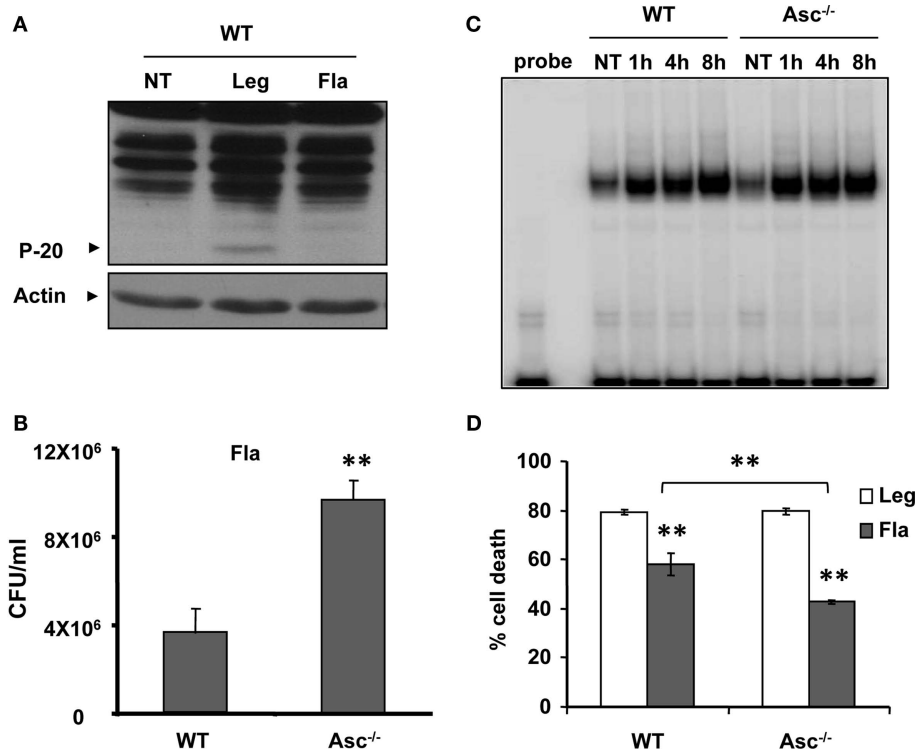


FIGURE 5 | Asc^{-/-} macrophages are permissive to flagellin mutant. (A) WT macrophages were infected with *L. pneumophila* (Leg), with corresponding flagellin mutant (Fla), or left untreated (NT). Active caspase-1 (p-20) was then detected in the cell extracts. **(B)** WT and Asc^{-/-} macrophages were infected with Fla mutant and CFUs were scored 72 h after infection. **(C)** WT and Asc^{-/-}

macrophages were infected with Fla mutant for 1, 4, and 8 h then, nuclear extracts were processed for determination of NF-κB activation using electrophoretic mobility shift assay (EMSA). **(D)** WT and Asc macrophages were infected with Leg or Fla for 24 h then LDH release was determined and presented as percent cell death on the Y axis.

threshold. Our data agree with recent work by Monack group although the interpretation differs. They suggested that caspase-1 can be active without being cleaved and independently of Asc as suggested by the absence of cleaved caspase-1 in culture supernatants (Broz et al., 2010). However, it is possible that caspase-1 was modestly cleaved within the cytosol (which was not examined) and not released in supernatants. Irrespective of its release, the active cytosolic caspase-1 is required and sufficient to restrict *L. pneumophila* growth in Asc^{-/-} macrophages.

On the other hand, the *L. pneumophila* mutant lacking flagellin did not activate cytosolic caspase-1 in macrophages and replicated efficiently. These findings support the idea that cytosolic cleavage of caspase-1 is sufficient to restrict *L. pneumophila* growth within macrophages and requires flagellin. The flagellin mutant replicated more effectively in Asc^{-/-} macrophages than in WT ones although both cells lacked cytosolic caspase-1 activation and activated NF-κB at comparable levels. However, Asc^{-/-} macrophages survive more than WT ones allowing more time for Fla replication. This was because caspase-3 was activated in WT macrophages during late stages of Fla infection but not in Asc^{-/-} macrophages. It is possible that *L. pneumophila* activates caspase-3 directly or through the Bax/Bak pathway which is governed by Asc (Abu-Zant et al., 2005; Fischer et al., 2006). Taken together, these data suggest that Asc is involved in caspase-3 activation and apoptosis induction during *L. pneumophila* infection. Altogether, our data demonstrate that the host employs more than one mechanism to prevent *L. pneumophila* infection and that many of these are governed by Asc.

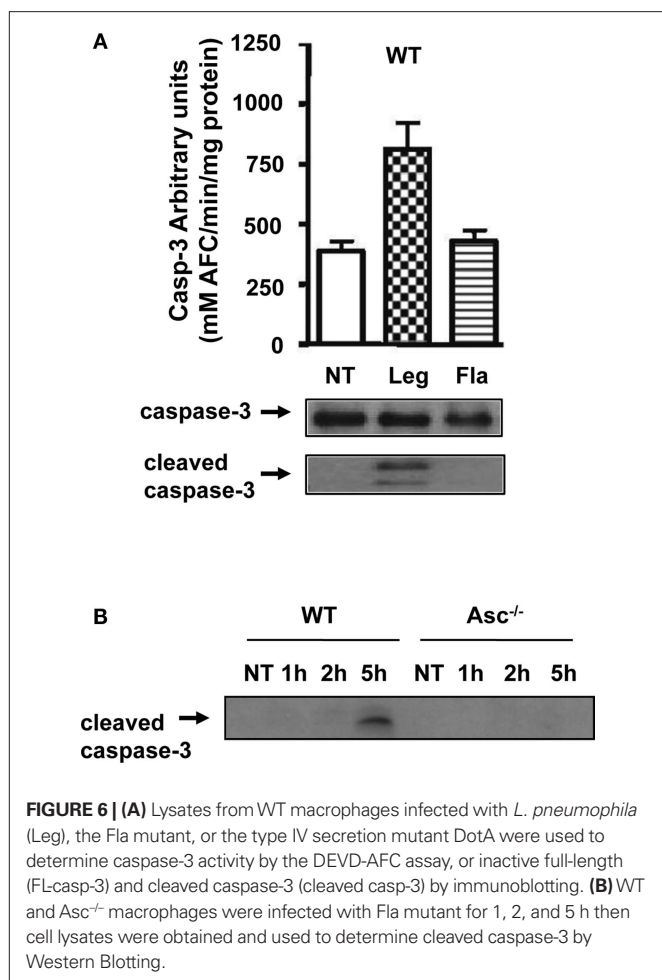
MATERIALS AND METHODS

MICE AND MACROPHAGES

Wild type C57BL/6 (B6) and Asc^{-/-} mice were previously described (Mariathasan et al., 2004). Caspase-1^{-/-} mice were from Dr. Amy Hise at Case Western University. All knockout mice were in a C57BL/6 background. Bone marrow-derived macrophages (BMDMs) were isolated from femurs of 6- to 12-week-old mice and were cultured in IMDM containing 10% heat-inactivated FBS, 20% L cell-conditioned medium, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. After 5 days of incubation, cells were collected and plated in 6-well plates or in 24-well plates in IMDM containing 10% heat-inactivated FBS (Stanley, 1997; Amer et al., 2005, 2006; Akhter et al., 2009).

BACTERIAL STRAINS

Legionella pneumophila strain Lp02, is a thymine auxotrophic derivative of Philadelphia-1 (Brenner et al., 1979; McDade and Shepard, 1979). *L. pneumophila* flagellin (Fla) mutant was previously described (Albert-Weissenberger et al., 2010). *L. pneumophila* was cultured as described previously (Sturgill-Koszycki and Swanson, 2000; Akhter et al., 2009) All experiments were performed at a low MOI of 0.5, followed by centrifugation and rinsing of the wells after 30 min except when otherwise indicated (Derre and Isberg, 2004).



INTRACELLULAR GROWTH OF *L. PNEUMOPHILA*

All experiments were performed at an MOI ranging 0.1–1, followed by centrifugation and rinsing of the wells after 30 min except when otherwise indicated (Derre and Isberg, 2004). All experiments were performed in the absence of ferric nitrate and L-cysteine from the monocytes or macrophage culture medium, to allow *L. pneumophila* multiplication only intracellularly. At designated time points, macrophages were lysed and plated on AYE plates for CFUs (Amer et al., 2006; Abdelaziz et al., 2010). The quantification of the CFU *in vitro* was performed more than four independent times as described (Amer et al., 2006; Abdelaziz et al., 2010).

IMMUNOBLOTTING

Cell extracts of macrophages were prepared and immunoblotted with an antibody that recognizes Nlrc4, Asc (Alexis Biochemicals), caspase-1 (Santa Cruz), IL-1 β (National Cancer Institute), caspase-3 (Cell Signalling), actin (Abcam), followed by appropriate secondary antibody as described (Amer et al., 2006; Abdelaziz et al., 2010).

NF- κ B DNA BINDING ACTIVITY ASSAY

Nuclear extracts of *L. pneumophila* treated or untreated BMDMs from WT and *Asc*^{-/-} mice were prepared as previously described (Akhter et al., 2009). EMSA was used to measure NF- κ B DNA binding activity as described (Sarkar et al., 2006).

QUANTITATIVE PCR

Total RNA was extracted from cells lysed in Trizol (Invitrogen Life Technologies) and 1–2 μ g of the RNA was converted to cDNA by ThermoScript RNase H⁻ Reverse Transcriptase (Invitrogen, Life Technologies). 20–60 ng of the converted cDNA was then used for quantitative PCR with SYBR Green I PCR Master Mix using the StepOne Plus Real Time PCR System (Applied Biosystems). The target gene C_t values were normalized to the C_t values of two housekeeping genes (mouse GAPDH and CAP-1) and expressed as relative copy number (RCN), as described earlier (Gavrillin et al., 2006; Zakharaova et al., 2010). Primers used in RT-PCR are presented in **Table A1** of Appendix. We also evaluated expression of an around 600 genes with Open Array Mouse Inflammatory Panel (BioTrove, Life Technologies). All individual C_t values were normalized to the average of 18 housekeeping genes used in this array, and also expressed as RCN.

CASPASE-3 ACTIVITY ASSAY

Active caspase-3 was determined by the AFC assay, as previously described (Gonzalez-Mejia et al., 2010). Lysates were incubated in a cyto-buffer (10% glycerol, 50 mM Pipes, pH 7.0, 1 mM EDTA, containing 1 mM DTT) containing 20 mM of the tetrapeptide substrate DEVD-AFC. The tetrapeptide was obtained from Enzyme Systems Products (Livermore, CA, USA). Release of free AFC was determined using a Cytofluor 4000 fluorometer (Perseptive Company, Framingham, MA, USA; Filters: excitation; 400 nm, emission; 508 nm).

LDH RELEASE ASSAY

LDH release into cell culture medium was used as an indicator of cell death using NAD⁺ reduction assay, according to the manufacturer (Roche Applied Science). Cells were plated in 12-well plate at the density 0.5 \times 10⁶, and 0.5 MOI of both wild type *L. pneumophila* (Leg) and its flagellin mutant (Fla) were added. Cell culture medium was collected 24 h post infection; clarified from floating bacteria by centrifugation; and used for LDH assay. To determine spontaneous cell death, referred as a negative control, we collected medium from cells incubated the same time without bacteria. To measure total LDH content in cells, referred as a positive control, cells were lysed by adding TritonX-100 (1% final concentration) to the well. Media alone was used as a blank. LDH concentration in the medium was detected at OD 490 nm. Cell death was calculated by the following formula: cytotoxicity (%) = (sample–blank/positive control–blank) \times 100.

STATISTICAL ANALYSIS

Data are displayed as mean of three independent experiments \pm SD. *P* value \leq 0.05 was considered significant.

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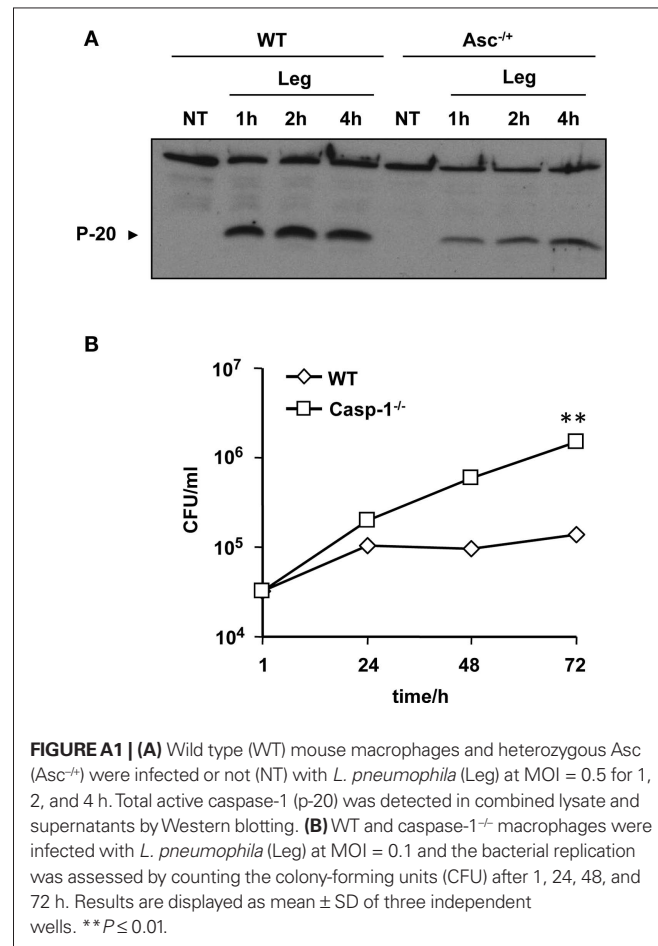
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APPENDIX



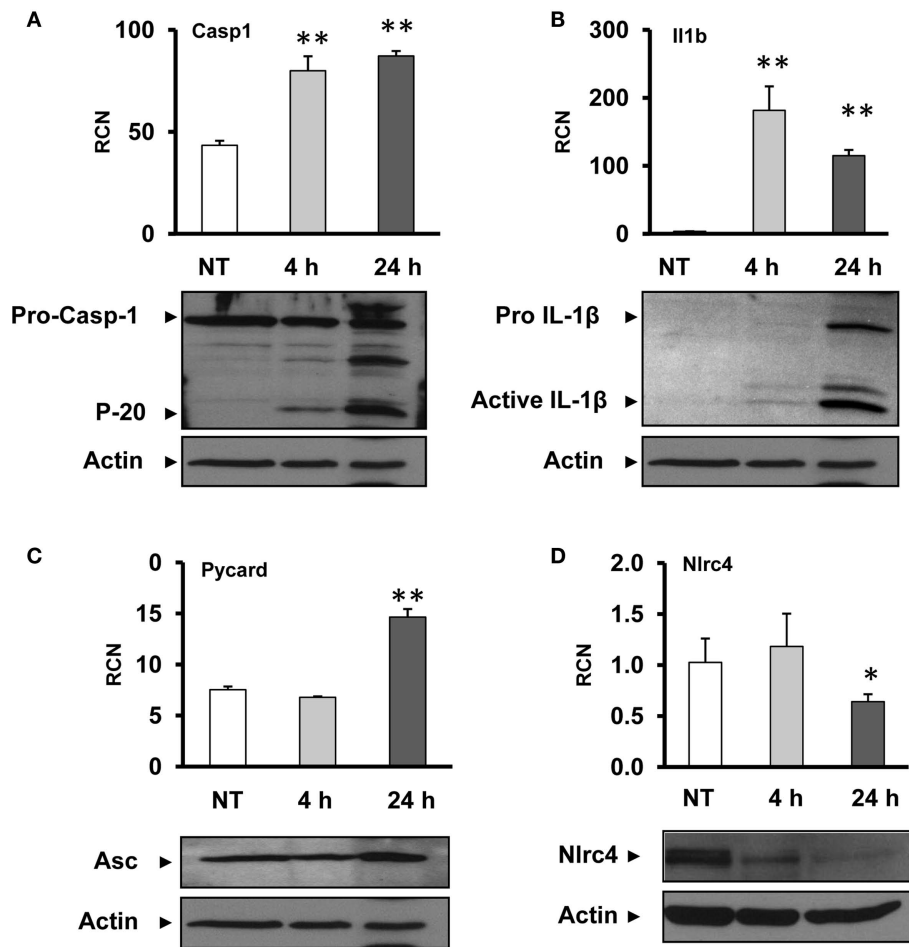


FIGURE A2 | *Legionella pneumophila* differentially regulates the expression of several components of the inflammasome. WT mouse macrophages were infected or not (NT) with *Legionella pneumophila* (Leg) at MOI = 0.5 for 4 and 24 h, then the expressions of (A) pro-caspase-1, (B) pro-IL-1 β , (C) Pycard (Asc), and (D)

Nlrc4 (IpaF) were then assessed on both mRNA (upper panels) and protein levels (lower panels) using RT-PCR and Western blots, respectively. The data of RT-PCR are displayed as mean relative copy numbers (RCN) \pm SD of three independent experiments. * $P \leq 0.05$, ** $P \leq 0.01$. Actin was used as loading control.

Table A1 | Mouse primers used in RT-PCR.

| Gene | Size | | Sequence |
|--------|------|---|-------------------------|
| Casp1 | 145 | F | ACCCTCAAGTTTGGCCCTTT |
| | | R | CCCTCGGAGAAAGATGTTGA |
| Cap1 | 96 | F | GAAGGCGGTGATTTTAACGA |
| | | R | TCCAGCGATTTCTGTCAGT |
| Gapdh | 128 | F | TGGCATTGTGGAAGGGCTCA |
| | | R | TGGATGCAGGGATGATGTTCT |
| Pycard | 173 | F | GCTCACAATGACTGTGCTTAG |
| | | R | TGACCCTGGCAATGAGTGCT |
| Il 1b | 153 | F | CCTGAACCTCAACTGTGAAATGC |
| | | R | GTGCTGCTGTGAGATTTGAAG |
| Nlrc4 | 152 | F | AGGACTTGCCAACTTGAT |
| | | R | TGAAGTAAAGCCATCCGTCAC |