Title

BBS821 research proposal

Abstract

The discovery of the inflammasome has lead to a greater understanding of how hosts detect and respond to pathogens. In most cases, once the inflammasome is activated by intracellular bacteria the host cell undergoes pyroptotic death (proinflammatory programmed cell death). Some organisms, such as *Chlamydia trachomatis,* are able to inhibit the host apoptotic response despite activating the inflammasome. Preventing the host apoptotic response may be one reason for the sever inflammation seen in this infection. In *C. trachomatis* it was recently discovered by Cheng et al. (2008) that knocking out an integral part of the inflammasome resulted in reduced inflammation without a significant increase in bacterial load. This finding may lead to novel therapies that could reduce the pathology of the infection without disabling an integral host defense at the same time.

Similarly, *Rickettsia* is also to prevent host apoptosis and infection results in severe inflammation. I propose *R. rickettsii* activates the inflammasome but concurrent activation of NF-kB by OmpA/OmpB prevents the host from inducing pyroptosis and fighting the infection, resulting in significant inflammation. My first aim is to determine the role of the inflammasome in *R. ricketsii* infection. The similarities between *Rickettsia* and *Chlamydia* suggest they may share a similar mechanism of immune activation. I will first determine if *R. ricketsii* activates the inflammasome upon infection of macrophages by western blot to detect mature caspase-1. IL-1B and IL-18 ELISAs will also be used to measure inflammasome cytokine production. To determine if *Rickettsia* infection is enhanced in the absence of caspase-1, I will measure bacterial loads in WT and caspase-1 knockout mice during Rickettsia infection. Next I will ascertain if inflammation is reduced in the vasculature of caspase-1 knockouts by looking at the histopathology.

Rickettsia prevents the host cells from undergoing apoptosis by activating NF-κB. This allows a robust inflammatory response, which may be mediated by the inflammasome, to cause significant damage. In my second aim I will determine whether Omp (outer membrane protein) A or OmpB is responsible for the activation of NF-κB. These two proteins are known to be immunostimulatory and are likely candidates for activating NF-κB. I will measure NF-kB activation through use of a reporter gene. The role of OmpA and/or OmpB will be determined through use of blocking antibodies and by using purified proteins. Identification of the molecule that induces NF-κB activation coupled with confirmation of inflammasome activation could lead to novel therapy development to treat *Rickettsia* infection.

Background

Rickettsia are obligate, intracellular, gram-negative bacilli. The two major groups that infect humans are the typhus group (*Rickettsia typhi* and *R. prowazekii*) and the closely related spotted fever group (*R. rickettsii*). The spotted fever group infects new cells through actin polymerization of tails they use to shoot themselves through the cytoplasm into a neighboring cell, similar to the mechanism used by *Listeria monocytogenes* or *Shigella flexneri*. The typhus group replicates extensively in the cytosol eventually causing the cell to lyse, thereby releasing the bacteria to infect neighboring cells. *Rickettsia* can infect virtually every cell line *in vitro*, but *in vivo* the main target is endothelial cells, especially vascular endothelial cells which results in widespread endothelial inflammation. The infection of small blood vessels creates widespread endothelial inflammation that can progress to a vasculitic process that may effect any organ (1). The widespread infection ability suggests that the host cell receptor is ubiquitous among cells or *Rickettsia* is able to use multiple receptors. The host cell receptor (s) is as yet unidentified, however the *Rickettsia* outer membrane protein, OmpA, has been implicated in adherence (2). Upon attachment the bacterium is phagocytosed. *Rickettsia* most likely induces phagocytosis since it is able to infect cells that do not normally phagocytose. Post phagocytosis, the bacteria escape the phagosome and enter the cytoplasm through a mechanism mediated by a phospholipase D or a phospholipase A (3). Once in the cytoplasm the bacteria are able to replicate freely.

The intracellular nature and cytosolic location of *Rickettsia* make it a likely candidate for activation of the inflammasome. A number of intracellular bacteria have recently been shown to activate the inflammasome. *L, monocytogenes* and *Salmonella* sp. are sensed through their flagella, while other intracellular bacteria (*Francisella tularensi, S. flexneri, and Chlamydia trachomatis*) activate the inflammasome by unknown bacterial molecules. *R. rickettsii* lacks flagella but may share some of the unknown bacterial components recognized by the inflammasome, or it may have a unique activating component.

The inflammasome is an innate immune complex in the cytosol that is responsible for inducing large amounts of inflammatory cytokines. There are currently four known inflammasomes: Nalp1, Nalp3, Naip5, and Ipaf, although there may also be a fifth that recognizes *F. tularensis*. Upon inflammasome activation, IL-1β and IL-18 are the major cytokines processed by caspase-1, although the inflammasome also induces IL-1α and IL-33 production (4, 5). IL-1B is one of the body's key mediators to microbial invasion and

inflammation (4). Production of the immature form of IL-1B and IL-18 is induced upon TLR stimulation (6). IL-1B secretion is greatly reduced in the absence of TLR2 in a L. monocytogenes infection even though caspase-1 processing is normal, because there is less pro-IL-IB produced (7). When the inflammasome is activated, caspase-1, cleaves the immature cytokine to form the mature cytokine which is then released.

The activity of all known Nalp3 activators can be blocked by inhibiting the efflux of K+ which suggests that a common trigger for this inflammasome is a decrease in intracellular K+, and in fact some of the most potent Nalp3 activators are K+ channels (nigercin, gramidicin, maitotoxin, alpha-toxin) (8). High concentrations of ATP can also stimulate the inflammasome by causing a rapid K+ efflux. It is thought that it mimics the rapid release of ATP from activated platelets, neurons, antigen-stimulated T-cells, and injured cells (5). However, not all inflammasome activation is preceded by TLR activation. It has been shown that induction of the inflammasome by intracellular bacteria containing flagella is independent of TLRs (7, 9-11). Perhaps a different cytosolic receptor for flagella is responsible for inducing production of the immature cytokines.

In most cases, once an intracellular bacterium is sensed by the inflammasome, the cell undergoes pyroptosis. Pyro, meaning fire, refers to the release of proinflammatory mediators and ptosis, meaning falling, is the term commonly used for cell death. Pyroptosis is mediated by caspase-1 instead of the apoptotic factor caspase-3 (12). Pyroptotic cell death shares characteristics with both apoptosis and necrosis. A cell undergoing pyroptosis looses its mitochondrial membrane potential, the DNA undergoes fragmentation, and nuclear condensation occurs while there is also a loss of plasma membrane integrity and release of cytoplasmic content (12). Pyropotic cells are positive for TUNEL staining which originally led people to believe these cells were undergoing apoptosis (12). The release of inflammatory cytokines in pyroptotic death is not what kills the cells. Caspase-1 substrates are involved in cytoskeletal maintenance and energy metabolism and cleavage eventually leads to cell death (13). However not all intracellular bacteria induce pyroptosis, *Chlamydia* prevents pyroptosis presumably through blocking of cytochrome c release from the mitochondria (14). *Rickettsia* also prevents host cell death, and although the mechanisms for blocking host cell death of *Rickettsia* and *Chlamydia* are different, the fact that they both do suggests a similar mechanism of action between these two organisms.

Rickettsia prevents cell death by activating NF-kB. Although it is known that *Rickettsia* activates IKK/NF-kB, the specific interactions between *Rickettsia* and the host

epithelial cell surface are as yet unknown. Rickettsia infection induces NF-kB by increasing the IkBα levels and increasing phosphorylation of IkBα which leads to the degradation of the NF-kB inhibitor (15). Although LPS is known to be a strong activator of NF-kB, it is not likely to be the activator in the case of *Rickettsia*. *Rickettsia* LPS is significantly less endotoxic than other bacterial LPS molecules and Clifton et al. (2005) have preliminary evidence that *Rickettsia* LPS is not involved in stimulating NF-kB activity.

The Outer membrane proteins of *Rickettsia* are likely candidates for the activation of NF-kB. First, the activator of NF-kB is a heat-sensitive protein present on the surface of *Rickettsia* bacteria (15). Also it has been shown that the outer membrane proteins of *Bartonella henselae*, another organism that targets endothelial cells, are responsible for the induction of the NF-kB-dependent upregulation of E-selectin and ICAM-1 (16). Although the outer membrane proteins are not the same between the two organisms, the fact that the *Bartonella* proteins are responsible for a NF-kB response suggests that OmpA and/or OmpB may be responsible for the activation in a *Rickettsia* infection. OmpA may be the more likely candidate since it has already been shown to be the attachment protein (2), but OmpB may also be involved, especially since typhus group *Rickettsia* do not have OmpA yet still activate NF-kB.

I propose *R. rickettsii* activates the inflammasome but concurrent activation of NF-kB through OmpA and/or OmpB prevents the host from fighting the infection through pyroptosis, resulting in the significant inflammation of the vascular endothelium seen in *Rickettsia* infections.

Specific Aim 1

My first goal is to determine the role of the inflammasome in a *Rickettsia ricketsii* infection.

Subaim 1: Determine if R. ricketsii infection induces the inflammasome.

Experimental Procedure:

It will be important to first show that *R. rickettsii* stimulates the inflammasome in vitro. To do this I will isolate macrophages from the bone marrow of wild type mice. These cells will then be primed with E. coli LPS to induce the cells to produce pro-IL-IB. Priming is generally necessary to see a response in vitro, but in this case the priming step may prove unnecessary since most of the intracellular bacteria shown to activate the inflammasome are able to do so without priming. Intracellular bacteria may prime the cells through some component of their outer membrane or something that is secreted making other priming unnecessary. Primed and unprimed cells will then be infected with *R. ricketsii* at 1

macrophage: 1 bacterium up to 1:1000. To evaluate whether or not the inflammasome is activated I will measure IL-1B and IL-18 cytokine secretion into the supernatant by ELISA. If the inflammasome is activated both of these cytokines will be excreted by the infected cells. A positive ELISA will be confirmed by western blot for the mature form of the cytokines since the ELISA will recognize the pro-form and it is possible that cells could lyse during the course of infection and release the pro-form into the supernantant.

For a negative control I will use a mock infection to show that there is no cytokine release in the absence of infection. For a positive control I will infect the cells with bacteria known to activate the inflammasome, *L. monocytogenes. L. monocytogenes* will produce a robust IL-1B and IL-18 response and show that the isolated macrophages are capable of producing an inflammasome response. Finally, to show that the production of IL-1B and IL-18 is due to the inflammasome, I will isolate bone marrow macrophages from caspase-1 knockout mice. These cells will then be infected with *R. rickettsii* or *L. monocytogenes* and cytokines will be measured as before. IL-1B and IL-18 production should be abolished in the caspase-1 KO if the production of these cytokines is dependent on the activation of the inflammasome.

Possible Results and Interpretations:

If *R. rickettsii* infection of primary macrophages activates the inflammasome then infection will induce release of IL-1B and IL-18. This phenotype will be dependent upon caspase-1 and cytokine production will be abolished in the caspase-1 KO. Another possible result is the infection stimulates cytokine production but the cytokines are independent of caspase-1. This could be due to an incomplete knockout and perhaps a residual effect from the remaining caspase-1. It could also be due to a different molecule being able to compensate for the lack of caspase-1 even though this is rather unlikely as it has been shown that caspase-1 is required for processing of IL-1B and IL-18. To address the possibility of a compensating caspase, a double knockout of caspase-1 and ASC to more fully block the inflammasome pathway could be used.

The most likely reason for cytokine production even in the absence of caspase-1 would be that *Ricketsia* infection elicits these proinflammatory cytokines through a different unknown mechanism. It is also possible that *Rickettsia* infection will result in no cytokine production. This would most likely be because *Rickettsia* does not activate the inflammasome. However, lack of detectable cytokines in the supernatant could also mean that *Rickettsia* activates the inflammasome but the bacterium has a mechanism to prevent the

release of mature cytokines. To test this I would lyse the infected cells and do a western blot on the cell supernatants to see if there is mature cytokine in the cytoplasm of the infected cells.

Subaim 2: Determine if a caspase-1 knockout effects Rickettsia rickettsii replication.

Experimental Procedure:

As shown by Cheng et al. (2008), *Chlamydia* activates the inflammasome but this activation does not negatively or positively effect bacterial replication. Given the similarities between *Rickettsia* and *Chlamydia* it seems likely that if *Rickettsia* is shown to activate the inflammasome, it may not actually negatively impact *Rickettsia* replication. To test if Rickettsia replication is influenced by inflammasome activation, I will infect epithelial cells and macrophages of wild type and caspase-1 knockout mice with *R. rickettsii*. I will also infect with *L. monocytogenes* or *C. trachomatis* as controls. I will then measure the bacterial load by plaque forming assay (17) at different time points to ascertain the impact of the presence of the inflammasome on bacterial replication. *C. trachomatis* should show no difference in the absence of caspase-1 whereas *L. monocytogenes* should have enhanced replication. If *Rickettsia* shares a similar mechanism with *Chlamydia* it also should not show a difference in bacterial replication in the absence of caspase-1.

Possible Results and Explanations:

The caspase-1 knockout could have no impact on the bacterial load, and if this bacterium is like *Chlamydia* this is the expected result. The most likely explanation would be that the inflammasome is not involved in controlling *Rickettsia* replication. It could also be that the inflammasome is involved but in the absence of caspase-1 another mechanism of defense can compensate and therefore there is no obvious phenotype.

If a caspase-1 knockout results in increased bacterial load, it could be because the inflammasome is an important component of the host cell defense against infection. Or it could be due to an indirect effect and the cells were less fit and therefore in general less able to fight the infection. The *Chlamydia* control should help distinguish between the two. *Chlamydia* bacterial load remaining unchanged in the caspase-1 knockout would favor the former explanation, but if the *Chlamydia* bacterial load is also increased in the knockout something besides the knockout of caspase-1 would likely be the cause.

A third possibility is that the caspase-1 knockout will result in a decreased bacterial load. The caspase-1 knockout could be involved in inhibiting another component that is better at combating *Rickettsia* infection, and without the negative regulation from the

caspase-1 molecule this other mechanism is able to quickly dispatch the invading Rickettsia bacteria. Although it may seem unlikely, perhaps a defense mechanism of this sort is too dangerous for the cell to leave unchecked and would harm the cell if there were no negative regulator. A decreased bacterial load could also arise inadvertently if the knockout somehow resulted in limiting a component that the bacterium needs to replicate effectively.

Finally, it is possible that a simplified in vitro model system will not show the expected results and the complexity of an in vivo model system will be needed. In this case bacterial replication could be monitored at different time points of an infection of both wild type and caspase-1 deficient mice.

Subaim 3: Determine the inflammatory role of caspase-1 in a Rickettsia ricketsii infection.

Experimental Procedure:

R. rickettsii activation of the inflammasome may not have any effect on bacterial replication. If this is the case I would expect that the inflammasome is involved in producing the robust inflammatory response that is seen in *Rickettsia* infections and is shown to be the case in *Chlamydia* infection (18). To test whether the inflammasome is involved in producing the inflammatory response I will infect wild type and caspase-1 knockout mice with R. rickettsii or *C. trachomatis* as a control. At various time points post-infection I will measure cytokine levels in the blood. To more directly elucidate the effect of the caspase-1 knockout on the inflammation of the vascular endothelium I will also look at the histopathology of the vascular endothelium of sacrificed mice late in the infection. If *Rickettsia* is similar to *Chlamydia* I expect that there will be a reduction in the inflammation of the endothelium. It is also quite possible that the caspase-1 knockout mice will be better able to survive the infection if it is shown that *Rickettsia* replication is not enhanced but inflammation is reduced in the knockout. This could lead to novel therapies directed against caspase-1 that would be effective in reducing the damage from a *Rickettsia* infection.

Possible Result and Interpretations:

If a caspase-1 knockout results in reduced inflammation and inflammatory cytokines in the blood, this could mean that the inflammasome is responsible for the robust inflammatory response that is caused in a *Rickettsia* infection and when the inflammasome is knocked-out the mechanism for the host inflammatory response is abrogated. Reduced inflammation could also be because of a defect in the knockout and maybe the mouse is simply unable to produce an inflammatory response irregardless of whether it acts through

caspase-1 or not. To test this I would stimulate the mice with a compound or pathogen such as LPS to stimulate TNFα production. This would show that the mouse is still able to cause inflammation independent of the inflammasome and there is not a broad defect.

It is possible that there will be no cytokine production in the blood or any inflammation of the vascular endothelium after *Rickettsia* infection. However, this is quite unlikely since *Rickettsia* infection known to cause significant inflammation. If there is no inflammation then something else would likely be wrong with the system that has little to do with caspase-1 and the inflammasome. It is possible that there will be no cytokines in the blood, but if the previous experiments show that *Rickettsia* does stimulate the inflammasome in-vitro it would be expected to stimulate in-vivo.

It is also possible that the caspase-1 knockout will have no impact on the histopathology. This could mean that the inflammasome is not activated and perhaps an in-vitro data showing it was does not apply to in vivo conditions. It could also mean that in vivo there is activation of the inflammasome but it is too weak to measure the response. Whereas in vitro, with the concentrated cells the response is easier to measure, but does not necessarily simulate the real life situation. The caspase-1 knockout could also result in increased inflammation. Again, perhaps caspase-1 is involved in a negative feedback loop, even though it seems rather unlikely that caspase-1 is involved in preventing inflammation when it has been shown to be a potent cause of inflammation.

Specific Aim 2:

My second aim is to ascertain if either OmpA or OmpB, or both, is responsible for activating NF-kB.

Experimental Procedure:

Rickettsia induces the activation of NF-kB to prevent the host cell from undergoing apoptosis. This allows the bacterium enough time to replicate and thereby infect other cells as opposed to dying with the first cell it infects. Although it is known that *Rickettsia* induces NF-kB activation by increased phosphorylation of IkBα (15), it is unknown just what interactions on the surface of the cell lead to this activation. Given that the NF-kB stimulator is a heat-sensitive protein on the surface of *R. rickettsii* (15), the two outer membrane proteins OmpA and OmpB are the most likely molecules. To test whether one or both of these proteins is responsible for the NF-kB activation I would first infect epithelial cells, that have been transfected with a NF-kB luciferase reporter gene that has five tandem repeats of a NF-kB binding element, with *R. rickettsii* that had been preincubated with specific antibodies

to either OmpA or OmpB or *R. conorii* as a control. The antibodies will block interaction of the Omp proteins with any protein on the surface of the epithelial cells. If one of them or both of them is involved then there should be no activation of NF-kB when they are blocked which would mean there would be no measurable luciferase activity. Whereas the *R. rickettsii* that was preincubated with an antibody to *R. conorii* should show activation. If Omps are not involved then blocking should not prevent NF-kB activation.

To further confirm either OmpA, OmpB or both proteins are involved in stimulating NF-kB activation I will isolate OmpA and OmpB through antibody purification as described in Li and Walker (1998). These purified proteins will then be used to stimulate the epithelial cells as before and measure NF-kB activation via reporter. This experiment will confirm the previous experiments results if one or both of the proteins are found to be responsible for NF-kB activation.

Possible Results and Interpretations:

One possible result is OmpA will be shown to activate NF-kB. If blocking of OmpA prevents NF-kB activation and purified OmpA is enough to induce NF-kB activation, it is most likely that OmpA is the protein responsible. However, blocking of OmpA has been shown to prevent attachment and therefore blocking may inadvertently prevent activation of the NF-kB pathway. If the bacteria unable to attach to the host cell, it will be less likely to interact with a surface protein that activates the NF-kB pathway. If the purified protein is unable to stimulate NF-kB then this is most likely the case.

Alternatively OmpB could be shown to activate NF-kB if blocking it prevents activation and the purified protein induces activation. Although this protein was not implicated in attachment, it is still a likely candidate because *R. typhi* that lacks OmpA still activates NF-kB. It is also possible that it is not one or the other of the Omps that activate NF-kB but both. This may be shown if blocking one or the other reduces the NF-kB activation but does not block completely. Blocking both at the same time would show if both proteins are involved, although it is possible there is a third unknown molecule involved in NF-kB activation that is also contributing something. Also both purified proteins stimulating NF-kB may mean they both stimulate or that one stimulates and there is contamination of the other in the purification. To look for contamination, I would run the purified protein on a gel and blot for both OmpA and OmpB. Hopefully there will be no evidence of contamination, but if there were I would complete another cycle of purification to try and get rid of the contaminant.

Of course it is possible that neither of the proteins is involved. However, it seems likely that at least one of the proteins is involved. Blocking OmpA or OmpB may not inhibit NF-kB activation, but it could be that the antibodies used to block them do not block completely and the unblocked protein was sufficient to activate the pathway. If the purified proteins are enough to activate NF-kB there would be evidence that the proteins are involved. If neither of the proteins is involved, a search for the responsible protein would have to be conducted. Since the activator appears to be a protein located on the surface of *Rickettsia*, I would test fractions of membrane preparations until one induced NF-kB activity and then attempt to identify the responsible molecule through additional fractionation and eventually identification by sequencing.

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