Published in final edited form as:

Shock. 2012 January ; 37(1): 47–55. doi:10.1097/SHK.0b013e318234f7ff.

A PROTECTIVE ROLE FOR INFLAMMASOME ACTIVATION FOLLOWING INJURY

Akinori Osuka^{*,§}, Marc Hanschen^{*}, Veit Stoecklein^{*}, and James A. Lederer^{*}

^{*}Department of Surgery (Immunology), Brigham and Women's Hospital/Harvard Medical School, Boston, MA

[§]Submitted as 2011 Shock Society New Investigator Award Nominated Paper, Norfolk, VA

Abstract

The inflammasome is activated in response to pathogen or endogenous danger signals and acts as an initiator and mediator of inflammatory reactions. In this study, we wished to identify whether the inflammasome is activated *in vivo* by injury. And if so, we wanted to characterize the kinetics, the immune cell distribution, and the functional impact of inflammasome activation on the injury response. Since caspase-1 activation is the final product of the inflammasome pathway, we used cleaved caspase-1 p10 and p20 as a measure for inflammasome activation in cells. We first developed a procedure to stain for caspase-1 p10 and p20 by flow cytometry (FACS) in LPS + ATP stimulated spleen cells. This method for measuring caspase-1 activation was validated using FLICA, a fluorescently-tagged specific binding reagent for activated caspase-1. Once validated by *in vitro* studies, we measured caspase-1 activation by FACS in immune cell subsets prepared from the lymph nodes and spleens of sham- or burn-injured mice at different time points. Lastly, the functional significance of inflammasome activation following burn injury was tested in mice treated with the specific caspase-1 inhibitor, AC-YVAD-CMK. The results of *in vitro* studies indicated that ATP and LPS stimulation induced significant caspase-1 activation in dendritic cells, macrophages, and NK cells. This approach also revealed caspase-1 activation in CD4 and CD8 T cells as well as B cells. We then measured caspase-1 activation in cells prepared from the lymph nodes and spleens of sham- or burn-injured mice. Significant caspase-1 activation was detected in macrophages and dendritic cells by 4 hours after injury and peaked by day 1 after injury. FLICA staining confirmed that caspase-1 activation occurred in these cells at 1 day after injury. We also found significant injury-induced caspase-1 activation in NK cells, CD4 T cells, and B cells, but CD8 T cells did not demonstrate caspase-1 activation. Surprisingly, we found that blocking caspase-1 activation with AC-YVAD-CMK *in vivo* caused significantly higher mortality in burn-injured mice ($p < 0.01$). Taken together, these findings document that injury induces inflammasome activation in many immune cell subsets, but primarily in macrophages and that inflammasome activation plays a protective role in the host response to severe injury.

Keywords

Trauma; Danger; DAMPs; IL-1 β ; IL-18; IL-33

Correspondence: James A. Lederer, PhD, Department of Surgery (Immunology), Brigham and Women's Hospital/Harvard Medical School, 75 Francis Street, Boston, MA 02115, P: 617-732-7399 F: 617-264-5222, jlederer@rics.bwh.harvard.edu.

Potential conflict of interest: Nothing to report

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

Severe injuries induce an early and sustained inflammatory immune response that is detectable in more than 90% of patients following trauma (1). This post-injury hyper-inflammatory state, the systemic inflammatory response syndrome (SIRS), depends on the severity of the initial insult and can lead to immunological complications or death by shock and multi-organ failure (MOF) (1, 2). Although it is obvious that injury is the primary trigger of the host immune response to trauma, the molecular pathways responsible for initiating the host response to injury are not well defined. Given the observation that injury primes the innate immune system for both early and prolonged inflammatory phenotype, we hypothesized that activation of the inflammasome pathway might be a central feature of the injury response in mice.

The inflammasome is a multi-protein complex of signaling proteins that assembles following cellular responses to pathogen or host-derived danger signals, *e.g.* bacterial lipopolysaccharide (LPS) and extracellular ATP (3). Other signals that activate the inflammasome include uric acid crystals, silica, and aluminum or asbestos particles (4, 5). Some common protein elements of the inflammasome include ASC (apoptosis associated speck-like protein containing a caspase recruitment domain) and caspase-1, however, distinct inflammasome activating pathways trigger differing Nod-like Receptor (NLRs), including NALP1, NALP3, or IPAF (3). Thus, inflammasome assembly and activating pathways can be unique depending on the specific initiating signal. Importantly, caspase-1 activation is a pivotal element of all known inflammasome activation pathways. Following inflammasome pathway activation, pro-caspase-1 is processed into two cleaved products called p10 and p20. Cleaved caspase-1 p10 and p20 then assemble to form IL-1 converting enzyme (ICE) to process the IL-1 family cytokines, IL-1 β , IL-18, and IL-33, to active cytokines (6). Because the inflammasome is activated by pattern recognition receptors for pathogens or danger signals, it is believed that the inflammasome represents a common innate immune system recognition pathway for pathogen- or tissue-driven inflammatory responses (3, 7).

We initiated this study to find out if a severe injury caused by full-thickness 25% total body surface area burn might activate the inflammasome pathway in mice. To accomplish this, we first developed a flow cytometry method for measuring caspase-1 activation in cells by measuring the cleaved caspase-1 products, p10 and p20, in cells stimulated with LPS, ATP, or LPS + ATP. After establishing this new method, burn-injured mice were tested for caspase-1 activation in spleen and lymph node immune cell subsets at early (1, 2, or 4 hours) and later (days 1, 3, or 7) time points after burn injury. We discovered that burn injury does indeed signal inflammasome activation by 1 day post injury. Moreover, the kinetics of injury-induced inflammasome activation correlated with detectable increases in circulating IL-1 β . Inflammasome activation following burn injury returned to normal levels by 3 days post injury suggesting that injury does not lead to sustained inflammasome activation. To measure the functional importance of injury-induced inflammasome activation, mice were treated with the irreversible caspase-1 inhibitor, AC-YVAD-CMK (YVAD), to measure phenotypic changes in the response to burn injury. We unexpectedly discovered that inhibiting caspase-1 activity *in vivo* caused significant mortality in burn-injured mice. This suggests that injury-induced inflammasome activation may be a protective mechanism for survival from severe injury. Collectively, the data reported here contributes new and fundamental information about the biology of inflammasome pathway activation following injury in a mouse model. The methods and information gained from this work could be applied to studies to investigate if trauma patients show inflammasome activation and whether the inflammasome activation phenotype or genotype affects the morbidity or mortality associated with trauma and its complications.

Materials and Methods

Animals

Male C57BL/6J mice were purchased from The Jackson Laboratories (Bar Harbor, ME). All mice were maintained in our full-barrier animal facility under controlled temperature, humidity and 12-hour light/dark regimen. Mice were acclimatized for at least 1 week prior to use with a diet of standard chow and water *ad libitum*. Experiments were performed on mice aged 6-8 weeks. All animal protocols were approved by the Harvard Medical School Standing Committee on Animal Research and were found to be in accordance with the guidelines of the USDA and the National Institutes of Health (NIH; Bethesda, MD).

Reagents

Cells were prepared in culture medium (C5); RPMI 1640 was supplemented with 5% heat-inactivated FCS, 1 mM glutamine, 10mM HEPES, 100uM nonessential amino acids, penicillin/streptomycin/fungizone, and 2.5×10^{-5} M 2-Mercaptoethanol, all purchased from Gibco-Invitrogen (Grand Island, NY, USA). Cells were fixed and permeabilized for FACS stains using phosphate-buffered saline (PBS) with paraformaldehyde (PFA) and methanol purchased from Sigma Chemical Company (St. Louis, MO). Non-specific binding was prevented by using mouse TruStain FcX reagent from BioLegend (San Diego, CA, USA). Antibodies specific for mouse CD3, CD4, CD8, CD11c, CD19, and F4/80 for FACS stains were purchased from BioLegend (San Diego, CA, USA). Primary antibodies to stain intracellular cleaved caspase-1 (p10 and p20) purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The detection of goat antibodies was performed by secondary incubation with PE-conjugated F(ab')₂, fragment of donkey anti-goat IgG, purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Ac-Tyr-Val-Ala-Asp-chloromethylketone (AC-YVAD-CMK) is a cell-permeable, irreversible inhibitor of caspase-1 and was purchased from Bachem Americas (Torrance, CA, USA). Fluorochrome Inhibitor of Caspase (FLICA) was purchased from Immunochemistry Technologies (Bloomington, MN, USA). FITC-conjugated annexin V is an apoptosis marker and was purchased from BD Biosciences (San Jose, CA, USA).

Mouse Injury Model

The mouse burn injury protocol was performed as described previously (8). Briefly, mice were anesthetized by i.p. injection with ketamine (125 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA, USA) and xylazine (10 mg/kg; Lloyd Laboratories, Shenandoah, IA, USA). The dorsal fur was shaved and mice were placed in a plastic mold that exposes only 25% of their total body surface area (TBSA). Injury was induced by immersing the exposed part of the dorsum to 90°C for 9 seconds in a water bath. This approach causes full-thickness and well-demarcated anesthetic burn injury, thus mice were not given analgesics post-injury. Sham-treated mice underwent the same procedure, but were exposed to water at 24°C for 9 sec. All animals were resuscitated with an i.p. injection of 1 mL of 0.9% pyrogen-free saline. The mortality from burn injury was less than 5%.

Lymph Node and Spleen Cell Preparation

At 1 hour, 2 hours, 4 hours, 1 day, 3 or 7 days post-injury, mice were sacrificed by CO₂ asphyxiation and the draining lymph nodes (axillary, brachial, inguinal) and the spleens were harvested. The lymph nodes and spleens were immediately minced using a sterile wire mesh and cell suspensions were prepared in C5 medium. Red blood cells in splenic cells preparations were lysed using an RBC lysis buffer and cell preparations were washed twice by centrifugation (200 x g, 10 min) in C5 medium, and then strained to remove debris. Cells

were plated in tissue-culture treated round-bottom 96-well plates (Corning Inc, Corning, NY, USA) at 5×10^5 cells/well and immediately fixed by adding 0.15% PFA buffer.

Flow Cytometry

Following 10 min fixation in 0.15% PFA, cells were centrifuged to pellet and then permeabilized by adding ice-cold methanol. Cells were washed by centrifugation and then incubated with 1:50 dilution of mouse TruStain FcX reagent. Fixed and permeabilized cells were stained with APC-labeled antibodies specific for CD11c, CD19, F4/80, or NK1-1. CD4 and CD8 T cells were identified by FITC-labeled anti-CD3 antibody with either APC-labeled CD4 or CD8 antibodies. Apoptotic cells were identified by FITC-labeled annexin V. Annexin V has high affinity for phospholipid phosphatidylserine which is translocated from the inner to the outer leaflet of the plasma membrane in apoptotic cells (9). Cells were incubated overnight with goat anti-mouse primary antibodies specific for cleaved caspase-1 p10 or cleaved caspase-1 p20. After washing by centrifugation, PE-labeled secondary donkey anti-goat IgG (F(ab')₂ fragment) was added as a last step in the staining procedure. Negative controls for cleaved caspase-1 detection were generated by performing the staining procedure without primary Ab. Stained samples were fixed in 0.3% PFA buffer, washed and reconstituted in PBS for flow cytometry using a Miltenyi MacsQuant instrument (Miltenyi Biotec, Auburn, CA, USA).

Goat anti-mouse p10- or p20-specific used in this study were tested *in vitro* to optimize staining conditions prior to use for *in vivo* studies. For these tests, whole spleen cell suspensions were cultured at 37°C, 5% CO₂ at 5×10^5 cells per well in a round-bottom 96 well plate. After one hour pre-incubation, LPS at 100 ng/ml and ATP at 5 mM were added. Cells were processed for intracellular p10 and p20 caspase-1 staining and analyzed by flow cytometry as described above.

Measurement of caspase-1 activity

Cells were stimulated for 1 hour with ATP and LPS before the addition of FAM-YVAD-FMK (5-carboxyfluorescein-Tyr-Val-Ala-Asp-fluoromethylketone, FLICA) according to the manufacturer's instructions (Immunochemistry Technologies, Bloomington, MN, USA). FLICA+ cells were detected and measured by FACS.

Cytokine assays

Culture supernatants were harvested at 48-hours after spleen cell stimulation and tested for IL-1 α , IL-1 β , IL-2, IL-4, IL-6, IL-10, IL-12, p40, IL-12p70, IL-13, IL-18, IL-33, IFN γ , and TNF α by our custom-made Luminex multiplex cytokine-detection bead assay using a Luminex 200 instrument (Luminex Corp., Austin, TX, USA). Blood samples were harvested by cardiac puncture in EDTA anti-coagulant solution (0.1 ml of 169 mM EDTA) from sham or burn mice and were centrifuged at 1000 x g for 10 minutes to prepare plasma. Plasma samples were also tested for cytokine levels using our Luminex bead assay platform. Our cytokine assays routinely attain sensitivity ranges of 1–25,000 pg/ml.

Statistical analysis

The results presented in the study were analyzed by two-way ANOVA and Tukey's multiple comparison tests or Mann-Whitney test using Prism 5.0 software (Graph-Pad Software, Inc., San Diego, CA, USA). Survival differences were evaluated by the log rank test. $P < 0.05$ was considered significant. Data are plotted as mean values $\pm$ SEM.

Results

Optimization of intracellular cleaved caspase-1 detection by flow cytometry

Since caspase-1 cleavage is a central component of inflammasome activation, we needed to develop a procedure to accurately measure and quantify caspase-1 activation in cells. Therefore, we developed and optimized a flow cytometry based method for detecting the cleaved caspase-1 end products, p10 and p20, in cells. We used an *in vitro* approach to develop this method. Spleen cells were prepared from mice and stimulated with LPS, ATP, or LPS + ATP – stimuli that are strong inducers of caspase-1 cleavage. After one hour stimulation, cells were fixed and stained for intracellular p10 and p20 levels as well as cell surface markers for specific immune cell subsets. The FACS histograms shown in Figure 1A illustrate the level of p10 and p20 staining in total splenocytes, CD11c⁺ dendritic cells, and F4/80⁺ macrophages. The plots include unstained controls, unstimulated cells, and cells stimulated with ATP + LPS for 1 hour. These representative histograms illustrate induced changes in p10 expression as measured by an increase in fluorescence intensity in unstimulated versus stimulated spleen cells and FACS-gated immune cell subsets – macrophages and dendritic cells. Moreover, they demonstrate our ability to detect caspase-1 cleavage and activation in cells that are stimulated to activate the inflammasome.

To validate our FACS method, we measured caspase-1 activation by an alternative method using a cell-permeable fluorescent probe called FLICA, which specifically binds to activated caspase-1(10). Spleen cells were activated with LPS and ATP for 1 hour in the presence of FLICA. Cells were then stained to identify macrophages or dendritic cells and analyzed by FACS. The FACS dot plots shown in Figure 1B illustrate that *in vitro* stimulation with LPS and ATP caused detectable caspase-1 activation by the FLICA method. Moreover, the FLICA stain data demonstrate a constitutive level of activated caspase-1 in macrophages and dendritic cells that is increased in response to LPS and ATP stimulation that is similar to what we observed by the FACS staining method. These data further validate the accuracy of our FACS method to detect activated caspase-1 in cells.

Using this FACS staining approach, we assessed the kinetics of caspase-1 cleavage in LPS and ATP stimulated spleen cells. Spleen cells were stimulated with LPS, ATP, or LPS + ATP for 15 min, 30 min, 1, 2, or 4 hours and then stained for p10 and p20 (Figure 2A). As shown, caspase-1 activation occurred when cells were stimulated with ATP, but not LPS alone. However, the combination of LPS and ATP caused sustained caspase-1 activation that was still detectable at 4 hours after stimulation, while ATP stimulation alone showed only early caspase-1 activation. The data plotted in Figure 2B shows caspase-1 p10 or p20 levels in macrophages, dendritic cells, NK cells, CD4 T cells (CD3⁺/CD4⁺ cells), CD8 T cells (CD3⁺/CD8⁺ cells) and B cells (CD19⁺ cells) at 1 hour after LPS + ATP. Surprisingly, we found evidence for significant caspase-1 activation in all of these immune cell subsets. However, p20 staining was not detected in CD4 T cells and B cells.

After finding *in vitro* conditions that induced significant caspase-1 activation, we wished to determine if activating caspase-1 in cells by ATP or LPS + ATP stimulation could induce caspase-1 dependent cytokine production. Spleen cells were stimulated with LPS, ATP, or LPS + ATP and supernatants were tested for cytokine levels by multiplex cytokine assays. The data plotted in Figure 3 illustrate that these different stimuli induced different levels and types of cytokines. As anticipated, LPS stimulation alone induced significant levels of TNF α and IL-6. In contrast, IL-1 β was not induced by LPS, but was significantly induced by ATP alone and combined stimulation with LPS + ATP. Surprisingly, the addition of ATP markedly suppressed TNF α and IL-6 production indicating that extracellular ATP stimulation suppresses TLR4-induced cytokines. Most important, the cytokine profiling data confirm that the experimental conditions that induced caspase-1 activation in cells also

specifically induced high-level production of the caspase-1-dependent cytokines, IL-1 β and IL-18.

Time course study for caspase-1 activation in innate immune cell subsets after burn injury

Following sham or burn injury, cells were prepared from the draining lymph nodes or spleens and FACS stained for CD11c, F4/80, NK 1.1, and cleaved caspase-1 p10 or p20 at 1, 2, 4 hours and 1, 3, 7 days after injury. We observed an increase in p10 in lymph node macrophages by 2 hours after injury, while significant increases in p10 in splenic macrophages and dendritic cells were seen at 1 day after injury (Figure 4A). Also, this injury-induced increase in p10 levels in splenic macrophages and dendritic cells peaked by day 1 after injury and returned to normal levels by 3 days post injury. Macrophages appeared to express the highest levels of p10 after injury as compared to dendritic cells and NK cells. Interestingly, burn injury also caused an increase in p20 levels in macrophages, dendritic cells, and NK cells, but the change was not statistically significant. To confirm and extend our findings, we measured caspase-1 activation in these spleen cell subsets with FLICA after sham versus burn injury. As shown in Figure 4B, we detected an increase in FLICA⁺ macrophages, dendritic cells, and NK cells prepared from the spleens of mice at 1 day after burn as compared to sham injury. The FLICA stains support the FACS results, but also show burn-induced caspase-1 activation in NK cells.

Next, we wished to determine which immune cell subsets show injury-induced caspase-1 activation. To accomplish this, we identified dendritic cells, macrophages, NK cells, CD4 and CD8 T cells (CD3⁺), and B cells by cell-surface staining and measured p10 or p20 levels by intracellular staining. As shown in Figure 5, these data indicate that burn injury induced significant caspase-1 activation in all but CD8⁺ T cells. Again, innate immune cell populations showed higher caspase-1 activation than CD4 T cells or B cells. Additionally, we examined whether cells showing caspase-1 activation were undergoing apoptosis by measuring annexin V binding on p10 or p20 expressing immune cell subsets. We observed significant increases in annexin V binding on most immune cell subsets prepared from the spleens of mice at 1 day after burn injury – the exception was CD8 T cells. However, annexin V positive cells did not show higher levels of p10 or p20 expression (Table 1). This finding suggests that the cells showing increased caspase-1 activation following burn injury are not undergoing apoptosis.

Finally, we wanted to sequentially measure circulating cytokine levels in sham and burn mice to determine if the kinetics of caspase-1 activation might coincide with the production of IL-1 β , IL-18, and IL-33. We prepared plasma samples from mice at 1, 2, 4 hours, and 1, 3, 7 days after sham or burn injury and these samples were screened for cytokine levels by multiplex cytokine assays. The multiplex assay approach allowed us to screen for many different cytokines, but only IL-1 β , IL-6, and TNF α were found to be induced by burn injury at these time points (Figure 6). Interestingly, we did not detect significant levels of these cytokines until 1 day post injury. The levels of IL-1 β and IL-6 returned to normal by 7 days after burn injury, but TNF α levels remained high. These data indicate that injury-induced IL-1 β coincides with the kinetics of burn-induced caspase-1 activation and with burn-induced increases in other pro-inflammatory mediators.

Functional significance of inflammasome activation following burn injury

To determine the functional significance of inflammasome activation after burn injury, we treated mice with an irreversible caspase-1 specific inhibitor called AC-YVAD-CMK (YVAD). This inhibitor blocks inflammasome assembly by binding specifically to caspase-1 subunits (11). Dose response studies were performed by treating mice with 1, 5, or 10 mg/kg at 2 hours before burn injury and measuring caspase-1 activation by FACS at 1 day after

injury. As shown in Figure 7A, treating mice with 10 mg/kg YVAD potently blocked caspase-1 activation as judged by p10 and p20 staining in spleen cells, while lesser doses did not effectively block caspase-1 activation. After determining an effective dose for YVAD, we tested the impact of blocking inflammasome activation on the burn injury response in mice. Unexpectedly, we found that YVAD treatment caused significantly higher mortality in burn-injured mice ($p < 0.01$) (Figure 7B). To confirm that inhibiting caspase-1 activation caused higher mortality in burn-injured mice, we performed an additional YVAD dose study comparing 1, 5, and 10 mg/kg treatments. We observed that 10 mg/kg treated mice showed high mortality, while mice treated with lower and less effective doses of 1 or 5 mg/kg showed no burn-injury induced mortality (Figure 7C). Taken together, these results support the conclusion that injury-induced inflammasome activation is protective.

Given the high mortality in YVAD treated burn mice, we wanted to determine why YVAD treated burn mice died. Blood was collected from YVAD-treated mice at 1 day after burn injury to test for evidence of bacteremia by performing blood cultures. No bacteria were detected in blood samples prepared at 1 or 2 days after burn injury in YVAD-treated or untreated mice (data not shown). In addition, we treated groups of YVAD-treated burn mice with oral antibiotics (sulfamethoxazole/trimethoprim) and found no significant difference in survival between untreated and antibiotic treated mice (data not shown, $n = 16$ mice per group). Lastly, we measured plasma cytokine levels at 1 day after burn injury in untreated versus YVAD-treated mice. As suspected, IL-1 β was significantly suppressed by YVAD treatment (Figure 8). However, IL-6 and IL-33 were significantly higher in plasma prepared from YVAD-treated as compared to untreated burn mice. Moreover, we found significantly lower levels of counter-inflammatory type cytokines, IL-4 and IL-13, in plasma from YVAD-treated burn mice as compared to untreated burn mice. These findings suggest that blocking inflammasome activation by YVAD treatment may have caused higher inflammation and organ damage in burn-injured mice by increasing inflammatory and reducing counter-inflammatory mediators. The combination of increased inflammation with no evidence of bacteremia suggests that the inflammasome plays a key role in controlling the immune response to severe injury.

Discussion

It is generally accepted that burn injury and trauma primes the innate immune system to enhance inflammatory responses to pathogen associated molecular signals (PAMPs), however, the molecular pathways responsible for injury-induced immune system activation are not well defined (12, 13). Research addressing how the immune system responds to injured and dead cells has identified an expanding list of molecules that signal inflammatory-type responses by receptor mediated signals (4, 14-16). These endogenous molecules are collectively referred to as danger molecules or damage associated molecular patterns (DAMPs) since they are generated when there is cell death or damage caused by pathogens or injury (17, 18). A central feature of danger signals is that they evoke an inflammatory-type response to initiate both innate and adaptive immune responses (19). The inflammasome was identified as a central feature of the danger response because most of the extracellular or intracellular signals that activate the inflammasome are considered danger molecules or alarmins (14, 20-22). This study was initiated to test the hypothesis that the inflammasome is activated in immune cells in an *in vivo* injury model since little is known about the *in vivo* nature of injury-induced inflammasome activation. Moreover, we wished to test the hypothesis that sustained inflammasome activation following burn injury in mice might contribute the development of SIRS and the two-hit response that occurs at later time points after injury. Our findings presented here establish that burn injury does induce inflammasome activation, but that its activation is shorter lived than anticipated - peak inflammasome activation occurred by 1 day post-injury and returning to normal levels at 3

and 7 days after injury. This observation is novel and indicates that injury induces early, but not sustained inflammasome activation. Thus, the inflammasome appears to be involved in initiating the host response to injury, but may not contribute to the SIRS and two-hit response phenotype that occurs after severe injury.

As part of this study, we developed a new FACS-based method to detect caspase-1 activation in cells. To our knowledge, this is the first study to characterize caspase-1 activation in cells by measuring changes in p10 and p20 levels by flow cytometry. A major advantage of using FACS to measure caspase-1 activation in cells versus Western immunoblot is that it allows for identifying the immune cell subsets showing caspase-1 or inflammasome activation. We used *in vitro* experiments to develop and validate our FACS-based method because it allowed us to stimulate inflammasome activation under conditions that are well known to activate the inflammasome. Preliminary studies were performed to optimize the cell fixation conditions, staining antibody concentrations, and stain incubation times. We found that light fixation with 0.15% PFA, followed by methanol fixation provided that best fixation conditions for these stains. Interestingly, we also found that overnight incubation with p10 or p20 antibodies was needed to generate the best staining signal. As illustrated in Figure 1, spleen cells stimulated with LPS + ATP showed detectable shifts in MFI for p10 and p20 by 1 hour after stimulation. FACS staining was then used to demonstrate that caspase-1 activation by LPS + ATP stimulation is not restricted to macrophages. Interestingly, we observed significant induction of either cleaved caspase p10 or p20 in dendritic cells, NK cells, CD4 T cells, CD8 T cells, and B cells at 1 hour post LPS + ATP stimulation. Macrophages, dendritic cells, and NK cells showed higher level constitutive p10 and p20 than T or B cells. The observed caspase-1 activation in NK cells, T cells, and B cells is novel and suggests that caspase-1 activation occurs in a wider assortment of immune cell types than previously appreciated. Burn injury also induced caspase-1 activation in these similar immune cell subsets. Again, the development of a FACS-based method to detect the p10 and p20 subunits of caspase-1 made it possible to discover that caspase-1 activation can occur in many different immune cell subsets.

Cytokine multiplex assays were used to measure ATP, LPS, or ATP + LPS induced cytokine production. LPS stimulation alone induced significant TNF α and IL-6 production by spleen cells, while IL-1 β and IL-18 were induced by ATP or ATP + LPS stimulation. This finding confirmed that activating caspase-1 *in vitro* caused significant production of the caspase-1 dependent cytokines, IL-1 β and IL-18. While performing these experiments, we also found that adding ATP to LPS-stimulated spleen cells blocked LPS-induced TNF α and IL-6 production. This result was surprising and suggests that extracellular ATP has potent anti-inflammatory activity that may be independent of its caspase-1 activating activity. The processing or degradation ATP to adenosine and adenosine receptor signaling may be responsible for suppressing TNF α and IL-6 (23).

The results from time course injury studies revealed that burn injury induces inflammasome activation as measured by caspase-1 activation. Given that inflammasome activation occurred early after burn injury, we anticipated that it may be involved in initiating the inflammatory response to burn injury. This observation also suggested to us that blocking inflammasome activation *in vivo* might evoke a beneficial response to injury by reducing inflammation-driven host responses as has been shown in other models of tissue injury (24-26). However, we found that treating mice with the caspase-1 specific inhibitor AC-YVAD-CMK showed a significant increase in injury-induced mortality by 4 days after burn injury. This finding led us to conclude that inflammasome activation and in particular caspase-1 activation plays an unanticipated protective role in the host response to injury. The *in vivo* dose of AC-YVAD-CMK used to inhibit caspase-1 activation was found to be an effective dose as judged by inhibition of caspase-1 activation and IL-1 β production

following burn injury. Furthermore, we observed that suboptimal inhibitory doses of less than 10 mg/kg did not cause increased mortality and did not inhibit caspase-1 activation in burn-injured mice. The results of these dose response studies further confirm that inflammasome activation by injury promotes better survival from severe injury. Other reports that used AC-YVAD-CMK to block caspase-1 *in vivo* did not describe changes in mortality, but did demonstrate that blocking caspase-1 activation reduced inflammatory responses to tissue injury (24, 27). Thus, our findings are unique among others that used AC-YVAD-CMK to block inflammasome activation *in vivo*.

We addressed potential causes for increased mortality in burn-injured, YVAD-treated mice. First, we suspected that these mice may die of infection due to reduced anti-microbial immunity. However, we did not detect bacteria in the blood of burn + YVAD-treated mice. In addition, antibiotic treatment did not significantly protect burn + YVAD-treated mice. These findings suggest that bacteremia was not the cause of death. Subsequently, we tested for changes in plasma cytokine levels in burn as compared to AC-YVAD-CMK treated mice. We discovered that YVAD treatment significantly blocked burn-induced IL-1 β as well as IL-4 and IL-13. In contrast, IL-33 and IL-6 levels were significantly higher in YVAD-treated burn mice. These cytokines have been shown to be important mediators of the acute phase response, tissue damage, and shock (28-30). The cytokine profile data suggest that mice may be dying of cytokine-induced shock or organ damage.

In summary, the studies presented here demonstrate that severe injury caused by burn injury induces early, but not late or sustained inflammasome activation in a variety of immune cell subsets. Thus, the inflammasome does not appear to play a direct role in mediating the SIRS and the two-hit response to injury. Instead, we discovered that injury-induced inflammasome activation is protective. Since we rarely observe greater than 5 percent direct mortality in our mouse injury model, this suggests that inflammasome-activating signals promote a beneficial response to severe injury. Therefore, it is possible that treatment strategies that target inflammasome activation pathways could be used to protect patients from organ damage and shock caused by severe injuries. For this reason, future work will seek to define the pathways and factors that induce inflammasome activation following injury.

Acknowledgments

This study was supported by grant funding from the National Institutes of Health R01GM035633-23, RO1GM57664-09, and R33AI080565-03.

This study was supported by grant funding from the National Institutes of Health 5R01GM035633-23 and 2R01GM57664-09

Abbreviations

MFI	mean fluorescence intensity
LPS	<i>lipopolysaccharide</i>
ATP	adenosine triphosphate

References

1. Hoover L, Bochicchio GV, Napolitano LM, Joshi M, Bochicchio K, Meyer W, Scalea TM. Systemic inflammatory response syndrome and nosocomial infection in trauma. *J Trauma*. 2006; 61(2):310–316. discussion 316-317. [PubMed: 16917443]
2. Sauaia A, Moore FA, Moore EE, Lezotte DC. Early risk factors for postinjury multiple organ failure. *World J Surg*. 1996; 20(4):392–400. [PubMed: 8662125]

3. Martinon F, Mayor A, Tschopp J. The inflammasomes: guardians of the body. *Annu Rev Immunol.* 2009; 27:229–265. [PubMed: 19302040]
4. Rock KL, Hearn A, Chen CJ, Shi Y. Natural endogenous adjuvants. *Springer Semin Immunopathol.* 2005; 26(3):231–246. [PubMed: 15609001]
5. Shi Y, Evans JE, Rock KL. Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature.* 2003; 425(6957):516–521. [PubMed: 14520412]
6. Arend WP, Palmer G, Gabay C. IL-1, IL-18, and IL-33 families of cytokines. *Immunol Rev.* 2008; 223:20–38. [PubMed: 18613828]
7. Zedler S, Faist E. The impact of endogenous triggers on trauma-associated inflammation. *Curr Opin Crit Care.* 2006; 12(6):595–601. [PubMed: 17077693]
8. Fujimi S, Lapchak PH, Zang Y, MacConmara MP, Maung AA, Delisle AJ, Mannick JA, Lederer JA. Murine dendritic cell antigen-presenting cell function is not altered by burn injury. *J Leukoc Biol.* 2009; 85(5):862–870. [PubMed: 19228816]
9. Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J Immunol Methods.* 1995; 184(1):39–51. [PubMed: 7622868]
10. Broz P, Newton K, Lamkanfi M, Mariathasan S, Dixit VM, Monack DM. Redundant roles for inflammasome receptors NLRP3 and NLRC4 in host defense against Salmonella. *J Exp Med.* 2010; 207(8):1745–1755. [PubMed: 20603313]
11. Rozman-Pungercar J, Kopitar-Jerala N, Bogyo M, Turk D, Vasiljeva O, Stefe I, Vandenabeele P, Bromme D, Puizdar V, Fonovic M, Trstenjak-Prebenda M, Dolenc I, Turk V, Turk B. Inhibition of papain-like cysteine proteases and legumain by caspase-specific inhibitors: when reaction mechanism is more important than specificity. *Cell Death Differ.* 2003; 10(8):881–888. [PubMed: 12867995]
12. Paterson HM, Murphy TJ, Purcell EJ, Shelley O, Kriynovich SJ, Lien E, Mannick JA, Lederer JA. Injury primes the innate immune system for enhanced Toll-like receptor reactivity. *J Immunol.* 2003; 171(3):1473–1483. [PubMed: 12874240]
13. Maung AA, Fujimi S, MacConmara MP, Tajima G, McKenna AM, Delisle AJ, Stallwood C, Onderdonk AB, Mannick JA, Lederer JA. Injury enhances resistance to Escherichia coli infection by boosting innate immune system function. *J Immunol.* 2008; 180(4):2450–2458. [PubMed: 18250454]
14. Li H, Ambade A, Re F. Cutting edge: Necrosis activates the NLRP3 inflammasome. *J Immunol.* 2009; 183(3):1528–1532. [PubMed: 19596994]
15. Gasse P, Riteau N, Charron S, Girre S, Fick L, Petrilli V, Tschopp J, Lagente V, Quesniaux VF, Ryffel B, Couillin I. Uric acid is a danger signal activating NALP3 inflammasome in lung injury inflammation and fibrosis. *Am J Respir Crit Care Med.* 2009; 179(10):903–913. [PubMed: 19218193]
16. Franchi L, Eigenbrod T, Munoz-Planillo R, Nunez G. The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis. *Nat Immunol.* 2009; 10(3):241–247. [PubMed: 19221555]
17. Matzinger P. The danger model: a renewed sense of self. *Science.* 2002; 296(5566):301–305. [PubMed: 11951032]
18. Seong SY, Matzinger P. Hydrophobicity: an ancient damage-associated molecular pattern that initiates innate immune responses. *Nat Rev Immunol.* 2004; 4(6):469–478. [PubMed: 15173835]
19. Bianchi ME. DAMPs, PAMPs and alarmins: all we need to know about danger. *J Leukoc Biol.* 2007; 81(1):1–5. [PubMed: 17032697]
20. Pedra JH, Cassel SL, Sutterwala FS. Sensing pathogens and danger signals by the inflammasome. *Curr Opin Immunol.* 2009; 21(1):10–16. [PubMed: 19223160]
21. Iyer SS, Pulsikens WP, Sadler JJ, Butter LM, Teske GJ, Ulland TK, Eisenbarth SC, Florquin S, Flavell RA, Leemans JC, Sutterwala FS. Necrotic cells trigger a sterile inflammatory response through the Nlrp3 inflammasome. *Proc Natl Acad Sci U S A.* 2009; 106(48):20388–20393. [PubMed: 19918053]
22. Goncalves GM, Zamboni DS, Camara NO. The role of innate immunity in septic acute kidney injuries. *Shock.* 2010; 34(Suppl 1):22–26. [PubMed: 20523275]

23. G, Hasko; Kuhel, DG.; Salzman, AL.; Szabo, C. ATP suppression of interleukin-12 and tumour necrosis factor- α release from macrophages. *Br J Pharmacol.* 2000; 129(5):909–914. [PubMed: 10696089]
24. Sozen T, Tsuchiyama R, Hasegawa Y, Suzuki H, Jadhav V, Nishizawa S, Zhang JH. Role of interleukin-1 β in early brain injury after subarachnoid hemorrhage in mice. *Stroke.* 2009; 40(7): 2519–2525. [PubMed: 19461019]
25. Liu HF, Liang D, Wang LM, Zhou N, Yao CW, Hong T, Tang DS, Chen XW. Effects of specific interleukin-1 β -converting enzyme inhibitor on ischemic acute renal failure in murine models. *Acta Pharmacol Sin.* 2005; 26(11):1345–1351. [PubMed: 16225757]
26. Karaoglan A, Kaya E, Akdemir O, Sagmanligil A, Bilguvar K, Cirakoglu B, Sahan E, Erdogan N, Barut AE, Colak A. Neuroprotective effects of Ac.YVAD.cmk on experimental spinal cord injury in rats. *Surg Neurol.* 2008; 69(6):561–567. [PubMed: 18262241]
27. Abreu MT, Fukata M, Arditi M. TLR signaling in the gut in health and disease. *J Immunol.* 2005; 174(8):4453–4460. [PubMed: 15814663]
28. Remick DG, Bolgos GR, Siddiqui J, Shin J, Nemzek JA. Six at six: interleukin-6 measured 6 h after the initiation of sepsis predicts mortality over 3 days. *Shock.* 2002; 17(6):463–467. [PubMed: 12069181]
29. Oboki K, Ohno T, Kajiwara N, Arae K, Morita H, Ishii A, Nambu A, Abe T, Kiyonari H, Matsumoto K, Sudo K, Okumura K, Saito H, Nakae S. IL-33 is a crucial amplifier of innate rather than acquired immunity. *Proc Natl Acad Sci U S A.* 107(43):18581–18586. [PubMed: 20937871]
30. Pushparaj PN, Tay HK, H'Ng SC, Pitman N, Xu D, McKenzie A, Liew FY, Melendez AJ. The cytokine interleukin-33 mediates anaphylactic shock. *Proc Natl Acad Sci U S A.* 2009; 106(24): 9773–9778. [PubMed: 19506243]

Figure 1. Detection of cleaved caspase-1, p10 and p20, by flow cytometry

Spleen cells were stimulated with ATP (5 mM) and LPS (100 ng/ml) for 1 hour, then stained for caspase-1 p10 and p20 as described in materials and methods. The plots in (A) are representative histograms showing the level of caspase-1 p10 or p20 detection in all cells (live cell gate), DCs (CD11c+ gate) or Macs (F4/80+ gate). As indicated, the solid line = negative control (no primary p10 or p20 antibody); the grey-filled line = unstimulated cells; the solid bold line = 1 hour stimulation with ATP + LPS. The FACS dot plots shown in (B) represent FLICA (Fluorescent-Labeled Inhibitor of Caspase: FAM-YVAD-FMK) staining intensity in all live cells, gated DCs (CD11c+) or Macs (F4/80+). The boxed region with the dot plots identifies the activated caspase-1 in these cells. These FACS plots are representative of 3 experiments using 3 mice per group.

Figure 2. Kinetics of caspase-1 activation by ATP + LPS stimulation

In (A), spleen cells were stimulated with LPS, ATP, or ATP + LPS for 15 minutes, 30 minutes, 1 hour, 2 hours or 4 hours and then stained for caspase-1 p10 and p20 by FACS. The data is plotted as mean $\pm$ SEM of mean fluorescence intensity (MFI) staining for p10 and p20 staining in all spleen cells and includes data from 3 independent experiments. The # indicates significant differences between unstimulated and ATP, the * indicates significant differences between ATP + LPS stimulated groups as determined by two-way ANOVA and Tukey's multiple comparison, $p < 0.05$. The data in (B) show the MFI staining for the indicated immune cell subsets in spleen cells stimulated with ATP + LPS for 1 hour. Antibodies specific for CD11c (DCs), F4/80 (Macs), NK1.1 (NK cells), CD3/CD4 (CD4 T cells), CD3/CD8 (CD8 T cells), and CD19 (B cells) were used to identify immune cell subsets to determine p10 and p20 staining. Data are plotted as mean $\pm$ SEM of MFI from 4 independent experiments. The * indicates significant differences between unstimulated and ATP + LPS stimulated cells by paired t-test.

Figure 3. Cytokine production in response to inflammasome activation by ATP + LPS
Spleen cells were stimulated with LPS, ATP or ATP + LPS as described in the materials and methods section. Culture supernatants were harvested at 48 hours and tested for IL-1 β , IL-18, TNF α , and IL-6 levels by multiplex cytokine assays. Data shown is from two independent experiments of 4 mice per group and is plotted as mean $\pm$ SEM. The * indicates significance, $p < 0.05$, between unstimulated cells.

Figure 4. Kinetics of *in vivo* inflammasome activation by burn injury

(A) Mice underwent sham or burn injury and the draining lymph nodes or spleens were harvested at 1 hour, 2 hours, 4 hours, 1 day, 3 days, or 7 days. Cells were stained with antibodies specific for F4/80 (Macs), CD11c (DCs), or NK1.1 (NKs) and for caspase-1 p10 or p20 as described in materials and methods. Data shown is the mean $\pm$ SEM of MFI from 3 independent experiments using 5 mice per group. The * indicates significance between sham and burn for Macs and the # indicates significance between sham and burn for DCs, $p < 0.05$ by two-way ANOVA and Tukey's multiple comparisons. The dot plots in (B) illustrate the FLICA staining patterns for caspase-1 activation in all cells, dendritic cells (DCs), macrophages (Macs), and NK cells (NKs) in spleen cells prepared from sham or burn mice at 1 day after injury. Plots are representative stains from 5 mice per groups. Burn injury induced increased FLICA staining intensity and percentages in dendritic cells, macrophages, and NK cells.

Figure 5. Caspase-1 activation profiles in immune cell subsets following burn injury

Mice underwent sham or burn injury and spleen cells were prepared at 1 day after injury. Cells were stained for the indicated immune cell subsets and p10 or p20 expression by FACS. The data are plotted as mean $\pm$ SEM of MFI from 4 mice per group and represent 2 independent experiments. The * indicates significant differences between sham and burn groups for the indicated immune cell subsets, $p < 0.05$ by Mann-Whitney test.

Figure 6. Kinetics of burn-induced cytokine production

Mice underwent burn injury and plasma samples were prepared at 1, 2, 4 hours, 1 day, 3 or 7 days after burn injury. Plasma from normal C57BL/6J mice was used as a baseline comparison control. Plasma samples were tested for cytokine levels by multiplex bead assays that detect IL-1 α , IL-1 β , IL-2, IL-4, IL-6, IL-10, IL-12p40, IL-12p70, IL-13, IL-18, IL-33, IFN γ , and TNF α . The data are plotted as mean $\pm$ SEM pg/ml of cytokines. The * indicates significant differences between normal serum cytokine levels and burn-induced cytokine levels, $p < 0.05$ by ANOVA and Tukey's multiple comparison test.

Figure 7. Blocking caspase-1 activation by AC-YVAD-CMK treatment causes burn-induced mortality

In (A), mice were given saline or AC-YVAD-CMK at 1 mg/kg, 5 mg/kg, and 10 mg/kg doses by i.p. injection at 2 hours before burn injury. Spleen cells were harvested and tested for caspase-1 p10 and p20 levels by FACS. The * indicates significant differences between saline and AC-YVAD-CMK treated mice, $p<0.05$ by two-way ANOVA and Tukey's multiple comparisons. The survival plot in (B) shows the effect of giving mice AC-YVAD-CMK at 10 mg/kg by i.p. injection. Mice were monitored daily for mortality for 14 days after sham or burn injury and the indicated treatments. The * indicates significant difference in survival between AC-YVAD-CMK-treated burn mice and all other groups, $p<0.05$ by log

rank test, n=10 mice per group for shams and n=22 mice per group for burns. The survival plot in (C) shows the dose effect of AC-YVAD-CMK (1, 5, or 10 mg/kg) treatment on burn injury survival. The ** indicates $p=0.08$ by log rank test for 6 mice per group.

Figure 8. Influences of AC-YVAD-CMK treatment of injury-induced cytokine levels in plasma
Mice were treated with saline or AC-YVAD-CMK (10 mg/kg) by i.p. injection 2 hours before sham or burn injury. Plasma samples were prepared at 1 day after injury and tested for cytokine levels by multiplex bead cytokine assays specific for IL-1 α , IL-1 β , IL-2, IL-4, IL-6, IL-10, IL-12p40, IL-12p70, IL-13, IL-18, IL-33, IFN γ , and TNF α . The data are plotted as mean $\pm$ SEM pg/ml of those cytokines that were detected and showed significant levels for $n=6$ mice per group. The * indicates significant differences between saline and AC-YVAD-CMK-treated burn-injured mice, $p<0.05$ by two-way ANOVA and Tukey's multiple comparisons.

Table 1

Annexin V positive cells in immune cell subsets and caspase-1 activation profiles in annexin V positive cells following burn injury. Mice underwent sham or burn injury and spleen cells were prepared at 1 day after injury. Cells were stained for the indicated immune cell subsets, annexin V, and p10 or p20. The data shown are mean (SEM) from 5 mice per group.

Cell Types	Injury	% Annexin V+	MFI p10	MFI p20
Macrophages	Sham	16.96 (0.67)	70.40 (8.88)	26.20 (6.16)
	Burn	25.02 (2.54)*	84.60 (12.48)	31.80 (6.88)
Dendritic Cells	Sham	25.13 (0.73)	75.60 (11.45)	30.20 (6.94)
	Burn	38.99 (2.96)*	104.2 (10.49)	38.80 (6.14)
NK cells	Sham	16.33 (0.80)	82.20 (9.55)	37.60 (6.52)
	Burn	25.91 (2.89)*	94.60 (9.13)	33.40 (4.37)
CD4 T cells	Sham	6.06 (0.15)	68.20 (6.62)	24.20 (4.10)
	Burn	8.22 (1.12)*	88.40 (9.70)	34.60 (5.52)
CD8 T cells	Sham	7.82 (0.26)	67.40 (7.67)	24.00 (4.76)
	Burn	9.76 (1.26)	72.40 (6.31)	22.40 (5.12)
B cells	Sham	6.99 (0.22)	68.00 (5.47)	27.60 (5.25)
	Burn	9.47 (1.35)*	83.30 (7.51)	28.20 (3.50)

* indicates significant differences between sham and burn groups, $p < 0.05$ by Mann-Whitney test.