Varicella-Zoster Virus Infection Triggers Formation of an IL-1β Processing Inflammasome Complex

Adel M. Nour¹, Mike Reichelt¹, Chia-Chi Ku², Min-Yin Ho², Thomas C. Heineman³ and Ann M. Arvin¹

¹ Departments of Pediatrics and Microbiology & Immunology, Stanford University School of Medicine, Stanford CA
² The Graduate Institute of Immunology, College of Medicine, National Taiwan University, Taipei, Taiwan, ROC
³ GlaxoSmithKline Biologicals, King of Prussia, PA

Correspondence:
* Adel M. Nour,
S-366, Stanford University School of Medicine, 300 Pasteur Drive, Stanford CA 94305-5208.
E-mail: adelmn@stanford.edu
Phone: 650-725-6555; Fax: 650-725-9828
VZV triggers inflammasome formation

Abstract

Innate cellular immunity is the immediate host response against pathogens and activation of innate immunity also modulates the induction of adaptive immunity. The NOD-like receptors (NLRs) are a family of intracellular receptors that recognize conserved patterns associated with intracellular pathogens but information about their role in the host defense against DNA viruses is limited. Here we report that varicella-zoster virus (VZV), an alphaherpesvirus which is the causative agent of varicella and herpes zoster, induces formation of the NLRP3 inflammasome and the associated processing of the proinflammatory cytokine, IL-1β, by activated caspase-1, in infected cells. NLRP3 inflammasome formation was induced in VZV-infected human THP1 cells, which is a transformed monocyte cell line, primary lung fibroblasts and melanoma cells. AIM2 (Absent in Melanoma gene-2) is an interferon-inducible protein that can form an alternative inflammasome complex with caspase-1 in virus-infected cells. Experiments in VZV-infected melanoma cells showed that NLRP3 protein recruits the adaptor protein ASC and caspase-1 to form an NLRP3 inflammasome complex independent of AIM2 protein and in the absence of free radical reactive oxygen species (ROS) release. NLRP3 was also expressed extensively in infected skin xenografts in the SCID mouse model of VZV pathogenesis in vivo. We conclude that NLRP3 inflammasome formation is an innate cellular response to infection with this common pathogenic human herpesvirus.

Introduction

Varicella-zoster virus (VZV), also known as human herpesvirus 3 (HHV3), is a highly contagious ubiquitous human herpesvirus that causes varicella (chickenpox) upon primary infection and zoster (shingles) upon reactivation of latent virus from sensory ganglia (1, 2). Herpesviruses have double-stranded DNA genomes; the VZV genome is the smallest (~125,000 bp) among the human herpesviruses, encoding about 70 open reading frames (ORF) and is closely related to herpes simplex viruses (HSV) 1 and 2.

VZV infection is known to elicit the production of cytokines associated with innate cellular responses (3, 4). Mammalian cells have pattern recognition receptors (PRRs) that recognize either conserved pathogen associated molecular patterns (PAMPS) or danger-associated molecular patterns (DAMPs) and trigger these intrinsic cellular responses. Many DNA viruses have been reported to be recognized by intracellular and extracellular PRRs (5-8). The two major groups of PRRs are the toll-like receptors (TLRs), which survey the extracellular space, and the nucleotide-binding oligomerization domain NOD-like receptors (NLRs), that sense PAMPS and DAMPS in the intracellular space (9, 10). The NLRs are a large family of cytosolic pattern recognition receptors; 23 NLRs have been identified in the human genome; the mouse genome has 34 NLRs (11, 12).

Caspase-1 activation is evidence of NLR signaling that has resulted in formation of a functional inflammasome complex within the cell. The inflammasome typically consists of the NLR, pro-caspase-1 and adaptor molecules (10). In addition to the signal provided by the pathogen, this multiprotein complex which is necessary to drive the activation of caspase-1, requires intracellular potassium ion pumping; when
VZV triggers inflammasome formation

Potassium efflux is prevented, inflammasome activation is abolished in response to most known signals (12,15). Only three NLRs family members are known to form inflammasomes in human cells; these are NLRP1 (NALP1), NLRP3 (NALP3), and NLRC4 (IPAF). Once activated, inflammasomes function in the innate response against microbes by catalyzing the processing of the inflammatory cytokines, interleukin (IL) IL-1β, IL-18 and probably IL-33, and target a broad range of cellular proteins (16-18). Depending on the cell type and the strength of the activation signal, active caspase-1 has functions that vary from promoting the survival pathway (17) to inducing cell death (19). IL-1β induction is an important initial host defense mechanism when cells encounter viral and bacterial pathogens. In the context of the innate cellular response to microbes, the proteolytic effect of active caspase-1 on IL-1β and its secretion is evidence of the enzymatic activity of the inflammasome in the infected cell.

The NLRP3 inflammasome, which is formed by multimerization of NLRP3, ASC protein (apoptosis-associated speck-like containing a caspase-activating and recruitment domain), CARDINAL, and procaspase-1, is the best characterized human inflammasome complex. NLRP3 contains a C-terminal leucine-rich repeat domain (LRR), homologous to that of the TLRs, a central nucleotide-binding and oligomerization domain (NACHT) and an N-terminal PYRIN (PYD) domain, which recruits ASC protein to the inflammasome. The adaptor protein ASC contains pyrin and CARD (caspase recruitment domain) domains that recruit caspase-1 to the NLRP3 complex (10, 20).

In addition to the pathway involving NLR-containing inflammasomes, caspase-1 activation can occur by a mechanism in which the Absent in Melanoma gene 2 (AIM2) protein functions as a sensor for cytosolic DNA in a complex with ASC (21). AIM2 is a type 1 interferon (IFN) induced protein. The AIM2/ASC mechanism is independent of NLR proteins but also results in caspase-1-mediated processing of pro-IL-1β to its mature form. AIM2/ASC inflammasome formation with caspase-1 activation has been described recently in cells infected with vaccinia virus, a large double-stranded DNA virus that replicates exclusively in the cytoplasm (8).

In this study, we report that VZV infection induces formation of the NLRP3 inflammasome and that this response is elicited in three different cell types that are permissive for VZV replication, including primary human lung fibroblasts, melanoma cells and the THP-1 monocyte cell line. Caspase-1 activation occurs via a mechanism that is independent of ROS release and type 1 interferon-mediated AIM2 expression. NLRP3 is induced during VZV infection of human skin xenografts in our severe combined immunodeficiency (SCID) mouse model of VZV pathogenesis, indicating that this innate response is triggered when human epidermal and dermal cells are infected with a human herpesvirus in vivo.

Experimental procedures

Cells and Viruses. Human lung embryonic fibroblasts (HELF) and human melanoma (MeWo) cells (ATCC) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS), nonessential amino acids (100 μM) and antibiotics (penicillin at 100 U/ml and streptomycin at 100μg/ml). The THP1 cell line (ATCC) was grown in RPMI medium supplemented with 50 μM β-mercaptoethanol, 10% fetal bovine serum, nonessential amino acids (100μM) and antibiotics (penicillin at 100 U/ml and
VZV triggers inflammasome formation

streptomycin at 100 µg/ml. VZV (recombinant parent Oka) (23) and rOka-ORF10-GFP were propagated in HELF, melanoma and THP1 cells. VZV infected cells were used to inoculate uninfected cells at ratios of 1:2, 1:5 or 1:10. Herpes simplex virus (HSV) 1 infection was done with cell free virus at a multiplicity of infection (MOI) of 10.

The recombinant VZV, rOka-ORF10-GFP, expressing ORF10 as an ORF10-GFP fusion protein was constructed from VZV cosmids (24). The 9145bp SpeI fragment from the pvFs4 cosmid was cloned into Bluescript to make pBS-9190. A BglII site was introduced into ORF10 in the pBS-9190 plasmid [CGCG (bp 13385-13388) to GATC] by site directed mutagenesis. To make the recombinant with GFP at the C-terminus of ORF10, enhanced green fluorescence protein (EGFP) with BglII linkers (generated by PCR) was inserted into the novel BglII site such that EGFP is expressed after amino acid 408 of ORF10. Transfection of the mutated cosmid with the three intact cosmids yielded rOka-ORF10-GFP; the recombinant virus had the same growth kinetics and plaque morphology as the parent. The expected ORF10-GFP insertion was confirmed by sequencing and the virus expressed GFP in melanoma cells.

For the inflammasome inhibition assay, HELF were mock-infected or infected with VZV for 24 hours and 1 µg/ml LPS (Sigma-Aldrich) was added for two hours before measuring IL-1β. Mock or VZV-infected cells treated with LPS were incubated with the caspase-1 inhibitor Boc-D-CMK (EMD Chemicals) at 50 µM (final concentration) for one hour, with 130 mM KCl (final concentration) for two hours or with 10 µM MG132 (EMD Chemicals) for two hours before measuring IL-1β.

For transfection with poly (dA:dT), THP1 and MeWo cells were grown in 6 well plates in RPMI and DMEM media respectively. THP1 cells were induced to differentiate with 50 ng/ml PMA (Sigma-Aldrich) for about 6 hours. Cells were washed with phosphate buffered saline (PBS) and transfected with 1 µg/ml poly (dA:dT) (Sigma-Aldrich) in Opti medium (Invirtogen) using lipofectamin 2000 (Invirtogen). For treatment with ATP/LPS, THP1 cells were grown in 6 well plates in RPMI, induced with 50 ng/ml PMA (Sigma) for about 6 hours before adding 10 µg/ml LPS (Sigma-Aldrich) overnight and 5 mM ATP (Sigma-Aldrich) was added for one hour. For interferon (IFN) α treatment, MeWo cells were grown in DMEM medium and treated with 1000 U/ml IFN-α (Sigma-Aldrich) for 12 hours.

IL-1β assays. Secreted IL-1β in supernatants from cells infected with VZV or HSV-1 was measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s recommendation (eBioscience). Samples were tested in triplicate and data were analyzed using Prism5 software.

Antibodies. Antibodies used for immunoblots were: rabbit polyclonal anti-caspase-1 (p20) (Cell Signaling), anti-caspase-1 (p10) (Santa Cruz biotechnologies), anti-ASC (Enzo Life sciences), anti-AIM2 (Abcam), anti-MxA ((25)); mouse anti-NLRP3 (Abcam); rabbit anti-IL-1β (Cell Signaling), mouse anti-β-Actin (Santa Cruz). Antibodies used for confocal microscopy and immunohistochemistry were, rabbit anti-NLRP1 (Abcam), mouse monoclonal anti-VZV-IE62 (Millipore), rabbit polyclonal anti-VZV-ORF23, rabbit anti-VZV-IE63 (a gift from William Ruyechan, University of Buffalo) and rabbit polyclonal anti-VZV-ORF29 (a gift from Saul Silverstein, Columbia University, New York). Antibodies for secondary detection were Alexa Fluor 488, 594 or 647 conjugated
VZV triggers inflammasome formation

donkey anti-mouse or donkey anti-rabbit antibodies (Invitrogen).

**Immunoaffinity purification.** Immunopurification of the caspase-1 complex was done as described previously (26). Briefly, 1 mg of the caspase-1 (p10) antibody was incubated with 1 ml protein A-Sepharose beads (Invitrogen), with continuous mixing at 4°C for 2 hours. The beads were then centrifuged and washed five times with PBS and once with 200 mM sodium borate, pH 9. Equal volumes of 40 mM dimethylsuberimidate (Sigma) in 200 mM sodium borate were mixed with the beads, and the suspension was incubated for 30 minutes with continuous mixing. The reaction was terminated by adding an equal volume of 400 mM ethanolamine, and the sample was incubated for an additional 30 minutes. The beads were packed into a 1-cm-diameter column (Bio-Rad) and washed three times with two bed volumes of PBS and once with four bed volumes of 100 mM glycine-HCl at pH 2.4 to remove non-cross-linked antibodies. Finally, the cross-linked antibody beads were washed with PBS until the pH reached 7 and stored at 4°C in PBS containing 0.02% sodium azide. To reduce nonspecific protein-protein interactions, test samples were pre-cleared with protein A beads cross-linked to total IgG from preimmune rabbit serum (Sigma-Aldrich) and purified rabbit IgG without exposure to human caspase-1 antigen. Infected and control uninfected cells were trypsinized and lysed with (1% NP40, 150 mM NaCl, 50 mM Tris pH 8.4) in the presence of protease inhibitors (Roche). Cell lysates were then centrifuged at 10,000 rpm for 15 minutes, and about 15 µg of protein was run on 4 to 20% SDS-polyacrylamide gradient gels (Bio-Rad). Immunoblot was performed with antibody dilutions recommended by the manufacturers.

**Flow cytometry,** Melanoma cells were infected with VZV ORF10-GFP virus (1 infected cell to 5 uninfected cells) for 24 hours and red CMPTX cell tracker was added (Invitrogen). THP1 cells were added to the monolayer of infected melanoma cells and incubated for 24 hours, collected and stained for cell viability (live/dead staining dye; Invitrogen). As a control, uninfected THP1 cells were stained with red CMPTX cell tracker. Cell tracker Green BODIPY was used to stain THP1 cells as a positive control for GFP signal. Using these markers, VZV infected THP1 cells were sorted, grown overnight in RPMI medium and checked for viability. The transmission of VZV from infected THP1 was assessed by adding infected THP1 to uninfected HELF (1:10 ratio). Cells were incubated for about four days and HELF infection was monitored by fluorescence microscopy.

**Fluorescence microscopy.** The immunostaining procedures were performed as described previously (26). Briefly, about 4x10^5 HELF cells/ml were grown overnight on coverslip Lab-Tek II chambers (Nalgene). Infected THP1 cells were induced with 50 ng/ml PMA for about 6 hours. Mock and VZV-infected melanoma and HELF cells were incubated with the biotinylated YVAD inhibitor (Ana spec) at a final concentration of 10 µM for 1 hour at 37°C. Cells were stained with Hoechst...
VZV triggers inflammasome formation

33342 (10 µg/ml) for 15 minutes, washed twice with PBS, fixed with 4% paraformaldehyde (PFD) for 10 minutes, and permeabilized with 1% Triton X100 in PBS. Fixed cells were incubated for about 30 minutes at room temperature in presence of 2 µg/ml streptavidin conjugated TEXAS Red (Immunoresearch Labs.), washed 5 times with PBS at room temperature and examined using a Leica TCS SP2 confocal laser scanning microscope (Heidelberg, Germany). For antibody immunostaining, fixed cells were blocked with 10% fetal bovine serum in PBS and then incubated with the desired primary antibody for 1 hour at room temperature. Cells were then washed 10 times with PBS, incubated with secondary antibodies (30 minutes), washed and mounted using Paramount (Invitrogen). Controls included nonmatching secondary antibodies and secondary antibodies alone. Cells were imaged with the Leica TCS SP2 confocal microscope.

**Electron Microscopy (EM).** EM was done as previously described (27). Briefly, THP1 cells were grown and infected with sorted, VZV infected, THP1 in a six well plate for 24 hours, cells were recovered, centrifuged, washed with PBS three times, gently pelleted and fixed in 4% PFA and 2% glutaraldehyde in phosphate buffer (0.1 M, pH 7.2). Cells were post-fixed with 1% osmium tetroxide (2 hours) and incubated in 1% aqueous uranyl acetate overnight. The samples were dehydrated in a series of increasing ethanol concentrations followed by a final propyleneoxide step. The samples were embedded in Embed812 (Electron Microscopy Sciences). Ultrathin sections (60 nm) were prepared with a diamond knife (Diatome) and an ultramicrotome (Ultracut, Leica). Sections were stained with 3.5% aqueous uranylacetate for 10 minutes and with 0.2% lead citrate for 3 minutes. The sections were analyzed using a JEOL 1230 transmission electron microscope (TEM) at 80kV and digital photographs were taken with a GATAN Multiscan 701 digital camera.

**ROS release assay.** Melanoma cells were infected with VZV for 24 hours, trypsinized, washed with PBS, stained with the ROS release sensor CM-H2DCFDA (Invitrogen) and evaluated for ROS release by FACS-Caliber (BD biosciences). As a positive control, uninfected melanoma cells were treated with 100 µM H2O2 (final concentration) for an hour.

**Analysis of VZV skin lesions.** VZV infection of human skin xenografts in SCID mice and the analysis of tissue sections were done as described previously (28). Briefly, skin xenografts were made in homozygous CB-17SCID/SCID mice using human fetal tissue obtained according to federal and state regulations; animal use was approved by the Stanford University Administrative Panel on Laboratory Animal Care. For immunohistochemistry analysis of NLRP3 expression, formalin-fixed, paraffin-embedded skin sections (5µm) were deparaffinized, rehydrated, treated with antigen retrieval reagent (Vector Laboratories) and treated with 3% hydrogenperoxidase. Sections were incubated with NLRP3 antibody, biotinylated anti-rabbit secondary antibody and HRP conjugated streptavidin (Lab Vision). Signals were developed with Vector VIP chromagen (purple) and counterstained with methyl green (Vector Laboratories). VZV was detected with a high-titer human polyclonal anti-VZV serum. For confocal microscopy to assess NLRP3 expression, 5µm sections of infected human skin were deparaffinized, rehydrated, and subjected to antigen retrieval using the pressure cooker method in presence of 10mM sodium citrate pH 6. Sections were blocked with 0.5% fish gelatin and stained with anti-NLRP3 (Abcam); secondary antibody alone was used as a control. The lesion area was
defined by cytopathic changes including syncytia formation and the expression of the VZV ORF23 capsid protein detected by staining with anti-VZV ORF23 polyclonal antibody. Tissue sections were imaged with a Leica TCS SP2 confocal microscope.

Results

**VZV activates caspase-1 and induces processing of IL1β in human fibroblasts.** HELF were inoculated with VZV-infected HELF for 24 hours and expression of activated caspase-1 was assessed by immunoblot when approximately 75% of HELF were infected. Activation of caspase-1 was shown by the detection of caspase-1 p20, which is one of the active caspase-1 subunits, in VZV-infected cells but not in control HELF (Fig. 1A). To further confirm that VZV activated caspase-1, HELF were infected with VZV and stained with biotinylated YVAD, which is a peptide inhibitor of activated caspase-1 and binds specifically to this form of the protein. As shown in Fig. 1B, activated caspase-1 was readily detected in HELF infected with VZV, using rOka-ORF10-GFP to identify infected cells. Release of IL-1β into culture supernatants demonstrates that caspase-1 is activated and functional (29, 30). IL-1β was detected in supernatants recovered from VZV infected cells but not from mock-infected HELF in the presence or absence of LPS (Fig. 1C), which was consistent with the detection of activated caspase-1 shown in Fig. 1A and Fig. 1B. IL-1β secretion from VZV infected HELF was enhanced in the presence of LPS (1 µg/ml). These findings of caspase-1 activation and IL-1β secretion provided evidence of inflammasome induction in response to VZV infection of HELF.

Several agents are known to interfere with inflammasome function. Most of these inhibitors block either active caspase-1, e.g. the peptide inhibitor, YVAD, and the chemical inhibitor, Boc-D-CMK, or prevent potassium ion efflux, e.g. glibenclamide (31), and extracellular KCl (32). Proteosomal inhibitors, such as MG132, have also been reported to block activation of the inflammasome (33), presumably by preventing proteosomal degradation of unidentified inflammasome regulatory protein(s). These agents were used to investigate whether IL-1β processing and release by VZV infected cells required caspase-1 activation, as expected for an inflammasome-mediated mechanism. HELF were infected with VZV for 24 hours and inhibitors were added as described in the Materials and Methods. As shown in Fig. 1C, treatment with MG132 (10 µM) reduced IL-1β concentration to levels found in supernatants from HELF that were mock-infected and treated with MG132. Treatment with extracellular KCL (130 mM) and Boc-D-CMK (50 µM) also prevented IL-1β processing in VZV infected HELF, reducing secretion to background levels (Fig. 1C). The higher background for HELF treated with different inflammasome inhibiting conditions is likely to reflect some associated cell toxicity. These results suggested that VZV induces an inflammasome complex with activated caspase-1 in HELF and requires potassium ion efflux and functional proteosomes in order to catalyze the processing of pro-IL-1β.

Lipopolysaccharide (LPS) is a potent stimulant of innate responses, inducing proinflammatory cytokines and enhancing IL-1β secretion (34-37). We compared the induction of IL-β and IL-1β processing in VZV infected HELF with and without LPS treatment (1 µg/ml) and in the presence and absence of the 50 µM Boc-D-CMK inhibitor of active caspase-1 (Fig. 1D). Pro-IL-1β was present at low levels in uninfected, untreated
VZV triggers inflammasome formation in HELF and was not increased by exposure to the relatively low concentration of LPS used in these experiments. VZV-infected cells had higher levels of expression of pro-IL-1β compared to uninfected HELF, and expression was increased somewhat more when infected cells were also treated with LPS. IL-1β processing also occurred in VZV infected cells, with and without LPS stimulation, as shown by detection of the p17 subunit of IL-1β (Fig. 1D). These results showed that VZV increased pro-IL-1β synthesis and confirmed that VZV alone, without the need for another trigger of inflammasome formation, was associated with processing of pro-IL1β in HELF.

**VZV induces formation of the NLRP3 inflammasome in human fibroblasts.** Since the NLRP3 inflammasome is reported to be involved in sensing of several viruses(7, 38, 39), we hypothesized that VZV induces this inflammasome complex in HELF. In order to test this hypothesis, caspase-1 was immunopurified from VZV infected and uninfected HELF using a cross-linked anti-caspase-1 antibody column and anti-NLRP3 and anti-ASC antibodies were used to probe the immunoblot for the other components of the complex. As shown in Fig. 2, both NLRP3 and ASC were detected in the lysate of VZV-infected HELF eluted from the anti-caspase-1 column but not from uninfected HELF lysate or the column with cross-linked preimmune rabbit IgG (N beads). The presence of activated caspase-1 (p20 subunit) in the VZV-infected cell lysate was confirmed. This result demonstrated that the formation of the active caspase-1 complex and suggested that NLRP3 is involved in sensing VZV in HELF.

**VZV activates caspase-1 in THP-1 cells.** THP-1 cells, a transformed human monocyte cell line, have been used extensively for studying human inflammasome formation (10). To determine whether VZV induction of the NLRP3 inflammasome could be investigated in these cells, we first assessed whether THP-1 cells were permissive for VZV replication. THP-1 cells were infected with rOka-ORF10-GFP and GFP positive and negative cells were sorted by flow cytometry. The VZV-positive sorted cells were examined for virus production in THP1 cells by electron microscopy (EM). As shown in Fig. 3A, VZV capsids at various stages of maturation, including empty capsids, capsids with a translucent core, and capsids containing packaged DNA, and enveloped virions (Fig. 3A, right panel) were detected in THP-1 cell nuclei. To confirm that THP-1 cells could produce infectious virus progeny, HELF were inoculated with the infected, sorted THP-1 cells (1:10 ratio). As shown in Fig. 3B, VZV plaques were formed in the HELF monolayer. Infected THP-1 cells were also analyzed for the expression of two immediate early VZV proteins, IE62 and IE63, the single stranded DNA binding protein, ORF29, which is a marker for VZV replication compartments in the infected cell nucleus, and the late ORF10 protein (Fig. 3C); as expected for permissive cells, all of these viral proteins were expressed in VZV infected THP-1 cells.

To investigate caspase-1 activation, VZV-infected THP-1 cells were used to inoculate uninfected THP-1 cells for 24 hours. Although phorbol-12-myristate-13-acetate (PMA) is commonly used to induce the differentiation of THP-1, PMA is an inducer of ROS release (40, 41), which could modify the cysteine residues of the active sites of many cellular enzymes including caspase-1 (42, 43). Moreover, PMA is also known to upregulate AIM2 expression (21), a cytoplasmic receptor of double-stranded DNA that activates caspase-1. Therefore, PMA was avoided in these experiments. As shown in Fig. 4A, VZV activated caspase-1 in THP-1 cells, as indicated by the presence of the active
VZV triggers inflammasome formation

caspase-1 subunit, p20, and by the detection of the processed form of IL-1β (p17) in infected cell lysates. HSV-1 also activated caspase-1 in THP-1 cells, as indicated by the presence of the intermediate active caspase-1 subunit, p35, and induced the processing of IL-1β in infected THP-1 cells (Fig. 4A). These results were confirmed by detection of IL-1β in infected cell supernatants from THP-1 cells infected with VZV and HSV (Fig. 4B, left panel). Since LPS and PMA were not used in these THP-1 cultures infected with VZV or HSV-1, the amount of secreted IL-1β was low. When sorted THP1 cells were used to infected uninfected THP1 cells followed with 1 µg/ml LPS treatment for 2 hours before measuring the secreted IL-1β in supernatants of the VZV infected and uninfected THP1 cells, more IL-1β was detected (Fig. 4B, right panel), which confirmed the results in HELF (Fig. 1D). This result confirmed that LPS is not required for the release of the processed IL-1β but it enhances its section. A possible explanation for why the amount of detected IL-1b secreted from VZV infected THP1 is low compared to VZV infected HELF cells (Fig.1C and 4B) is the binding of the secreted IL-1b by the IL-1 receptors on uninfected THP1 cells.

When the formation of the NLRP3 inflammasome in VZV-infected THP-1 cells was evaluated by immunopurification using an anti-caspase-1 column, NLRP3 was shown to be present in the caspase-1 complex but was not detected in uninfected THP-1 cells (Fig. 4C). Activated caspase-1, detected as the p20 subunit as well as processed IL1-β, detected as the p17 form of the protein, was confirmed to be present in the initial infected THP-1 cell lysates by immunoblot (Fig. 4C)

VZV triggers formation of an activated caspase-1 inflammasome complex in the absence of AIM2. The AIM2 protein recognizes dsDNA of several pathogens including vaccinia virus which, unlike VZV, replicates in the cytoplasm and caspase-1 is activated by an AIM2/ASC complex (19-21). In human monocytes, AIM2 expression requires induction by interferons (8). Since the activation of caspase-1 in VZV infected cells might occur through AIM2/ASC complex formation, we investigated whether AIM2 was induced in VZV infected THP-1 cells. As shown in Fig. 5A, VZV did not upregulate AIM2 in THP-1 cells although caspase-1 was active, as indicated by the presence of the p20 subunit. The secretion of IL-1β into supernatants of VZV infected THP-1 cell cultures was confirmed (Fig. 5B); positive controls included poly (dA:dT) transfected and ATP/LPS treated THP-1 cells. Since AIM2 is induced by type I IFNs, we analyzed cytokine release from VZV infected and uninfected THP1 using a multiplex (Luminex) assay. Type I IFN secretion did not differ between mock infected and VZV infected THP1 (data not shown). The human myxovirus resistance protein1 (MxA) is a sensitive marker for type I IFN induction (44). As shown in Fig. 6A, VZV infected THP1 cells did not show upregulation of the IFN-induced form of MxA in contrast to the control THP1 cells which were transfected with poly (dA:dT), a known IFN inducer (45). This result is consistent with our observation that VZV blocks IRF3 expression in HELF cells (22).

AIM2 expression has been reported to be absent in melanoma cells (46). In order to confirm that the melanoma cells (MeWo) used in these experiments did not express AIM2, we transfected these cells with poly (dA:dT). We avoided using VZV genomic DNA in this experiment because VZV DNA alone is infectious. As shown in Fig. 6B, poly (dA:dT) induced the type I IFN pathway, as indicated by upregulation of MxA protein expression; however, this condition failed to induce caspase-1 activation compared to THP1 cells.
VZV triggers inflammasome formation

transfected with poly (dT:dA). This result confirms that MeWo cells lack the AIM2-dependent sensor(s) of double-stranded DNA (dsDNA) that induces caspase-1 activation; however, the cells can respond to dsDNA to induce IFN signaling. Moreover, AIM2 expression was not detected in dsDNA transfected MeWo cells (data not shown). When caspase-1 activation was evaluated in VZV infected melanoma cells, activated caspase-1 was detected using the biotinylated caspase-1 peptide inhibitor, YVAD, in melanoma cells infected with VZV for 36 hours (Fig. 7A, upper panels), but not in the uninfected cells (Fig. 7A control, lower panel). When formation of the NLRP3 inflammasome was evaluated 24 hours after VZV infection of melanoma cells, NLRP3 and ASC were detected in the immunopurified complex (Fig. 7B). These results confirmed the observations from VZV infected HELFs and THP-1 cells, suggesting that the NLR protein, NLRP3, is involved in sensing VZV PAMPs or DAMPs. Because the NLRP3 inflammasome has been reported to be regulated by free radical reactive oxygen species (ROS) (47), we hypothesized that VZV might induce ROS release, which in turn would induce formation of the NLRP3 complex. To test this hypothesis, melanoma cells were infected with VZV for 24 hours and stained with CM-H2DCFDA, a fluorescence probe for ROS. No significant amount of ROS was detected (Fig. 7C). These experiments in melanoma cells suggest that VZV activates the NLRP3 inflammasome complex by a mechanism that is independent of AIM2 and oxidative stress.

**NLRP3 expression in VZV-infected skin xenografts in vivo.** Expression of NLR proteins is tissue restricted (48). NLRP3 has been reported to be weakly expressed in skin keratinocytes (48). Based on the results in cultured cells infected with VZV *in vitro*, we examined NLRP3 expression in human skin xenografts in SCID mice. Skin sections were tested for NLRP3 expression seven days after VZV infection. By immunohistochemistry, NLRP3 was induced after VZV infection (Fig. 8A) but was not expressed in mock-infected normal skin xenografts. NLRP3 was detected in cells expressing VZV proteins, identified by staining with a polyclonal human anti-VZV antiserum but not in the neighboring uninfected epidermal cells in the same sections. NLRP1 expression was not induced in VZV lesions (data not shown). NLRP3 up-regulation in lesions formed by VZV infection of skin xenografts was also detected by immunofluorescence, using ORF23 expression as the marker of VZV infection (Fig. 8B). Interestingly, NLRP3 was detected in the nucleus as well as the cytoplasm of cells within the skin lesions. It is not clear whether NLRP3 protein is actively translocated into the nucleus under these conditions or reaches the nucleus because of VZV disruption of the nuclear membranes of infected skin cells. However, these results indicate that VZV infection induces NLRP3 expression in lesions in human skin xenografts *in vivo*.

**Discussion**

Innate immune recognition of pathogens induces proinflammatory cytokines, type I IFNs or both. In this study, we report that VZV activates the NLRP3 inflammasome in three cell types, including fibroblasts, THP-1, and melanoma cells that are permissive for VZV replication *in vitro*. In these cells, VZV DNA is not required for caspase-1 activation. Further, activation of the NLRP3 inflammasome does not require ROS release in melanoma cells.

The NLRP3 inflammasome is activated by a broad range of microorganisms including influenza virus
VZV triggers inflammasome formation

(39), adenovirus (7), Candida albicans (49), Staphylococcus aureus (50), and Listeria monocytogenes (51). Microbial components such as muramyl dipeptide and bacterial pore-forming toxins can also activate the NLRP3 inflammasome (52, 53). In addition, host-derived stress signals such as extracellular ATP (54), monosodium urate (MSU) (55) and amyloid-β (56), as well as silica, asbestos, aluminum hydroxide, and many pollutants can elicit formation of the NLRP3 inflammasome (57, 58). Inflammasome formation leads to activation of procaspase-1 (p45), formation of heterotetramers of p10/p20 subunits of the procaspase-1, and the processing and the release of the inflammatory cytokines that regulate the adaptive immune response (59, 60).

Like many viral pathogens, VZV infection is characterized by local inflammatory reactions, which is obvious at the sites of replication in skin, and proinflammatory cytokines are present in the peripheral blood of infected subjects (61-64). Our experiments help to account for these observations by establishing that VZV triggers assembly of an inflammasome complex. As defined in human fibroblasts, this process requires potassium efflux and proteasome function. Moreover, VZV, like RNA viruses (38) and other DNA nuclear replicating viruses (7), is recognized by NLRP3 protein. Whether other NLR proteins also function in inflammasome complex formation in VZV-infected cells is not excluded and warrants further study.

The local inflammatory response recruits circulating monocytes, dendritic cells (DCs) and macrophages to sites of infection (65). DCs and macrophages isolated from human sources are known to be permissive for VZV replication (66). We used THP-1 cells to determine whether VZV infection of this cell type might be associated with inflammasome formation. THP-1 cells supported VZV replication and VZV infection was associated with formation of a functional NLRP3 inflammasome, as determined by the occurrence of IL-1β processing and secretion. HSV-1, another human alphaherpesvirus, also induced activation of caspase-1 and secretion of IL-1β by THP-1 cells. With regard to possible relevance for VZV pathogenesis, IL-1β secretion upregulates the surface expression of the adhesion molecules on both mesenchymal and endothelial cells. Surface expression of these adhesion molecules, along with secretion of chemokines, is required for recruitment of the circulating blood cells into infected tissues (59). We have shown that VZV infects T cells (28), allowing spread through the host and causes skin lesions; VZV infection is presumed to be amplified by entry of uninfected T cells into skin sites of replication. Since IL1β also has adjuvant properties that enhance the adaptive immune response, it is also likely to regulate the course of VZV infection so that it is rarely life-threatening to the host (10).

DNA viruses that replicate in the cytoplasm can activate caspase-1 through an alternative pathway involving formation of an AIM-2/ASC inflammasome that lacks NLRP3 (8, 21). AIM2, which is not expressed in melanoma cells, is a cytoplasmic protein that belongs to the HIN-200 family, induced by type I IFNs (8, 67). Several herpesviruses, including VZV, block type I IFNs (68-70, 22). Consistent with these studies, we found that VZV does not upregulate AIM2 or MxA proteins in THP1 cells, although it activates caspase-1. Moreover, caspase-1 was not activated in melanoma cells after transfection with dsDNA, whereas VZV infection activated the NLRP3 inflammasome, indicating that caspase-1 activation is independent of type I IFN induction and therefore of AIM2, in
these cells. Consistent with our results, AIM2 is not the sensor of HSV-1 in macrophages (71). How NLRP3 senses diverse stimuli is not understood. Free radical reactive oxygen species (ROS) have been proposed to activate the NLRP3 inflammasome (47, 72, 73). Possible ROS sources include xanthine oxidase, peroxisomal oxidases, or NADPH oxidases that may be altered in virus-infected cells. However, ROS release was not detected in VZV-infected melanoma cells at a time point corresponding to the activation of caspase-1 in these cells. We concluded that ROS is not required for caspase-1 activation during VZV infection in melanoma cells.Interestingly, knocking out superoxide dismutase, an enzyme required for deactivation of ROS, results in high intracellular ROS and impairment of caspase-1 function (43). Moreover, patients with mutated NADP oxidase, an enzyme required for the generation of ROS, activate caspase-1 normally and secrete IL-1β (74).

Taking advantage of our SCID mouse model of VZV pathogenesis, we found that NLRP3, unlike NLRP1, was induced in cells within VZV lesions in human skin xenografts, indicating that NLRP3 inflammasome formation occurs also in VZV infected skin in vivo. However, whether inflammasome activation is beneficial for the host or required for effective spread of the virus is not known. Treatment of HSV-1 infected mice with interleukin (IL)-18, one of the substrates of active caspase-1, has been reported to protect against HSV-1 infection (75). Though it seems likely to be beneficial for enhancing the host adaptive immune response, caspase-1 activation has been also shown to be required for successful infection by bacterial pathogens (76).

In summary, we report that VZV is sensed by the innate cellular NLR mechanism, causing caspase-1 activation in vitro and in infected skin in vivo. This mechanism is functional even though the viral DNA sensing proteins that are regulated by type I IFNs are blocked by VZV interference with the type IFN response within infected cells. In contrast, we found that VZV triggers NLRP3 inflammasome formation and IL-1β processing in different human cell lines that support VZV replication. NLRP3 inflammasome formation can be considered an immunomodulatory mechanism triggered by the virus that helps to support VZV persistence in the population by modifying the severity of infection in the individual. This proinflammatory mechanism is likely to be involved in the pathogenesis of infections caused by the other medically important human herpesviruses and might be targeted for enhancement by antiviral therapies.
VZV triggers inflammasome formation

**Acknowledgments.**

AMN and AA wrote the paper, AA supervised the project, MR did the EM, CK and MH did the immunohistochemistry staining of skin for NLRP3 and THC constructed the ORF10-GFP virus. AMN designed the experiments. We would like to thank Dr. Jurgen Brojatsch, Albert Einstein college of medicine, for his suggestions. We also would like to thank Kathy Crumpton and Tim Knaak from the FACS facility at Stanford University for their technical assistance in sorting of THP1 cells. Also, we would like to thank Yael Rosenberg-Hasson, human immune monitoring center facility at Stanford University, for her technical assistance to run the multiplex (luminex) assay. The work was supported by National Institute of Health grants (AI20459 and AI053846) and a National Cancer Institute grant (CA49605).
VZV triggers inflammasome formation

References:


VZV triggers inflammasome formation


VZV triggers inflammasome formation


Figure legends

Figure 1. Activation of caspase-1 in VZV infected HELF cells.
A, Immunoblot of caspase-1 in VZV (recombinant parent Oka) infected HELF cells. Active caspase-1 subunit (p20) was detected in VZV infected HELF but not in the mock-infected cells.
B, Active caspase-1 visualization with 10 µM biotinylated peptide caspase-1 inhibitor, YVAD, in the rOka-ORF10-GFP infected HELF cells. Active caspase-1 was stained with biotinylated YVAD and detected with streptavidin conjugated to Texas Red. Active caspase-1(red) was labeled in the VZV infected HELF cells but not the mock-infected. Hoechst staining was used to detect the nuclei (blue). The inset (orange square) indicate the red that is shown at higher magnification in the right panel. Uninfected cells, lower panel, were used as a negative control.
C, ELISA measurement of secreted IL-1β from HELF cells in presence/absence of LPS. VZV infected and uninfected HELF were treated with the caspase-1 inhibitor, Boc-D-CMK (50µM), the proteosomal inhibitor, MG132 (10µM), or the potassium efflux inhibitor, extracellular KCl (130mM), in the presence of 1µg/ml LPS. ELISA data represented two independent experiments, with samples measured in triplicate in each; the error bar represents the standard error of the mean (S.E.M.).
D, VZV infection alone induced pro-IL1β expression and processing in HELF cells. VZV infected and uninfected HELF were used to test for the effects of VZV infection, with and without LPS, on proIL-1β expression and processing. VZV infection upregulated and induced the processing of IL-1β in the presence and in the absence of LPS (1µg/ml), as indicated by the detection of the pro-IL-1β and the IL-1β processed form (p17). 50 µM caspase-1 inhibitor, Boc-D-CMK, was sufficient to block the caspase-1 enzymatic activity and to prevent the processing of IL-1β in VZV infected LPS treated and LPS untreated HELF cells, as indicated by the detection of the processed form (p17) of IL-1β.

Figure 2. Immunopurification of caspase-1 complex from VZV infected HELF cells. HELF cells infected with VZV, recombinant parent Oka, activated caspase-1, as indicated by the presence of p20 in the infected lysate. Using cross-linked anti-caspase-1 antibody, both NLRP3 and the adaptor protein ASC were pulled down with the immunopurified caspase-1 complex from the VZV infected HELF lysate. Uninfected HELF cells and normal rabbit IgG, preimmune IgG cross-linked to agarose beads (N beads) were used as negative controls.

Figure 3. VZV replication and production of the virus progeny in THP1 cells.
A, Electron microscopy of VZV infected THP1. THP1 infected with the ORF10-GFP expressing rOka virus were examined 24 hours after infection. The left panel shows an overview of THP1 infected cell (low magnification, scale bar is 1µm); (N) indicates the nucleus and (V) indicates vacuoles. The middle panel shows capsids maturation in an infected THP1 nucleus. Arrows indicate different stages of VZV capsid maturation, including empty capsids, capsids with a translucent core, and capsids containing packaged DNA. The right panel shows an enveloped VZV virion at higher magnification (scale bar is 50 nm). The area in the white square of the middle panel (scale bar is 0.2µm) is shown at higher magnification in the right panel.
B, rOka-ORF10-GFP infected THP1 transmitted rOka-ORF10-GFP virus to uninfected HELF cells. Infected THP1 cells were used to inoculate uninfected HELF cells (1 infected THP1 cells to 10 uninfected HELF cells). GFP protein was expressed in HELF cells after 4 days of incubation, indicating VZV infection; infected cells (green), and uninfected cells (grey; phase contrast)
VZV triggers inflammasome formation

C, Detection of VZV protein expression in the infected THP1 cells. Sorted THP1 cells, infected with rOka-ORF10-GFP, were subjected to immunostaining for VZV immediate early proteins (IE62 and 63), and ORF29, a single-stranded DNA binding protein, as an early protein. The late protein, ORF10, was expressed as GFP fusion protein. Infected THP1 cells expressed IE62 and 63 (red), ORF29 (red), and ORF10 (green). Hoechst staining (blue) was used to detect the nuclei. Uninfected cells served as staining control.

**Figure 4. Activation of caspase-1 in VZV infected THP1 cells.**

A, Detection of caspase-1 activity in VZV and HSV-1 infected THP1 cells. THP1 cells were infected with the sorted VZV ORF10-GFP positive cells for 24 hours; for HSV-1 infection, THP1 were infected with HSV-1 (MOI of 10) for 12 hours. Immunoblot of THP1 infected with VZV or HSV1 using anti-caspase-1 antibody detected p35, an intermediate of active caspase-1 in the infected THP1, but not in the mock uninfected THP1 cells. The processed form of IL1β (p17) was detected in the cell lysates of VZV and HSV-1 infected THP1 cells, indicating formation of an active caspase-1 complex in these infected cells.

B, ELISA measurement of IL-1β secretion in the supernatants of VZV and HSV-1 infected THP1 cells in the absence (left panel) of LPS and for the VZV, in the presence (right panel) of 1µg/ml LPS. ELISA data represent two independent experiments, with triplicate samples in each; the error bar represents S.E.M.

C, Immunopurification of caspase-1 complex from THP1 lysate. A cross-linked anti-caspase-1 antibody column was used to immunopurify caspase-1 complex from VZV infected and uninfected THP1 lysates. NLRP3 protein was detected in the eluate only of the VZV infected THP1 lysate. The activity of caspase-1 in the lysate was judged by the presence of the p20 subunit of caspase-1 and the processed form of IL-1β (p17). Uninfected THP1 lysate and preimmune rabbit IgG, normal antibody cross-linked to agarose beads (N beads) were used as negative controls.

**Figure 5. AIM2 protein expression in VZV infected THP1 cells.**

A, Immunoblot detection of the AIM2 protein expression in VZV infected THP1 cells. Sorted THP1 cells infected with VZV, rOka-ORF10-GFP, were probed for AIM2 protein expression. PMA differentiated THP1 treated with ATP/LPS and PMA differentiated THP1 cells transfected with the poly (dA:dT), a ligand of AIM2, were used as positive controls. VZV infection did not induce AIM2 upregulation in THP1 cells, unlike the PMA differentiated THP1 cells transfected with poly (dA:dT) and those treated with ATP/LPS. Caspase-1 activity in the lysates was detected by the anti-caspase-1 (p20) antibody. B, ELISA measurement of the secreted IL-1β in the supernatant of VZV infected, poly (dA:dT) transfected, and ATP/LPS treated THP1. ELISA data represent two independent experiments, with triplicate samples in each; the error bar represents S.E.M.

**Figure 6. Double stranded-DNA (dsDNA) transfection activates caspase-1 in THP1 but not in melanoma cells.**

A, Immunoblot detection of MxA protein expression in VZV infected THP1 cells. Sorted THP1 cells infected with VZV, rOka-ORF10-GFP, were probed for MxA protein expression. PMA differentiated THP1 cells transfected with 1µg/ml poly (dA:dT), a ligand for AIM2 and a robust inducer of type I interferon, were used as a positive control. Mock infected THP1 used as a negative control. No MxA protein expression was detected in VZV infected THP1 as a consequence of blocking type I interferon response however, THP1 transfected with dsDNA activates caspase-1 and upregulates MxA protein.
VZV triggers inflammasome formation

B, Immunoblot detection of caspase-1 activity in melanoma cells after transfection with double-stranded DNA. Melanoma cells were transected with 1µg/ml poly (dT:dA). No caspase-1 activity was detected in melanoma cells transfected with dsDNA though the cells upregulated MxA protein; indicating that dsDNA trasfection induced type I secretion and upregulated MxA but not AIM2 protein. Melanoma cells treated with 1000 U/ml interferon α (INFα) used as a positive control for MxA expression. THP1 transfected with 1 µg/ml poly (dT:dA) used as a positive control for MxA upregulation and caspase-1 activation. Untreated melanoma cells served as a negative control.

Figure 7. Caspase-1 activity in VZV infected melanoma cells.
A, Visualization of active caspase-1 in VZV infected melanoma cells. Melanoma cells infected with rOka-ORF10-GFP for 36 hours were stained with the biotinylated caspase-1 peptide inhibitor, YVAD, and detected with streptavidin conjugated to Texas Red. Active caspase-1 (red) was detected only in infected melanoma cells. VZV infection was indicated by the expression of the late protein ORF10-GFP (green). Hoechst staining was used to detect the nuclei (blue).

C, Flow cytometry analysis of VZV infected melanoma cells stained with the CM-H2DCFDA, a detector of ROS release. VZV did not induce significant amount of ROS release from VZV infected melanoma cells compared to the uninfected cells and the positive control. Melanoma cells treat with 1% H2O2 were used as a positive control. The uninfected cells were used as a negative control.

Figure 8. NLRP3 expression in human skin xenografts in the human SCID mouse model of VZV pathogenesis.
A, Immunohistochemistry of NLRP3 expression in infected human skin xenografts in human SCID mouse model. Formalin-fixed, paraffin-embedded sections (5µm) of VZV-infected skin xenografts were stained with human polyclonal anti-VZV serum, detected with Vector VIP (purple) as shown in the left panel (magnification, x200). Methyl green was used as the counterstain, visible in uninfected surrounding cells. NLRP3 antibody staining of a VZV lesion showed NLRP3 expression (purple) in the nuclei and the cytoplasm of VZV infected skin xenografts. NLRP3 expression was not detected in uninfected areas (middle panel). The right panels are a higher magnification (x400) of the NLRP3 stained lesion in the middle panel.

B, Immunostaining of NLRP3 expression in infected human skin xenografts. The left panel shows the control staining with Alexa Fluor 488 conjugated anti-mouse secondary antibody alone. The middle panel showed the expression of the VZV protein, ORF23 (red) in the lesion. The right panel showed the cytoplasmic and nuclear staining of the NLRP3 (green) in the infected lesion. Nuclei were stained with Hoechst (blue).
Figure 1. Activation of caspase-1 in VZV infected HELF cells.

A

Control  VZV

-Procaspase-1 (p45)
- Caspase-1 (p20)
- β-actin

B

VZV (rOka-ORF10-GFP)  Biotinylated YVAD  Merged

uninfected  Biotinylated YVAD  Merged

C

IL-1β (pg/ml)

Mock  VZV

0  50  100  150  200  250

- - + + + + + + + + LPS  VZV  MG132  KCl  Boc-D-CMK

D

VZV  LPS  Boc-D-CMK

-pro-IL-1β  IL-1β (p17)
- β-actin
Figure 2. Immunopurification of caspase-1 complex from VZV infected HELF cells.

<table>
<thead>
<tr>
<th>Method</th>
<th>N beads</th>
<th>control</th>
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<tr>
<td>IP: anti-caspase-1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NLRP3</td>
<td>ASC</td>
<td>caspase-1(p20)</td>
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<tr>
<td>IB: anti-p20</td>
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</tr>
<tr>
<td></td>
<td>β-actin</td>
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| IB: anti-β-actin |         |         |      |
|                  | β-actin |         |      |
Figure 3. VZV replication and production of the virus progeny in THP1 cells.
Figure 4. Activation of caspase-1 in VZV infected THP1 cells.

A

B

C

Procasae-1 (p45)
Caspase-1 (p35)
Caspase-1 (p20)
IL-1β (p17)
β-actin

IP:anti-caspase-1
IB:anti-caspase-1
IB:anti-IL-1β
IB:anti-β-actin

N beads
control
VZV

← NLRP3
← Caspase-1 (p20)
← IL-1β (p17)
← β-actin

Mock
VZV
HSV1

IL1β (pg/ml)

Mock
VZV
HSV1

IL1β (pg/ml)

Mock
VZV
Figure 5. AIM2 protein expression in VZV infected THP1 cells.

(A) Western blot analysis of AIM2, Caspase-1(p20), and β-actin in control, Poly(dA:dT), VZV, and ATP/LPS treated cells.

(B) Graph showing IL1β (pg/ml) levels in control, Poly(dA:dT), VZV, and ATP/LPS treated cells.
Figure 6. Double stranded DNA transfection activates caspase-1 in THP1 cells but not in melanoma cells.
Figure 7. Caspase-1 activity in VZV infected melanoma cells.

A

VZV (rOka-ORF10-GFP)  Biotinylated YVAD  Merged

Uninfected  Biotinylated YVAD  Merged

B

IP: anti-caspase-1  NLRP3
IP: anti-caspase-1  ASC
IP: anti-caspase-1  Caspase-1(p20)
IB: anti-NLRP3  NLRP3
IB: anti-ASC  ASC
IB: β-actin  β-actin

C

Uninfected  + H₂O₂  24 hours post-VZV infection

CMH2DCFDA  FL1-H  CMH2DCFDA  FL1-H  CMH2DCFDA  FL1-H

negative control.001  positive control.002  mel39 vzv.003

1.3%  61.55%  2.19%
Figure 8. NLRP3 expression in human skin xenografts in the human SCID mouse model of VZV pathogenesis.
Varicella-Zoster virus infection triggers formation of an IL-1β processing inflammasome complex
Adel M. Nour, Mike Reichelt, Chia-Chi Ku, Min-Yin Ho, Thomas C. Heineman and Ann M. Arvin

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