An Essential Role for SHARPIN in the Regulation of Caspase 1 Activity in Sepsis

Madalina-Viviana Nastase, † Jinyang Zeng-Brouwers, † Helena Frey, † Louise Tzung-Harn Hsieh, † Chiara Poluzzi, † Janet Beckmann, † Nina Schroeder, † Josef Pfeilschifter, † Jaime Lopez-Mosqueda, † Jan Mersmann, † Fumiyo Ikeda, † Renato V. Iozzo, † Ivan Dikic, † and Liliana Schaefer †

From the Pharmacenter Frankfurt/ZAFES,* the Institute of General Pharmacology and Toxicology, the Institute of Biochemistry II, ‡ the Institute for Chemical-Pharmaceutical Research and Development, ‡ the National Institute for Chemical-Pharmaceutical Research and Development, ‡ Bucharest, Romania; the Institute of Molecular Biotechnology of the Austrian Academy of Sciences, † Vienna, Austria; and the Department of Pathology, Anatomy and Cell Biology, † Sidney Kimmel Medical College, Thomas Jefferson University, Philadelphia, Pennsylvania

Sepsis is a complex systemic inflammatory response to invading pathogens and remains one of the leading causes of death worldwide.1,2 Sensing of the initial trigger by innate immune receptors stimulates the NF-κB pathway and synthesis of proinflammatory cytokines, including tumor necrosis factor (TNF) and IL1β.3 Although overexpression of TNF represents an early response, IL1β abounds throughout sepsis.4–7 However, clinical trials in septic patients treated with anakinra, an IL1 receptor antagonist, have not been encouraging.8 In contrast, combined inhibition of IL1β and IL18 appears to be a more promising strategy.9

The active cysteine protease caspase 1, positioned upstream of IL1β and IL18, is required for proteolytic cleavage and maturation of both cytokine pro-forms and induces an inflammatory type of cell death, pyroptosis.10 Therefore, it is conceivable that inhibiting caspase 1 activation, rather than blocking downstream cytokines, could be a potent therapeutic approach in sepsis. Activation of caspase 1, in turn, is triggered by the assembly of multiprotein complexes, the inflammasomes,11 and by caspase 11.12 This process ultimately leads to the conversion of pro–caspase 1 into its cleaved active form, a heterotetramer harboring two p20 and two p10 subunits.13 The formation of p20/p10 heterotetramer

Disclosures: None declared.

Supported by the German Research Council [SCHA 1082/6-1, SFB 815, projects A5 (L.S.), A7 (J.P.), and A17 (J.M.); SFB 1039, project B2 (L.S.); and LOEWE program Ub-Net (L.S. and M.-V.N.)].

Copyright © 2016 American Society for Investigative Pathology. Published by Elsevier Inc. All rights reserved.

http://dx.doi.org/10.1016/j.ajpath.2015.12.026

Accepted for publication December 16, 2015.
Address correspondence to Liliana Schaefer, M.D., Pharmacenter Frankfurt/ZAFES, Institute of General Pharmacology and Toxicology, Haus 74, 3.108a, Theodor-Stern-Kai 7, 60590, Frankfurt am Main, Germany. E-mail: schaefer@med.uni-frankfurt.de.
is the last step in caspase 1 activation independent of the initial trigger. However, the mechanisms of p20/p10 dimer disruption of caspase 1 are not known.

Ubiquitination is a crucial post-translational regulator of the inflammatory response. The linear ubiquitin chain assembly complex (LUBAC) consists of heme-oxidized iron regulatory protein 2 ligase-1 (HOIL-1L), HOIL-1L—interacting protein (HOIP), and Shank-associated regulator of G-protein signaling homology domain—interacting protein (SHARPIN). In humans, bi-allelic mutations of HOIL (RBCK1) and homoygous missense mutations of HOIP (RNF31) cause autoinflammation, immunodeficiency, and amylopectinosis. Linear ubiquitination and the activation of the proinflammatory pathway NF-κB are impaired in the fibroblasts of these patients. However, monocytes present a proinflammatory phenotype, underlining that LUBAC components might have relevance.

Mice

C57BL/6 mice were bought from Charles River Laboratories (Wilmington, MA). Homozygous and heterozygous Cpdm and Cpdmc wild-type (WT) mice were from the C57BL/KaLawRij colony and were raised in the specific pathogen-free breeding facility at the Goethe University animal facility. NOD.129S2(B6)-Casp1tm1Sesh/Lij mice (Casp1fl/fl) were bought from Jackson ImmunoResearch Laboratories (Sutherland, UK). Casp1$^{-/-}$, Casp1$^{-/}$, double knockout, and Casp1$^{-/}/$Casp1$^{-/}$Tg mice (a gift from Drs. Vishva M. Dixit and Nobuhiko Kayagaki), from C57BL/6 background, have been previously described. As the caspase 1 and caspase 11 genes are adjacent in the mouse genome, Casp1$^{-/}/$Casp11$^{-/}$Tg mice were generated by transgenic overexpression of caspase 11 in a Casp111 double-knockout background. Cpdmc/Casp11$^{-/-}$, Cpdmc/Casp11$^{-/-}$, and Cpdmc/Casp1$^{-/-}$/Casp11$^{-/-}$Tg mice were obtained by intercrossing (see Mouse Strain Generation and Genotyping). All mice were housed in a pathogen-free facility.

Mouse Strain Generation and Genotyping

Cpdmc/Casp111$^{-/-}$Tg mice were generated by intercrossing of heterozygous Cpdmc mice with Casp111$^{-/-}$Tg mice and were then back-crossed into the C57BL/6 background. The genotyping of the Cpdmc/Casp111$^{-/-}$Tg mice for the Cpdmc mutation was made at Varionostics GmbH (Ulm, Germany). The genotype of the resulting mice was identified by PCR using genomic DNA purified from tail and the following primers for the Casp1$^{-/-}$ allele: forward, 5′-GCGCCTCCCCTACCCGG-3′; reverse, 5′-AAATCATCAGTAGAGGGAATGAT-3′; and C: forward, 5′-TCGCTCCCCTACCCGG-3′ (mutant). The resulting band size was 600 bp for both WT and mutants. Due to the fact that caspase 1 and caspase 11 are adjacent in the mouse genome and are separated by approximately 1500 bp, the presence or absence of the caspase 1 gene implies the same for caspase 11.

Cpdmc/Casp11$^{-/-}$ mice were generated by intercrossing of heterozygous Cpdmc mice with Casp11$^{-/-}$ mice. The genotyping for the caspase 11 gene was performed by PCR using genomic DNA isolated from tail and the following primers: A: forward, 5′-TCRD1 forward, 5′-CAAGG-3′; B: reverse, 5′-CTGCAUGCCTA-3′; and C: forward, 5′-GCGCCTCCCCTACCCGG-3′, yielding a 475-bp WT DNA fragment (by using B and C primers) and a 337-bp mutant DNA fragment (by using A and B primers). Cpdmc/Casp1$^{-/-}$/Casp11$^{-/-}$Tg mice were generated by intercrossing of Cpdmc/Casp11$^{-/-}$ with Casp1$^{-/-}$/Casp11$^{-/-}$Tg mice. Generation of Casp1$^{-/-}$/Casp11$^{-/-}$Tg mice was described previously. The presence of caspase 11 gene was identified by PCR using genomic DNA isolated from tail and the following primers: casp11, forward, 5′-CTGCAUGCTA-GTGTGCCATT-3′; reverse, 5′-GCGCCTCCCCTACCCGG-3′. A band of 411 bp was predicted. As an endogenous control, an additional PCR was performed by using the following primers: TCRD1: reverse, 5′-GCGCCTCCCCTACCCGG-3′; and TCRD2: forward, 5′-CAAGG-3′. A band of 211 bp resulted. The genotyping for the Cpdmc mutation was performed at...
Varionotics GmbH. All primers were bought from Sigma-Aldrich (Hamburg, Germany). The PCR reactions were performed by using a Primus 96 Plus Thermal Cycler (MWG-Biotech AG, Eurofins Genomics, Ebersberg, Germany).

The genomic DNA was isolated from the tail by using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) and the PCR reaction for caspase 11 genotyping was performed by using a HotStarTaq DNA Polymerase Kit (Qiagen, Hilden, Germany) from the 10× Hotstart PCR-Master Mix B2 (Bio&SELL, Feucht, Germany) for the Ccde mutant mice. In the case of the Ccde mutant mice, a KAPA Mouse Genotyping Hot Start Kit (Peqlab, Erlangen, Germany) was used to identify the presence of caspase 11.

In Vivo Mouse Model of Sepsis

Mice were injected i.p. with 50 mg/kg body weight of LPS from Salmonella minnesota (Sigma-Aldrich). LPS was reconstituted in 1× Dulbecco’s phosphate-buffered saline (PBS) buffer (Gibco, Thermo Fisher Scientific, Schwerte, Germany) at 40 μg/μL. This solution was used to obtain 50 mg/kg body weight in 300 μL of final volume of 0.9% NaCl solution (B. Braun, Melsungen, Germany). At 2 hours after injection, mice were sacrificed by using 4 μL/g body weight of Merial Narcare 16% solution (Henry Schein Animal Health, Hallbergmoos, Germany) i.p., to collect the respective samples. Caspase 1 inhibitor (Ac-YVAD-CMK) (ALX-260-028-M005; Enzo Life Sciences, Farmingdale, NY) was injected 30 minutes before LPS injection. The corresponding dimethyl sulfoxide volume was injected as a control. Mice were randomly assigned to the control or LPS-injected group. The investigators (M.-V.N., J.Z.-B., H.F., L.T.-H.H., and J.B.) were not blinded to the study groups or analyses.

Cell Culture, Transfection, and in Vitro Caspase 1 Cleavage Assay

THP-1 cell lines (Sigma-Aldrich) were maintained at 37°C, in 5% CO2 in RPMI 1640 (Gibco, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Gibco, Thermo Fisher Scientific), 100 U of penicillin and streptomycin (Gibco, Thermo Fisher Scientific), 2 mmol/L L-glutamine (Gibco, Thermo Fisher Scientific), and 10 mmol/L HEPES (Sigma-Aldrich). Cells tested negative for Mycoplasma contamination. For transfection, 10^5 cells were plated in 1 mL of RPMI + 10% fetal bovine serum in a 12-well cell culture plate. A total of 0.5 μg of Strep-SHARPIN-HApDNA5 plasmid and the empty vector were used in 200 μL of OptiMEM (Gibco, Thermo Fisher Scientific). Transfection was performed using Lipofectamine (Life Technologies, Carlsbad, CA). After 18 hours, the medium was changed, and 20 hours after transfection the cells were stimulated with 1 μg/mL Escherichia coli LPS for 16 hours. Serum-free media were changed, and the cells were pulsed with 5 mmol/L ATP (Sigma-Aldrich) for 1 hour. The supernatant was collected and proteins were precipitated by using trichloroacetic acid. The cells were subjected either to lysis for Western blot analysis or to fluorescence-activated cell sorting analysis after staining with carbocytosine-tyrosyl-valyl-valyl-aspartyl-fluoromethylketone (FAM-YVAD-FMK) (FAM-FLICA Caspase-1 Assay Kit; ImmunoChemistry Technologies, Bloomington, MN). For IL-β measurements in the supernatant, THP-1 cells were differentiated with 40 ng/mL phorbol 12-myristate 13-acetate for 18 hours before transfection. For the cleavage assay, THP-1 cells were treated overnight with 4 μg/mL phorbol 12-myristate 13-acetate, fresh media were added, and the cells were differentiated for 3 days. After that, they were stimulated with 1 μg/mL E. coli LPS for 4 hours. The cells were washed with cold PBS and lysed with 20 μL of cleavage buffer (see SDS-PAGE and Western Blot) per 10^6 cells. The lysate was warmed at 30°C for 30 minutes. Before warming, the lysate was incubated with 0.5 μg of glutathione S-transferase (GST)—SHARPIN or GST. After warming, the lysates were subjected to Western blot analysis.

SDS-PAGE and Western Blot

Lung tissue was lysed in buffer containing 50 mmol/L Tris-HCl (pH 8), 150 mmol/L NaCl, 0.02% Na3VO4, 1% SDS, 0.1% SDS, 1 μg/mL aprotinin, 1% Nonidet P-40, 0.5% Na-deoxycholate, 100 μg/mL phenyl methyl sulfonyl fluoride, and protease inhibitor mix (10 mmol/L e-amino-n-capric acid, 0.5 mmol/L benzamidine hydrochloride hydrate, 1 mmol/L EDTA, 1 mmol/L N-ethyl maleimide).

THP-1 cell lysates used for the cleavage assay were prepared by using the cleavage buffer HEPES 20 mmol/L (Tris pH 7.5), 10 mmol/L KCl, 1.5 mmol/L MgCl2, 1 mmol/L EDTA, and 1 mmol/L EGTA, supplemented with 2 mmol/L dithiothreitol, 2 μg/mL leupeptin, 100 μg/mL phenylmethylsulfonyl fluoride, and 2.5 μg/mL aprotinin.

Skin was cut into 1-cm² pieces, frozen in N2(l), and pulverized using a mortar and pestle. It was then lysed in 1 mL of lysing buffer containing 10 mmol/L Tris-HCl (pH 8), 2.5 mmol/L EDTA, 68.5 mmol/L NaCl, 5% glycerol (v/v), 0.5% Triton X-100, 1 mmol/L dithiothreitol, 10 mmol/L NaF, 2 mmol/L Na3VO4, 1 mmol/L phenyl methyl sulfonyl fluoride, 5 μg/mL aprotinin, 5 μg/mL leupeptin, and 50 mmol/L okadaic acid.

For SDS-PAGE and Western blot analysis, 100 μg (total protein) of sample was mixed with protein loading buffer [250 mmol/L Tris-HCl (pH 6.8), 8% sodium dodecyl sulfate, 40% glycerol, 8% β-mercaptoethanol, and 0.02% bromophenol blue] and boiled at 95°C for 5 minutes. SDS-PAGE was performed as previously described. After electrophoresis, the proteins were subjected to transfer to a
nitrocellulose membrane (Hybond Amersham, GE Healthcare, Freiburg, Germany) at 250 mA for 40 minutes followed by 350 mA for 15 minutes. The membrane was blocked in either 5% milk in Tris-buffered saline with 0.1% Tween 20 or 1× Roti-Block (Carl Roth, Karlsruhe, Germany).

Primary antibodies used were mouse monoclonal anti-β-actin (A5491; Sigma-Aldrich), rat purified anti-caspase 1 (645102; BioLegend, Fell, Germany), caspase 1 p10 antibody (M-20) (sc-514; Santa Cruz Biotechnology, Heidelberg, Germany), caspase 1 p10 antibody (sc-515; Santa Cruz Biotechnology, Hamburg, Germany), caspase 1 p20 antibody (#2225; Cell Signaling Technology, Leiden, the Netherlands), cleaved caspase 3 (45A1E) (5A1E) rabbit monoclonal antibody (#9664; Cell Signaling Technology), caspase 11 antibody [17D9] (GTX10454; GeneTex, Irvine, CA), mouse II1β/II1F2 affinity purified polyclonal antibody (AF-401-NA; R&D Systems, Wiesbaden, Germany), rabbit anti-RBCK1 (Ras-related nuclearprotein binding protein 2-type and C3HC4-type zinc finger containing protein ligand-1; HOIL-1L; Ab38540; Abcam, Cambridge, UK), anti-RNF3 (ring finger protein 3)/HOIP antibody (Ab38540; Abcam), and SHARPIN antibody (14626-1-AP; Proteintech, Manchester, UK).

Secondary antibodies used were donkey anti-IgG antibody horseradish peroxidase labeled (NA934V; GE Healthcare), polyclonal sheep anti-IgG antibody horseradish peroxidase labeled (NA931V; GE Healthcare), peroxidase-AffiniPure donkey anti-goat IgG (705-035-147; Jackson ImmunoResearch Laboratories), and goat anti-rat IgG (112-005-003; Jackson ImmunoResearch Laboratories). Detection was performed using chemiluminescence with either ECL Plus Western blot analysis detection reagents (GE Healthcare) or SuperSignal West Femto chemiluminescent substrate (Thermo Fisher Scientific).

Enzyme-Linked Immunosorbtent Assay

Tissue samples were treated as described in SDS-PAGE and Western Blot. Blood collected from mice was transferred in heparin (Ratiopharm GmbH, Ulm, Germany) prewashed micro centrifuge tubes, incubated for 15 minutes at room temperature, and centrifuged for 15 minutes at 2000 × g.

The following enzyme-linked immunosorbent assay (ELISA) kits were used: mouse II1β/II1F2 DuoSet (DY401), mouse TNF-α DuoSet (DY410), mouse CCL2/JE/MCP-1 DuoSet (DY479), human IL18/II1F4 ELISA (7620), and human II1β/II1F2 DuoSet (DY201), all from R&D Systems (Wiesbaden, Germany), and mouse II18 ELISA kit (7625; MBL International, Woburn, MA).

RNA Isolation, Real-Time Quantitative PCR, and Ethidium Bromide Detection PCR

Total RNA was isolated using TRI Reagent (Sigma-Aldrich). cDNA was reverse-transcribed using the a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany).

Real-time quantitative PCR was performed using an ABI Prism 7500 Sequence Detection System (Applied Biosystems).

TaqMan real-time quantitative PCR was performed using TaqMan Fast Universal PCR Master Mix (Applied Biosystems) and the following primers: caspase 1 (Mm00438023_m1), Ccl2 (Mm00441242_m1), Gapdh (4352932E), II1β (Mm00434228_m1), II18 (Mm00434225_m1), and Tnf (Mm010443260_g1) (Applied Biosystems).

Alternatively, RT2 SYBR Green qPCR Master Mix (Qiagen) was used, with the following primers: caspase 11: forward, 5’-GATGCCAAAAGAAACACACGC-3’ and reverse, 5’-CTCCATTTCACAGTAGCTTCACC-3’; Gapdh: forward, 5’-CATGGCCTTCGTGTTCTCA-3’ and reverse, 5’-CCTGCTTACACCTCTTGAT-3’.

Caspase 1 cDNA was also amplified using a HotStarTaq Master Mix Kit (Qiagen) and visualized with ethidium bromide after agarose gel electrophoresis. The following primers were used: caspase 1: forward, 5’-GATTCTAAAGGAGGAGACATCC-3’ and reverse, 5’-GTAATGAACTGGGAGAGACG-3’; Gapdh: forward, 5’-CATCTCCAGAGCGAGCACC-3’ and reverse, 5’-CTGTTGTCATGAGCCCTTCC-3’.

GST Protein Purification

GST fusion proteins of human SHARPIN and mutant variant SHARPIN ΔC (1-232) containing only the first 232 N-terminal amino acid residues of SHARPIN were purified essentially as previously described. In brief, transformed E. coli BL21(DE3) cells were grown in Luria-Bertani medium until OD600 0.7 to 0.8 and induced with 0.5 mmol/L isopropyl-β-d-thiogalactoside. After 3 hours, bacteria were harvested and lysed by sonication in cold 0.01% Triton X-100 in PBS containing protease inhibitors. The lysate was incubated on glutathione sepharose beads for 3 hours and afterward washed successively with cold PBS. The beads immobilized with the GST fusion proteins were further used for GST pull-down assays.

GST Pull-Down Assays

Purified recombinant human active caspase 1 (Enzo Life Sciences, Lausen, Switzerland) was incubated with GST proteins conjugated to glutathione sepharose beads at 4°C for 1 hour. After washing four to five times with PBS containing 300 mmol/L NaCl, the beads were lysed in protein loading buffer and analyzed via SDS-PAGE and Western blot.

Competition Experiment

Five micrograms of anti-cleaved caspase 1 p20 antibody (sc22163; Santa Cruz Biotechnology, Heidelberg, Germany) was immobilized on beads (Pierce Co-Immunoprecipitation Kit; Thermo Fisher Scientific). A total of 0.75 μg (100 nmol/L)
of recombinant active caspase 1 consisting of p20/p10 dimers (approximately 30 kDa) were retained on the p20 antibody-based columns. Purified SHARPIN ΔC (1-232) (approximately 23 kDa) was added onto the active caspase 1–based columns at different concentrations (0.1, 0.5, and 1 μmol/L). After washing, the samples were eluted and subjected to Western blot analysis.

Histologic Analysis

Tissue samples were fixed and embedded in paraffin. For analysis, 4-μm-thick slides were treated in xylol for paraffin removal, then rehydrated using successive treatment with 100%, 95%, 70%, and 50% ethanol solution and H2O for 3 minutes each.

For periodic acid-Schiff staining, slides were incubated for 5 minutes with 0.8% periodic acid to achieve the oxidation reaction. Afterward the slides were rinsed with distilled water three times and incubated for 15 minutes with Schiff reagent (Carl Roth) at room temperature. The slides were washed with tap water two times for 5 minutes. Counterstaining was performed by using Mayer’s Hematoxylin Solution (Sigma-Aldrich, Munich, Germany). After the final washing step with tap water, the slides were covered with glycerol/gelatin (Sigma-Aldrich, Munich, Germany).

Confocal Microscopy

THP-1 cells (1 × 10^6) were grown on 0.2% gelatin-coated eight-chamber slides (Nunc; Thermo Fisher Scientific). After LPS + ATP treatment, cells were washed with PBS and fixed for 30 minutes in 4% paraformaldehyde at 4°C. After permeabilization with 0.1% Triton X-100 for 30 seconds and blocking in PBS, 5% bovine serum albumin, cells were incubated overnight with the goat anti-cleaved caspase 1 p20 (sc22163; Santa Cruz Biotechnology, Heidelberg, Germany) or rabbit anti-SHARPIN antibody (14626-1-AP; Proteintech), washed in PBS, and then incubated with donkey anti-goat IgG Alexa Fluor 488 or donkey anti-rabbit IgG Alexa Fluor 594, for 1 hour. Nuclei were visualized with DAPI (Vector Laboratories, Burlingame, CA). A Zeiss LSM-510 confocal laser-scanning microscope (Carl Zeiss, Oberkochen, Germany) was used for the acquisition of confocal images with a ×63, 1.3 oil-immersion objective. Merged images represent single optical sections (<0.8 μm), collected with the pinhole set to 1 airy unit for the red channel, and adjusted to give the same optical slice thickness in the green and blue channels. Images were acquired in single confocal planes to determine colocalization using Zeiss LSM-510 software version 5.0.0.267 (Carl Zeiss), with filters set at 488/594 nm for dual-channel imaging. All images were analyzed using ImageJ software version 1.43.67 (NIH, Bethesda, MD; http://imagej.nih.gov/ij) and Adobe Photoshop CS6 (Adobe Systems, San Jose, CA).

Line scanning was utilized to further quantify colocalization of SHARPIN and caspase 1. This technique gives measurements of the pixels along a single defined axis along the specimen to define localization of two differentially labeled fluorophores. A qualitative assessment of a proximity-dependent localization between the two potentially interacting molecules is obtained from measuring the extent of overlap, defined as two different fluorescent labels displaying independent emission wavelengths that occupy the same pixel.

Colocalization analysis was performed using the ImageJ colocalization color map plugin. Colocalization was performed on single, segmented cells. The index of colocalization is represented on the y axis.

Co-Immunoprecipitation

Co-immunoprecipitation was performed by using a Pierce Co-Immunoprecipitation Kit (Thermo Fisher Scientific). Five micrograms of antibody recognizing the bait protein were immobilized. A total of 6.5 mg of lysate was incubated with the antibody resin overnight at 4°C. After washing, the complex bait–prey proteins were eluted by using 50 μL of elution buffer containing primary amines (pH 2.8). The following antibodies against bait proteins were used: rat purified anti–caspase 1 (645102; Biolegend), caspase 11 antibody [17D9] (GTX10454; GeneTex, Herford, Germany), cleaved caspase 1 p20 antibody (sc22163; Santa Cruz Biotechnology, Heidelberg, Germany).

Microscale Thermophoresis

To study the binding between SHARPIN and caspase 1, purified human GST-SHARPIN was labeled with the red fluorescent dye NT-647 by using a Monolith Protein Labeling Kit Red (NanoTemper Technologies, Munich, Germany). As a control, GST protein was labeled in the same manner. Human caspase 1 protein (Enzo Life Sciences, Lausen, Switzerland) was titrated in concentrations in the range of 0.488 to 1000 nmol/L. Before loading onto the capillaries, the tubes were centrifuged for 5 minutes at maximum speed. For measurements the Monolith NT.115 device (NanoTemper Technologies) was used, and for data analysis the NT Analysis software version 1.427 (NanoTemper Technologies) was used. Parameters used were: laser power, 100%; LED, 80; laser on-time, 30 seconds; laser off-time, 5 seconds; and temperature, 25°C.

The normalized fluorescence,

\[ F_{\text{norm}} = \frac{F_{\text{hot}}}{F_{\text{cold}}} \]  

is correlated to the fraction of molecules existing in the bound and unbound forms by:

\[ F_{\text{norm}} = (1 - x)F_{\text{norm(unbind)}} + xF_{\text{norm(bound)}}, \]

where \( x \) is the fraction of molecules bound to their targets, \( F_{\text{norm(unbind)}} \) is the normalized fluorescence of unbound labeled molecules, and \( F_{\text{norm(bound)}} \) is the
normalized fluorescence of labeled molecules bound to their unlabeled targets. $F_{\text{norm}}$ [\%] was plotted against the concentration of the unlabeled molecule, resulting in a sigmoidal dependence if the unlabeled and labeled molecules interact; from this graph the dissociation constant was directly obtained.

**Statistical Analysis**

Data are presented as means ± SEM. Results are representative of three or more individual experiments. For comparison of two groups, an unpaired two-tailed $t$-test was used, and results were considered significant at $P < 0.05$.

**Results**

**SHARPIN Deficiency Results in Enhancement of Mature II1β/II18 and Active Caspase 1 in the Lung and Plasma of Endotoxemic Cpd m Mice**

We investigated the role of Sharpin in the regulation of the caspase 1/II1 pathway in Cpd m mice. We analyzed maturation of II1β and II18 as well as activation of caspases 1 and II in plasma and lungs of nontreated and LPS-treated mice. LPS is an in vivo trigger of both II1β transcription through NF-κB as well as caspase 1 activation. We found higher levels of mature II1β/II18 in the plasma and lungs of LPS-treated Cpd m mice.
Endotoxemic Sharpin-Deficient and G) was markedly enhanced in plasma (Figure 1F) and lungs (Figure 1G) compared to WT. This finding was associated with enhanced pulmonary caspase 11 (Figure 2, E and F) in Cpdm mice, in nontreated and LPS-treated mice. Thus, Sharpin deficiency results in an increment of active caspase 1 and mature II1β/18, which is pronounced in endotoxic shock.

Caspase 1 Is the Main Inducer of II1β/II18 in Endotoxic Sharπn-Deficient Mice

To provide genetic proof of the role of caspase 1 in the generation of mature II1β/18 in Cpdm mice and to define the role for caspase 11 in this process, we crossed Cpdm mice with mice deficient in caspase 1 (Casp1<sup>−/−</sup>/II1Tg), caspase 11 (Casp11<sup>−/−</sup>), or both enzymes (Casp11Tg double knockout). We investigated caspase 1-dependent enhancement of mature II1β/18 during LPS-induced endotoxemia. Under these conditions, Cpdm;Casp11<sup>−/−</sup> Tg mice exhibited significantly lower levels of circulating (Figure 1A) and pulmonary (Figure 1, B–D) II1β compared to those in Cpdm mice. Plasma II1β levels of Cpdm;Casp11<sup>−/−</sup> Tg mice were comparable to those of Cpdm;Casp1<sup>−/−</sup>/II1Tg mice (Figure 3A). Cpdm;Casp11<sup>−/−</sup> Tg mice also displayed reduced plasma II1β levels, but these did not reach statistical significance (Figure 3A). Similar variations in II1β levels were also observed among septic control mice (WT, Casp11<sup>−/−</sup>, Casp1<sup>−/−</sup>/II1Tg and Casp11Tg double knockout) without a Cpdm background (Figure 3A). Consequently, we confirmed that caspase 1 is the main contributor of II1β maturation in Cpdm mice during endotoxemia. In comparison, lack of caspase 11 in Cpdm mice decreased II1β plasma levels insignificantly compared to the ablation of only caspase 1 (Cpdm;Casp1<sup>−/−</sup>/II1Tg mice) or both caspases 11 and 11 (Cpdm;Casp11Tg double knockout). However, it is notable that absence of both caspases 1 and 11 did not reduce completely the II1β levels in Cpdm mice. This finding might be explained by the existence of other enzymes that were shown to be involved directly in the maturation of II1β cytokine (eg, metalloproteinases or granzyme A).

Under nonstimulated and LPS-treated conditions, plasma (Figure 1F) and pulmonary (Figure 1G) levels of II18 in Cpdm mice were significantly reduced when both caspases 1 and 11 were absent. II18 plasma levels compared to WT. The lung was chosen as a target organ as it is one of the most affected in endotoxic shock. II18 (Figure 1, F and G) was markedly enhanced in plasma (Figure 1F) and lungs (Figure 1G) of nontreated Cpdm mice. However, pulmonary II1β (Figure 1E) and II18 (Figure 1H) mRNA levels were reduced in Cpdm mice. The discrepancy between protein and mRNA levels was specific for II1β/18, whereas the NF-kB–induced cytokine Tnf was down-regulated in plasma (Figure 1I) and lungs (Figure 1J) of Cpdm mice.

Thus, we investigated whether enhancement of active caspase 1 and its potential upstream activator, caspase 11, could be linked to the elevation of mature II1β/18 in Cpdm mice under LPS-induced endotoxemia in lung. We found a substantial increase in the pulmonary active caspase 1 in LPS-treated Cpdm mice and a slight elevation in untreated Cpdm (Figure 2, A and B), even though Casp1 mRNA was decreased (Figure 2, C and D) compared to WT. This finding was associated with enhanced pulmonary caspase 11 (Figure 2, E and F) in Cpdm mice, in nontreated and LPS-treated mice. Thus, Sharpin deficiency results in an increment of active caspase 1 and mature II1β/18, which is pronounced in endotoxic shock.

Figure 2 Increased levels of active caspase (Casp) 1 and 11 in lungs from Cpdm mice. WT, white bars; Casp1/11 Tg, light blue bars; Cpdm, dark blue bars; Cpdm;Casp1/11<sup>−/−</sup>, red bars. A and B: Representative immunoblot of pro- and active Casp1 in lungs from mice with the indicated genotypes (A) and densitometric quantification of the bands representing active Casp1 normalized to β-actin (B). C and D: Casp1 and Gapdh gene expression in lungs derived from mice with the indicated genotypes (E) and densitometric quantification of these two Casp11 isoforms normalized to β-actin (F). Sepsis was induced with lipopolysaccharide (LPS) 50 mg/kg for 2 hours. Data are expressed as means ± SEM. n = 3 (B and F); n = 4 mice per group (D). *P < 0.05 versus WT or controls (unpaired two-tailed t-test). dKO, double knockout.
in LPS-treated and -nontreated 

Cpdm:Casps1111/−/Tg mice were comparable to those in 

Cpdm:Casps1111/−/11Tg mice, whereas 

Cpdm:Casps1111/−/11Tg mice did not show any rescue in Il18 levels as compared to 

Cpdm mice (Figure 3B). Thus, as opposed to caspase 11, caspase 1 is the main inducer of Il18 in both control and endotoxemic 

Cpdm mice. However, as the levels of Il18 were not completely reduced, we presume that other enzymes shown previously to process Il18 are involved in Il18 maturation in 

Cpdm mice (eg, granzyme G, elastase).36

Caspase 1 Deficiency but not Caspase 11 Prevents Inflammation in 

Cpdm Mice

Having obtained mutant mice with deficiencies in the 

Sharpin gene and caspase 1/11 genes, we tested the effects of Casps1111 double deficiency on multiorgan abnormalities in 

Cpdm mice.20,37–39 Ablation of caspase 1, either autonomously or in combination with caspase 11 in 

Sharpin-deficient mice, allowed the mice to reach sexual maturity and to breed. It rescued the macroscopic skin lesions (Supplemental Figure S1A) and normalized their lifespan (Supplemental Figure S1B). Lifespan monitoring had to be interrupted in 

Cpdm and 

Cpdm:Casps1111/−/11Tg mice at 23 weeks due to severe skin lesions (Supplemental Figure S1B). Additionally, reduction in body weight (Supplemental Figure S1C) and splenomegaly in 

Cpdm versus WT mice (Supplemental Figure S1D) were significantly rescued only in 

Cpdm:Casps1111/−/11Tg but not in 

Cpdm:Casps1111/−/11Tg and 

Cpdm:Casps1111/−/ mice.

Furthermore, the deficit in IgG (Supplemental Figure S1E) and surplus of IgM (Supplemental Figure S1F) in 

Cpdm mice38 were restored to normal by either caspase 1 or both caspases 1 and 11 deficiency for IgG and by double ablation for IgM. In contrast to caspase 11 loss, which did not affect the hepatitis in 

Cpdm mice, caspase 1 loss rescued the hepatic phenotype (Supplemental Figure S1G).

Caspase 1 but not Caspase 11 Ablation Rescues the Skin Phenotype in 

Cpdm Mice

Cpdm skin showed immune cell infiltrates in the dermis with para- and hyperkeratosis in the epidermis (Supplemental Figure S2A), as previously reported.20 Lack of both caspases 1 and 11 reduced both the dermal infiltrates and the epidermal thickness (Supplemental Figure S2A). Unlike caspase 11, the absence of caspase 1 alone rescued the inflammatory skin phenotype in 

Cpdm mice (Supplemental Figure S2A). ELISA showed high levels of 

Il1β (Supplemental Figure S2B) and Il18 (Supplemental Figure S2C) in the skin of 

Cpdm mice; these levels were significantly attenuated by loss of both caspases 1 and 11, or caspase 1 or 11 individually (Supplemental Figure S2, B and C). In 

Cpdm mice, depletion of caspase 11 alone led to a decrease in Il1β/18 (Supplemental Figure S2, B and C). However, this effect was significantly smaller than the reductions observed in 

Cpdm:Casps1111/−/11Tg and 

Cpdm:Casps1111/−/ mice.

Secondary Lymphoid Organ Development Is Not Rescued by Caspase 1 Knockout

Not all immunologic defects in 

Sharpin-deficient mice are rescued by caspase 1 loss. The abnormal splenic architecture in 

Cpdm mice22,32,33 was not affected by ablation of caspase 1 and/or caspase 11 (Supplemental Figure S3A). Peyer’s patches were absent in 

Cpdm mice40 and did not recover after loss of caspase 1 and/or caspase 11 (Supplemental Figure S3B).

Pharmacologic Inhibition of Caspase 1 Prolongs Survival and Reduces Splenic Apoptosis in 

Sharpin-Deficient Endotoxemic Mice

Next, we determined whether caspase 1 inhibition could improve the poor survival in endotoxemic 

Cpdm mice.41 To this end, we treated LPS-injected 

Cpdm mice with 10 mg/kg of Ac-YVAD-CMK caspase 1 inhibitor on a 2-hourly basis. Ac-YVAD-CMK is an irreversible inhibitor of caspase 1 used previously in several reports to reduce the LPS lethality in vivo in WT animals.32,42 We analyzed the survival rate (Figure 4A), splenic apoptosis (Figure 4B), and Il1β plasma levels (Figure 4C) to see whether the inhibition of caspase 1 might improve the outcome of endotoxic shock in 

Cpdm mice in a fashion similar to genetic ablation (Figure 4, A–C). After LPS...
injection, the Cpdm mice survived for a much shorter time period than WT mice did. Notably, caspase 1 inhibition prolonged survival in Sharpin-deficient endotoxemic mice to an extent comparable to the genetic loss of caspase 1 (Figure 4A). Moreover, caspase 1 inhibition blocked splenic apoptosis in Sharpin-deficient controls and endotoxemic mice, as detected by a reduction in cleaved caspase 3 (Figure 4B).

Figure 4  Pharmacologic inhibition of caspase 1 in Cpdm endotoxemic mice and correlation of SHARPIN and active caspase 1 levels in peripheral blood mononuclear cells (PBMCs) from septic patients. A: Survival of the wild-type (WT), Cpdm, Cpdm + caspase 1 inhibitor (Ac-YVAD-CMK), and Cpdm;Casp1−/−/11Tg mice after a lethal dose (50 mg/kg) of lipopolysaccharide (LPS). B: Representative immunoblot of active caspase 3 in spleens from WT and Cpdm mice after inhibition with Ac-YVAD-CMK under control and endotoxemic (2 hours) conditions. C: Plasma levels of Il1β after inhibition with Ac-YVAD-CMK in endotoxemic WT and Cpdm mice as determined by enzyme-linked immunosorbent assay. D–K: Analysis of PBMC lysates from healthy control subjects (open circles) and septic patients (closed squares). Lysates were probed by Western blots (D), followed by densitometric analysis of the resulting bands normalized to β-actin (E–G). Levels of SHARPIN (E), HOIP (F), and HOIL-1L (G) in septic patients vis-à-vis healthy controls. Levels of cleaved caspase 1 in septic patients were plotted against levels of SHARPIN (H), HOIP (I), and HOIL-1L (J). Plasma levels of IL1β were plotted against levels of SHARPIN (K). Data are expressed as means ± SEM. n = 3 mice per group (B); n = 4 or 5 (C; n = 6 (D–K, healthy subjects); n = 11 (D–K, septic patients). *P < 0.05 (unpaired two-tailed t-test). DMSO, dimethyl sulfoxide.
Levels of SHARPIN in Peripheral Blood Mononuclear Cells from Septic Patients Negatively Correlate with the Levels of Active Caspase 1

To provide a link between the studies using mutant mice and human inflammatory conditions, we studied in septic patients a possible correlation between the expression levels of SHARPIN in peripheral blood mononuclear cells (PBMCs) and levels of active caspase 1 and whether this effect is specific for SHARPIN only or also for the LUBAC components HOIL-1 and HOIP. Accordingly, we evaluated by Western blot the expression of SHARPIN, HOIL-1L, HOIP, and active caspase 1 in PBMCs isolated from late-stage septic patients, whose clinical characteristics are listed in Supplemental Table S1. First, we observed that the levels of all three LUBAC components, SHARPIN (Figure 4, D and E), HOIP (Figure 4, D and F), and HOIL-1L (Figure 4, D and G), varied among the septic patients. Of these, levels of SHARPIN and HOIL-1L were significantly down-regulated vis-à-vis healthy volunteers, whereas levels of HOIP, while slightly lower in septic patients, did not reach statistical significance. Only SHARPIN levels negatively correlated with active caspase 1 levels in PBMC lysates ($R^2 = 0.8107$) (Figure 4H). In contrast, active caspase 1 levels weakly and positively correlated with HOIP (Figure 4I) and positively correlated with HOIL-1L (Figure 4J). These findings might support the potential role of SHARPIN as a negative regulator of inflammation through caspase 1. The different correlation pattern observed for active caspase 1/HOIL-1L confirms rather its role described previously as an activator of the inflammasome/caspase 1 pathway.44 We also analyzed levels of SHARPIN expression in PBMC lysates with serum levels of mature IL18 (Figure 4K) and found a negative correlation, but with a much lower correlation factor ($R^2 = 0.2726$) (Figure 4K) than with levels of active caspase 1 in PBMCs. It is notable that although secreted IL18 was found to be abundant in the serum of septic patients compared to healthy volunteers, the levels of IL1β were very low, close to the lower limit of detection (data not shown). These independent and unbiased observations in humans corroborate the conclusions drawn from our murine studies regarding the role of SHARPIN as a negative regulator of caspase 1 activation. However, the same is valid for neither HOIL-1L nor HOIP, which positively correlate with caspase 1 activation.

SHARPIN Binds to Caspase 1 in Vivo in Lungs

Given the role of SHARPIN deficiency in modulating caspase 1 activation in the mutant Cpdm mice, we tested the physical interaction between SHARPIN and caspase 1/11 using co-immunoprecipitation of lung extracts. We found SHARPIN protein in complex with caspase 1 (Figure 5A)
SHARPIN colocalizes with caspase 1 and influences caspase 1 activation. A–C: Colocalization of caspase 1 and SHARPIN in THP-1 cells unstimulated and stimulated with 1 μg/mL lipopolysaccharide (LPS) for 30 minutes and pulsed with 5 mM ATP for 30 minutes. A: Confocal images for caspase 1 (green) and SHARPIN (red) show their colocalization after LPS + ATP treatment indicated by white arrows. B: Nuclei (blue) were stained with DAPI. The line scanning profiles, next to each confocal image, show the relative signal distribution for each fluorescent channel between the white arrows (SHARPIN in red and caspase 1 in green). C: Dot plots indicate the data set distribution and correlation index (ICorr).

D and E: Influence of SHARPIN overexpression on levels of cleaved caspase 1 probed by immunoblot (D) and on caspase 1 activity shown by fluorescence-activated cell sorting analysis using FAM-YVAD-FMK FLICA (E) in THP-1 cells after stimulation with LPS + ATP. F: Influence of SHARPIN overexpression on levels of IL1β in culture media of THP-1 cells after stimulation with LPS + ATP. G and H: Inhibition of caspase 1 cleavage by SHARPIN at 30°C in LPS-stimulated THP-1 cells. Immunoblot for caspase 1 (G) and the densitometric quantification (H) of cleaved caspase 1 shown in G in THP-1 cells after incubation of THP-1 lysates with either glutathione S-transferase (GST)-SHARPIN or GST. I and J: Disruption of p20/p10 dimer of active caspase 1 by SHARPIN ΔC(1-232) after immunoprecipitation of caspase 1 p20. Immunoblot for caspase 1 p20 and p10 (I) and the densitometric quantification (J) of the p10/p20 ratio. Data are expressed as means ± SD (C–G) or as means ± SEM (H and J). n = 3 (A, B, D–J); n = at least 10 cells per condition (C). *P < 0.05 (unpaired two-tailed t-test). Scale bar = 10 μm. FAM-YVAD-FMK, carboxyfluorescein-tyrosyl-valyl-alanyl-aspartyl-fluoromethylketone; IP, immunoprecipitation.
SHARPIN Inhibits Caspase 1 in Sepsis

SHARPIN Inhibits Caspase 1 in Sepsis

SHARPIN Colocalizes with Caspase 1 and Inhibits Caspase 1 Activation in THP-1 Cells

To confirm that SHARPIN and caspase 1 are found in complex we primed THP-1 cells, immune cells of human origin, with LPS and pulsed with ATP to activate the inflammasome and caspase 1 and found colocalization of SHARPIN and caspase 1 (Figure 6, A–C). To determine SHARPIN effects on caspase 1 activation, we overexpressed SHARPIN in human monocytic THP-1 cells, stimulated with LPS + ATP, and showed that SHARPIN overexpression led to a decrease in the amount of secreted active p20 form of caspase 1 compared to control after LPS + ATP stimulation. In contrast, intracellular pro–caspase 1 remained unchanged (Figure 6D). Next, we utilized FAM-YVAD-FMK (FLICA), a fluorescent substrate peptide, which selectively binds to cleaved (active) caspase 1 enzyme. Under these conditions, SHARPIN-overexpressing cells showed reduced FLICA (Figure 6E). The effect of SHARPIN on caspase 1 activation was further confirmed by its ability to block mature IL1β induction evoked by LPS + ATP stimulation (Figure 6F). Additionally, we found that SHARPIN inhibited the cleavage of pro–caspase 1 in vitro (Figure 6, G and H). Finally, we utilized affinity chromatography whereby active caspase 1, consisting of p20/p10 dimers, was bound to a column containing antibody against the p20 form. Increasing amounts of purified SHARPIN ΔC (1–232), shown to bind active caspase 1 (Figure 5D), could efficiently prevent binding of the p20 form of active caspase 1 to the p10 form (Figure 6, I and J).

Discussion

This is the first study showing SHARPIN as an endogenous inhibitor of caspase 1 activation. Of particular importance is our finding that SHARPIN blocks the p20/p10 dimer formation, the last and common step of caspase 1 activation. The multilayer caspase 1 activation is secured by various mechanisms. It is generally thought to occur after assembly of an inflammasome scaffold, with several inflammasomes existing, each activated in turn by specific stimuli and inflammatory caspase 11, which can potentiate the inflammasome or directly cleave caspase 1.12,30 Following these pathways, pro–caspase 1 is cleaved into the active p10 and p20 subunits that assemble into a heterotetramer harboring two p20 and two p10 subunits.13,45 With this in mind, it is conceivable that blocking the p20/p10 dimer formation could be the most powerful pharmacologic approach, as the inhibition of any particular upstream inflammasome or caspase 11 will not completely block active caspase 1. Moreover, by inhibiting one pathway, the other alternate pathways might increase their potential to activate caspase 1 due to compensatory effects.

Here we discovered that SHARPIN is found in a molecular complex with caspase 1 after co-immunoprecipitation in mouse lungs. Further, we show that through its N-terminal domain, SHARPIN directly binds to the active caspase 1 tetramer inhibiting the p20/p10 interaction and its activity, consequently preventing the release of mature cytokines. A
isolated from them have lower levels of SHARPIN and ablation of caspase 1, survival in induced endotoxemia. After either genetic or pharmacologic SHARPIN in the negative regulation of caspase 1 in LPS-autonomous of LUBAC. A correlation between SHARPIN and caspase 1 levels is tory role of SHARPIN is LUBAC independent. Further-
components HOIL-1L and HOIP, showing that the inhibi-
tation of caspase 1 complex does not contain the other LUBAC
abundance of SHARPIN that negatively correlated with
in nuclear cells from human septic patients, we found a low
kines and caspase 1 levels is autonomous of LUBAC.

Importantly, our study has unveiled a novel role for SHARPIN in the negative regulation of caspase 1 in LPS-induced endotoxemia. After either genetic or pharmacologic ablation of caspase 1, survival in Cpdm mice exposed to endotoxic shock is considerably increased. Thus, from a clinical translational perspective, our results indicate that natural (eg, SHARPIN) or synthetic caspase 1 inhibitors might provide a novel therapeutic approach for septic patients. Up to now only downstream effectors of caspase 1 have been targeted in sepsis (eg, by treatment with the natural IL1 receptor inhibitor anakinra), revealing quite disappointing clinical outcomes.\(^1\) As the effects of caspase 1 extend beyond processing IL1\(\beta\) and IL18, in addition to antibiotic therapy, it is conceivable that the inhibition of caspase 1 enzymatic activity could be a more effective therapeutic strategy. Indeed, we show that treatment with caspase 1 inhibitor not only decreases IL1\(\beta\)/IL18 levels but also prevents inflammatory death of splenic cells.

Our mechanistic and in \(\textit{vivo}\) experimental findings are strongly underlined by clinical data. In circulating mono-
uclear cells from human septic patients, we found a low abundance of SHARPIN that negatively correlated with high levels of active caspase 1. On the other hand, active caspase 1 levels positively correlated with HOIL-1L levels, consistent with the reported findings that HOIL-1L is required for inflammasome activation.\(^1\) These findings suggest a role for SHARPIN in controlling caspase 1 activation, opposite to and independent of the role played by other LUBAC components. To date, the only known case of SHARPIN deficiency in humans is the report of three patients with familial cold autoinflammatory syndrome.\(^1\) These patients display an autoinflammatory syndrome and experienced pyogenic bacterial infections, whereas cells isolated from them have lower levels of SHARPIN and HOIP proteins.\(^1\) Despite recent advances in medical science, effective new therapies for sepsis have not been forthcoming.\(^4\) Our findings suggest that using pharmacologic caspase 1 inhibitors, in particular SHARPIN-derived peptides blocking the p20/p10 dimer formation, could be beneficial in septic patients with low SHARPIN levels. We think that septic patients with a deficit in SHARPIN gene/protein could be more efficiently treated by inhibiting caspase 1 than by other anti-inflammatory therapies. A number of caspase 1 inhibitors have been tested in humans. For example, the VX-765 inhibitor can effectively inhibit caspase 1 in cells from patients with familial cold auto-inflammatory syndrome\(^2\) and in a mouse model of epilepsy.\(^4\) In a Phase IIa trial, the administration of VX-765 was found to be and well-tolerated.\(^3\)

Our findings add new light to the understanding of the inflam-
matory Cpdm mouse phenotype. We demonstrate for the first time that genetic ablation of Casp1 gene alone, but not of Casp11, rescues the Cpdm phenotype in various organs. Recently, higher levels of active caspases 1 and 11 were shown in the skin of unchallenged Cpdm mice, with concomitant ablation of caspases 1 and 11 reducing skin inflammation.\(^4\) In contrast to our findings, caspase 1/11−deficient Cpdm mice reported by Douglas et al\(^5\) still presented inflammatory skin lesions that developed before 14 weeks.\(^6\) Cpdm;Casp11/11−/− mice generated in our laboratory were free of any skin lesions at any age up to 1 year of continuous monitoring. In addition, we observed a pronounced reversal of the inflammatory lung phenotype in Cpdm mice lacking either Casp1 or Casp11/11 genes. This apparent discrepancy can be partially explained by different experimental protocols, ours involving an extensive backcrossing of the mutant mice, ensuring uniform genetic background. Thus, in contrast to Douglas et al,\(^5\) we determined which of the two inflammatory caspases, caspase 1 or 11, is the key player in the development of the Cpdm inflammatory phenotype.

Overall, we demonstrate that SHARPIN plays an essential role as a negative regulator of caspase 1 activation in inflammatory conditions, and propose novel therapeutic avenues for treating sepsis and other inflammatory conditions in which SHARPIN is decreased or absent.

**Acknowledgments**

We thank Riad Haceni for performing the IHC analysis, Anja Kirchhof for the help with genotyping and breeding of the mice, Drs. Vishva M. Dixit and Nobuhiko Kayagaki for kindly gifting mice deficient in caspase 1 and caspase 11, and Dr. Patrick Meybohm, Victoria Buderus, and Karin Pense for their help with patient enrollment.

M.-V.N., J.Z.-B., J.B., H.F., L.T.-H.H., C.P., and N.S. performed the experiments; J.L.-M. and J.M. provided research materials and analyzed the data; and M.-V.N., R.V.I., J.P., F.I., I.D., and L.S. designed the research and analyzed the data. All of the authors contributed to writing the manuscript and provided final approval of the submitted and published versions.

**Supplemental Data**

Supplemental material for this article can be found at \(\text{http://dx.doi.org/10.1016/j.ajpath.2015.12.026} \).

**References**


ajp.amjpathol.org ■ The American Journal of Pathology


