Supporting Information

Mezzaroma et al. 10.1073/pnas.1108586108

SI Materials and Methods

Experimental Acute Myocardial Infarction (AMI). All animal experiments were conducted under the guidelines on humane use and care of laboratory animals for biomedical research published by National Institutes of Health (no. 85-23, revised 1996). The study protocol was approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee.

Caspase-1. Hearts collected 3 and 7 d after surgery (n = 4–6 per group) were homogenized in RTL buffer (Qiagen) using lysing-matrix D beads (MP Biomedicals). Total mRNA was extracted using RNeasy extraction kit (Qiagen) and was converted to cDNA using the reverse transcription kit (Applied Biosystems). Procaspase-1 mRNA expression was determined using SYBR-green real-time PCR (Applied Biosystems) with the following couples of primers: caspase-1 forward 5′-TCCGCGGTTGA-ATCCTTTTCAGA-3′; caspase-1 reverse 5′-ACCACAATTG-CTGTGTGTGCGCA-3′ and GAPDH forward 5′-ACTGAG-CAAGAGAGGCCCTA-3′; GAPDH reverse 5′-TGTGGGTG-CAGCGAACTTTA-3′.

Caspase-1 protein levels were determined in clarified homogenates of the whole hearts explanted 7 d after surgery (n = 4–6 per group) and immediately frozen in liquid nitrogen. The samples were homogenized using RIPA buffer (Sigma-Aldrich) containing a mixture of protease inhibitors (Sigma-Aldrich) and were centrifuged at 16,200 × g for 20 min. The supernatants were collected and the protein contents were quantified using the Bradford assay. Fifty micrograms of proteins for each sample were analyzed by Western blot. Proteins were denatured for 10 min at 97 °C and subjected to SDS/PAGE in 15% acrylamide gels to allow for separation of the bands. The proteins were then transferred onto nitrocellulose membranes and incubated with a rabbit polyclonal antibody (C4851; Sigma-Aldrich) that hybridizes with both pro- and cleaved caspase-1 (p20). A mouse anti–β-actin monoclonal antibody (clone C-2; Sigma-Aldrich) was used for the normalization following enhanced-chemiluminescence (ECL) analysis and autoradiography. The protein bands were compared by densitometric analysis (Scion Image) and the results were adjusted to the β-actin quantity in the samples. Caspase-1 activity in clarified homogenates of heart tissue was determined in the whole hearts collected at different time points (1, 6, and 24 h and 3, 7, and 14 d) after surgery (n = 4–6 per time point) and immediately frozen in liquid nitrogen. The activity was determined by cleavage of a fluorogenic substrate (CaspACE; Promega. From each sample, 75 μg of proteins were used for the assay according to the supplier's instructions. The fluorescence was measured 60 min later and was expressed as arbitrary fluorescence units produced by 1 μg of sample per min (fluorescence/μg/min) and shown as fold change compared with sham.

Expression of the Component of the Inflammasome in the Heart During AMI. The whole hearts collected at 3 and 7 d after surgery were processed as previously described. For the quantification of the apoptosis speck-like protein containing a caspase-recruitment domain (ASC) mRNA levels, the hearts were processed as described above. ASC mRNA expression was determined using SYBR-green real-time PCR, performed using the following primers: ASC forward 5′-CAGAGTACAGCCAGAA-CAGGACAC-3′; ASC reverse 5′-GTGGTCTCTGCACGAA-CTGCCTG-3′ and GAPDH forward and reverse the same as above.

ASC protein expression in the heart during AMI was evaluated using Western blot as described above using a commercially available antibody (Sigma-Aldrich). Formalin-fixed paraffin-embedded heart tissue slides were used. Heart sections were deparaffinized and rehydrated. After antigen retrieval with 0.01 M citrate buffer (pH 6.0) for 20 min, slides were blocked with 1% normal swine serum in TBS for 15 min. For characterization of cell type-specific expression of the inflammasome, a double immunofluorescence technique was used: After antigen retrieval, slides were incubated with primary antibody for cryopyrin (1:50; Santa Cruz), ASC (1:25; Sigma-Aldrich) or caspase-1 (1:50; Sigma-Aldrich) overnight at 4 °C. Antigoat or antirabbit Alexa Fluor 594- or 488-conjugated secondary antibody (1:100) was applied for 4 h at room temperature, then slides were incubated with primary antibody for ASC (1:25; Sigma-Aldrich), caspase-1 (1:50; Sigma-Aldrich), cardiac actin (1:200; Sigma-Aldrich), CD45 (1:2; BD Bioscience), S100A4 (1:50; Abcam or 1:3; Sigma-Aldrich), caveolin-1 (1:50; Cell Signaling or 1:20; AbD Serotec) overnight at 4 °C. Then, Alexa Fluor 488- or 594-conjugated secondary antibody (1:100; Invitrogen) was applied for 4 h at room temperature. Counterstaining was performed with 4′,6-diamidino-2-phenylindole (DAPI) 1:20,000 for 5 min and the slides were coverslipped with SlowFade Antifade (both Invitrogen). Negative controls with nonspecific IgG were run in parallel. Images were acquired with an IX70 microscope and MagnaFire 1.1 software (both Olympus) using a 40× objective (400× magnification). Color composite images were generated with ImageJ software (National Institutes of Health, http://imagej.nih.gov/ij/, 1997–2011).

Expression of the components of the inflammasome was quantified by three different investigators using a semiquantitative scale ranging from 0 (no expression) to 1+ (minimal expression meaning either few aggregates (<1 per high power field) or mild diffuse stain without aggregates), 2+ (moderate expression meaning either 1–5 aggregates per high power field or diffuse stain with few aggregates), 3+ [diffuse intense staining with many cytoplasmic aggregates (>5 per high power field)], and expressed as mean and SEM.

Formation of the Inflammasome in Isolated Cardiomyocytes in Vitro. Cells were subjected to "simulated ischemia" for 6 h by replacing the Claycomb medium with an "ischemia buffer" (118 mM NaCl, 24 mM $NaHCO_3$, 1.0 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 20 mM sodium lactate, 16 mM KCl, pH adjusted to 6.2) and exposure to a hypoxic environment in a 5% CO_2 95% N_2 atmosphere in a gas chamber, and incubated at 37 °C.

Assessment of Cell Death in Isolated Cardiomyocytes in Vitro. Cell death in HL-1 cardiomyocytes in vitro was assessed determining loss of membrane integrity using Trypan blue as well as determining nuclear DNA fragmentation using in situ end labeling (TUNEL). HL-1 cells treated as described above were harvested and, after being resuspended in 1 ml of Claycomb medium, 100 μL of 0.4% Trypan blue stain (Gibco; Invitrogen) were added and incubated at room temperature for 5 min. The number of HL-1 cells that did not exclude Trypan blue were deemed nonviable and counted as percentage of all cells per each field. Trypan-blue positivity reflected loss of membrane integrity as seen in necrotic/oncotic cell death or in pyroptosis (1). TUNEL was performed according to the supplier's instructions (2). Nuclear DNA fragmentation is a hallmark of programmed cell death, namely apoptosis or pyroptosis (1). Pyroptosis

is caspase-1–dependent cell death, to determine whether HL-1 cardiomyocytes death was caspase-1 dependent, a caspase-1 inhibitor (1 μM benzyl-oxycarbonyl-Trp-Glu(OMe)-His-Asp(OMe)-fluoromethylketone; R&D Systems) was added in vitro.

1. Kroemer G, Galluzzi L, Vandanabeele P, J Abrams, ES Anemri, et al. (2009) Classification of cell death: Recommendations of the Nomenclature Committee on Cell Death 2009. *Cell Death Differ* 16:3–11.

2. Abbate A, et al. (2008) Anakinra, a recombinant human interleukin-1 receptor antagonist, inhibits apoptosis in experimental acute myocardial infarction. *Circulation* 117:2670–2683.

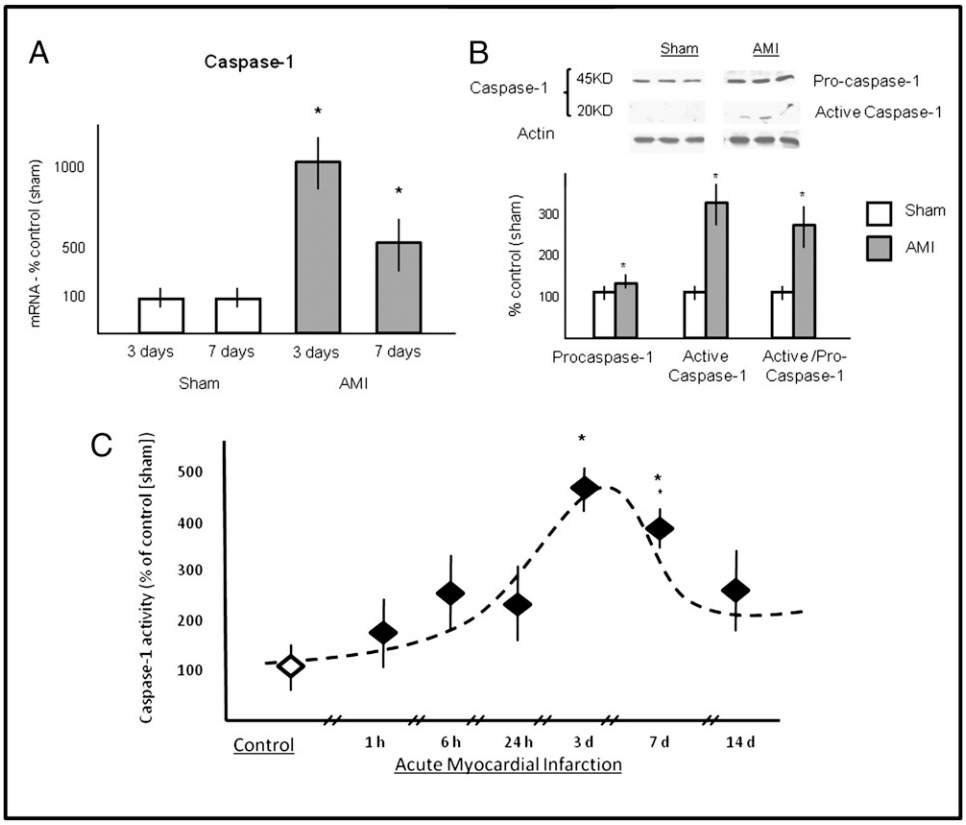

Fig. S1. Caspase-1 activation in acute myocardial infarction. Mean ± SEM values are reported as percentage of control values given for the sham-operated mice (n = 4–6 per group) in *A*–*C*. (*A*) Ten- and fivefold increase in caspase-1 mRNA (real-time PCR) in the heart after acute myocardial infarction (AMI) on days 3 and 7, respectively, vs. sham-operated mice. (*B*) Increase in procaspase-1 and in caspase-1 cleavage in the heart during AMI (Western blot) vs. sham-operated mice 72 h after surgery. (*C*) Caspase-1 activity in the heart at several time points after AMI.

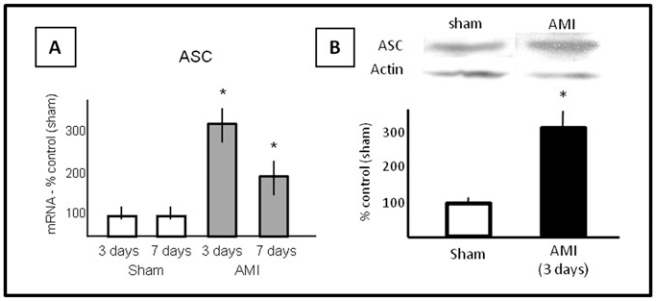

Fig. S2. ASC expression in acute myocardial infarction. Mean ± SEM values are reported as percentage of control values given for the sham-operated mice (n = 4–6 per group) in *A* and *B*. (*A* and *B*) Increased ASC mRNA and protein, respectively, after AMI.

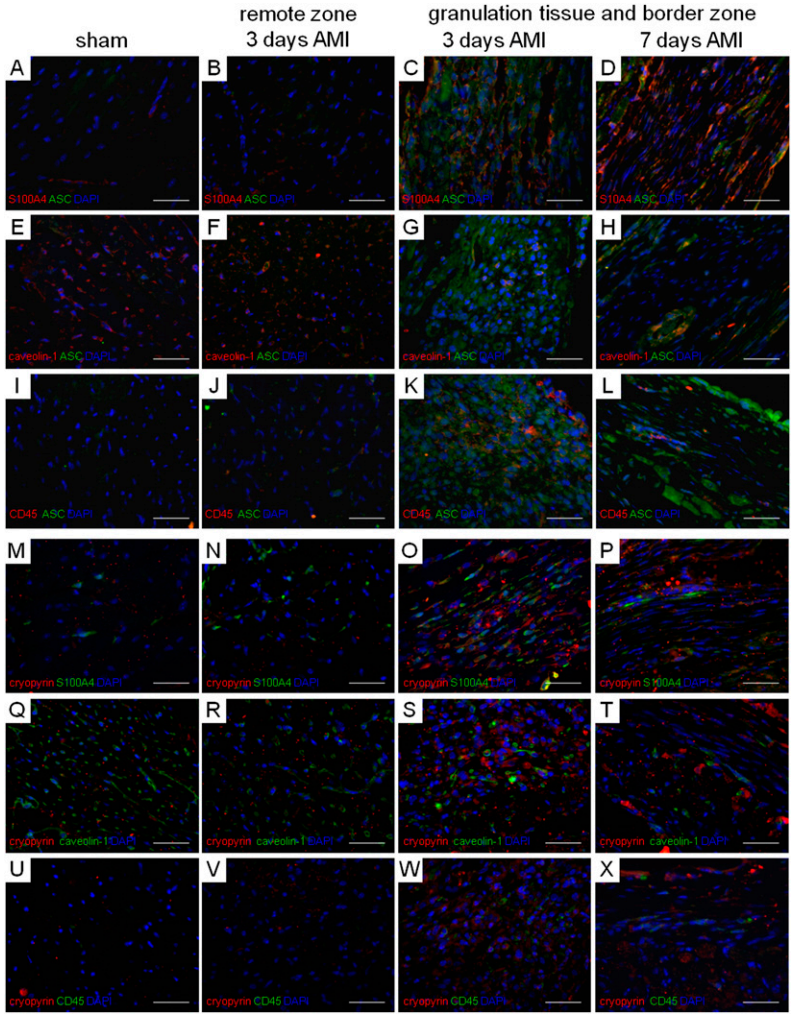

Fig. S3. Formation of the inflammasome in fibroblasts, endothelial cells, and leukocytes in the granulation tissue in acute myocardial infarction. Overlap of ASC (a key structural component of the inflammasome) and S100A4 (a marker used to detect fibroblasts) is presented in *A–D*, showing an intense staining and some overlap in the granulation tissue and border zone, 3 and 7 d after AMI (*C* and *D*) and minimal staining in sham (*A*) and remote myocardium (*B*). Overlap of ASC and caveolin-1 (a marker used to detect endothelial cells) is presented in *E–H*, showing staining and overlap in the granulation tissue and border zone, 3 and 7 d after AMI (*G* and *H*) and no overlap in sham (*E*) and minimal overlap in remote myocardium (*F*). Overlap of ASC and CD45 (a marker used to detect leukocytes) is presented in *I–L*, showing staining and overlap in the granulation tissue and border zone, 3 and 7 d after AMI (*K* and *L*) and minimal or no staining in sham (*I*) and remote myocardium (*J*). (*M–X*) Similar distribution of cryopyrin (another key structural component of the inflammasome). Overlap of cryopyrin and S100A4 (a marker used to detect fibroblasts) is presented in *M–P* showing staining and some overlap in the granulation tissue and border zone, 3 and 7 d after AMI (*O* and *P*) and minimal staining in sham (*M*) and remote myocardium (*N*). Overlap of cryopyrin and caveolin-1 (a marker used to detect endothelial cells) is presented in *Q–T*, showing staining and overlap in the granulation tissue and border zone, 3 and 7 d after AMI (*S* and *T*), and no overlap in sham (*Q*), and minimal overlap in remote myocardium (*R*). Overlap of cryopyrin and CD45 (a marker used to detect leukocytes) is presented in *U–X*, showing staining and overlap in the granulation tissue and border zone, 3 and 7 d after AMI (*W* and *X*) and minimal or no staining in sham (*U*) and remote myocardium (*V*). Counterstaining with DAPI (blue). Original magnification, 40×. (Scale bar, 20 μm.)

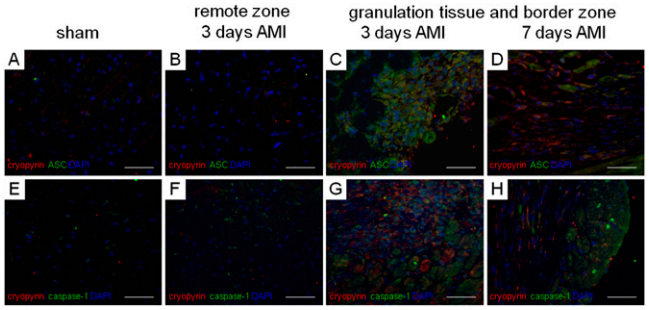

Fig. S4. Inflammasome components in the granulation tissue and cardiomyocytes. Overlap of the different components of the inflammasome [cryopyrin (red) and ASC (green) in *A–D*; and cryopyrin (red) and caspase-1 (green) in *E–H* is presented, showing intense expression of the individual components and strong overlap in the in the granulation tissue and border zone, 3 d (*C* and *G*) and 7 d after AMI (*D* and *H*) and minimal staining and overlap in sham (*A* and *E*) and remote myocardium (*B* and *F*). Counterstaining with DAPI (blue). Original magnification, 40×. (Scale bar, 20 μm.)

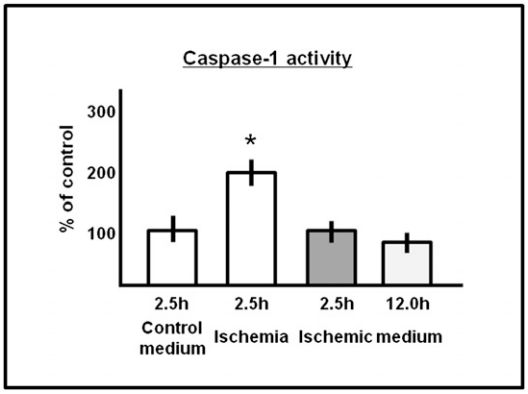

Fig. S5. Effects of "simulated ischemia" or "ischemic medium" on caspase-1 activation in HL-1 cardiomyocytes. Caspase-1 activity assessed using an enzymatic assay in cultured adult HL-1 cardiomyocytes exposed to simulated ischemia obtained by exposing cells to hypoxia and an ischemic buffer for 2.5 h, to the ischemic medium removed from HL-1 cells treated with simulated ischemia for 2.5 h or 12 h, or to control medium. As expected, simulated ischemia induced caspase-1 activation in HL-1 cardiomyocytes. Ischemic medium, however, failed to induce caspase-1 activation in HL-1 cardiomyocytes, suggesting that in HL-1, in vitro ischemia induces caspase-1 activation by a mechanism other than release of intracellular content.