Hyperlipidemia, a risk factor for cardiovascular disease, is defined as pathologically elevated plasma concentrations of cholesterol and other lipids, which are commonly found in patients with atherosclerosis. We and others previously reported that hyperlipidemia, proinflammatory mediators, and other risk factors promote endothelial cell (EC) activation and atherosclerosis via several mechanisms, which include inducing endothelial activation and injury, increasing monocyte recruitment and differentiation, and decreasing regulatory T cell population.

ECs that line the inner surface of vessel wall are the first cells exposed to metabolite-related endogenous danger signals in the circulatory system. Endothelial activation, therefore, defined as the initial event responsible for monocyte recruitment in atherogenesis. However, questions such as how hyperlipidemia can be sensed by ECs and how hyperlipidemia-induced vascular inflammation is initiated remain largely unanswered.

The cellular receptors, which can recognize the risk factors for atherogenesis, such as hyperlipidemia, have been under intensive search. The role of receptors for pathogen-associated molecular patterns has been characterized recently as bridging innate immune sensory systems for exogenous infectious agents and endogenous metabolic danger signals associated with molecular patterns (DAMPs) to initiation of inflammation. The toll-like receptors, mainly located in the...
plasma membrane, recognize a variety of conserved micro-
bial pathogen-associated molecular patterns and metabolic DAMPs and promote inflammatory gene transcription. As we described previously, for inflammation-privileged tissues in which inflammasome component genes are not constitu-
tively expressed, toll-like receptors also work in synergy with cytosolic sensing receptor families, including nod-like recep-
tors (NLRs; NOD [nucleotide binding and oligomerization domain]-like receptors) in recognizing endogenous DAMPs and in mediating upregulation and activation of a range of inflammatory genes.11 Caspase-1, a member of the cysteine protease family of caspases, is present in the cell cytosol as pro-caspase-1, an inactive zymogen, and requires the assem-
bly of an NLR family member–containing protein complex called inflammasome for activation. Activated caspase-1 is 
required for cleaving/processing pro-interleukin-1β (IL-1β) and pro-IL-18 into mature proinflammatory cytokines IL-1β and IL-18, respectively, and activation of other inflammatory pathways. However, it remains unclear whether in early ath-
erosclerosis, the caspase-1-inflammasome pathway in ECs can sense elevated lipids as a DAMP and promote endothelial activation.

Previous reports showed that cholesterol crystals activate NLRP3 inflammasome in macrophages, suggesting that NLRP3 inflammasome in macrophages can sense chole-
sterol crystals formed in advanced stage of atherosclerosis. However, monocyte migration into the aorta after 3 weeks of high fat (HF) diet feeding is detected in atherosclerotic apo-
lipoprotein E (ApoE)−/− mice, suggesting that before cho-
lesterol crystal formation in the vessels, ECs may respond to hyperlipidemia and activate caspase-1 precedent for mono-
ocyte recruitment. It has been reported that in response to vari-
ous proinflammatory stimuli, including lipopolysaccharide, human ECs secrete IL-1β, resulted from the cleavage of pro-
IL-1β by activated caspase-1. However, IL-1β secretion from human ECs, detected by ELISA, are 70.6-folds lower than that secreted from human monocytes, suggesting that IL-1β role in ECs as functional consequence of caspase-1 activation may not be as significant as that in monocytes. Thus, addi-
tional roles of caspase-1 in ECs need to be further explored. Although proatherogenic functions of caspase-1, NLRP3, IL-1β, and IL-18 have been reported, important knowledge gaps remain, such as (1) whether caspase-1 sensing system in ECs can sense early hyperlipidemia (noncholesterol crystals lipid stimulus) and (2) whether caspase-1 activation in ECs can promote endothelial activation, monocyte recruitment, and atherogenesis.

Our previous report showed that caspase-1 can have >70 protein substrates, the list of which is getting longer. A recent report showed that caspase-1 specifically cleaves sirtuin 1 (Sirt1), a nicotinamide adenine dinucleotide-dependent protein/class III histone deacetylase, in adipose tissue during metabolic stress. However, the question of whether cas-
pase-1 cleaves Sirt1 in aortic ECs remains unanswered.

In this study, we examined a novel hypothesis that caspase-1 in ECs can sense hyperlipidemia in mice fed a HF diet for 3 weeks and that caspase-1 activation in ECs, potentially via the caspase-1–Sirt1 pathway, can promote endothelial activation, monocyte recruitment, and atherogenesis. We generated double gene knockout (KO) mice that are deficient of caspase-1 and ApoE (ApoE−/−/caspase-1−/−) by crossing caspase-1−/− mice into ApoE−/− mouse background. Our results demonstrate that caspase-1 activation significantly contributes to endothelial activation, monocyte recruitment, and athero-
genesis via the caspase-1–Sirt1–activator protein-1 (AP-1) pathway. Therefore, our results indicate a role for caspase-1 activation in sensing hyperlipidemia as a DAMP and promoting endothelial activation.

**Materials and Methods**

Materials and Methods are available in the online-only Data Supplement.

**Results**

**Hyperlipidemia Induces the Upregulation of Caspase-1 Expression and Caspase-1 Activation in ApoE−/− Aorta**

To examine our hypothesis that early hyperlipidemia activates the caspase-1 in the aortic tissue, we performed Western blot analysis with caspase-1 antibodies on mouse aortic protein lysates collected from wild-type (WT) mice and ApoE−/− mice fed a HF diet for 0, 3, and 6 weeks. Plasma lipid profiling data (Figure 1A) showed that 3-week HF diet feeding signif-
ically increased plasma cholesterol levels and triglyceride levels in ApoE−/− mice, reaching hyperlipidemic conditions (>200 mg/dL). More importantly, pro-caspase-1 expression levels (Figure 1B) were significantly upregulated in ApoE−/− mouse aorta after feeding a HF diet for 3 (122%) and 6 weeks (160%), respectively. Because catalytic activation of procasp-
ase-1 (45 kDa) into 2 smaller subunits, p20 and p10, in a protein complex termed inflammasome is required for its pro-
tease activity, we also examined the expression of activated caspase-1 p20 subunit. The results (Figure 1B) showed that activ-
atated caspase-1 was increased in ApoE−/− mouse aorta fed with a HF diet for 3 weeks (604%) and 6 weeks (818%), respectively. Of note, upregulation of pro-caspase-1 induced by 6 weeks of HF diet feeding in ApoE−/− mouse aorta was 2-folds higher than that of WT mouse aorta. In contrast, activ-
atated caspase-1 p20 expression in HF diet–fed ApoE−/− mouse aorta was 8 folds higher than that of WT mouse aorta. With the lipid profiling data, we performed regression analysis of the lipid data against expression data of p20-activated caspase-1 detected by Western blot in Figure 1B. We found that

<table>
<thead>
<tr>
<th>Nonstandard Abbreviations and Acronyms</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP-1</td>
</tr>
<tr>
<td>DAMPs</td>
</tr>
<tr>
<td>ECs</td>
</tr>
<tr>
<td>HAEcs</td>
</tr>
<tr>
<td>HF</td>
</tr>
<tr>
<td>MAECs</td>
</tr>
<tr>
<td>ROS</td>
</tr>
<tr>
<td>Sirt1</td>
</tr>
<tr>
<td>WT</td>
</tr>
</tbody>
</table>
Deficiency of Caspase-1 in ApoE−/− Background Results in Decreased Atherosclerotic Lesion in the Early Stage of Atherogenesis

To examine the hypothesis that caspase-1 plays an important role in early atherogenesis, ApoE−/−/caspase-1−/− double gene KO mice were generated. The protein expression of pro-caspase-1 in mouse aorta (Figure 1A, right panel in the online-only Data Supplement) was absent in the double KO mice, which were verified with the mouse tail genomic DNA analysis (Figure 1A, left panel in the online-only Data Supplement). General health, body weight, heart and spleen weights (Figure 1B in the online-only Data Supplement), and plasma cholesterol and triglyceride levels (Figure 1C in the online-only Data Supplement) of ApoE−/−/caspase-1−/− mice were not significantly different from those of ApoE−/− mice. More importantly, after 3 weeks of HF diet, the atherosclerotic lesions in the aortic sinus area, the most sensitive atherogenic area in the aorta, of the double KO mice were significantly decreased by 44% (lesion area mean±2SD=3.92%±1.42%) compared with that of ApoE−/− mice (6.98%±2.67%; 

Deficiency of Caspase-1 in ApoE−/− Background Results in Decreased Expression of Proinflammatory Cytokines and Chemokines in the Aorta

Because proinflammatory cytokines and chemokines play essential roles in recruiting inflammatory cells into the aorta during atherogenesis,22 to determine the molecular mechanism underlying the reduction in atherosclerotic lesion formation in ApoE−/−/caspase-1−/− mice, we examined the hypothesis that the decrease in atherosclerotic lesion may be a result of the decreased generation of proinflammatory cytokines and chemokines in mouse aorta. We used an antibody array to compare simultaneously the expressions of 40 cytokines and chemokines in ApoE−/−/caspase-1−/− mouse aorta and ApoE−/− mouse aorta (Figure II in the online-only Data Supplement). The results showed that the expressions of 17 cytokines and chemokines out of 40 examined in ApoE−/− mouse aorta were higher than those of ApoE−/−/caspase-1−/− mice. More importantly, a recent study showed that caspase-1 is activated in aortic residential cells at the early stage of atherosclerosis.

Activated caspase-1 p20 expression in ApoE−/− mouse aorta was correlated well with increased plasma cholesterol levels (R²=0.8096; P=0.0004) and increased triglyceride levels (R²=0.7469; P=0.0013; Figure 1C), suggesting that caspase-1 activation is tightly associated with elevated cholesterol and triglycerides levels, as early as 3 weeks of hyperlipidemia. Of note, the expression of pro-caspase-1 in non–HF diet fed ApoE−/− mouse aorta was not significantly higher than that in non–HF diet fed WT mouse aorta, suggesting that upregulation of procaspase-1 in HF diet–fed ApoE−/− mouse aorta was not because of deficiency of the ApoE gene. The results showed that hyperlipidemia also upregulated the expression of caspase-1 mRNA 2-folds (Figure 1D), which was similar to the upregulation of pro-caspase-1 detected by Western blots. These results suggest that upregulation of pro-caspase-1 induced by hyperlipidemia in HF diet–fed ApoE−/− mouse aorta results from the hyperlipidemia-induced transcriptional mechanism and the posttranslational mechanism. As the substrate of activated caspase-1, cleaved and activated IL-1β was induced after 3 weeks of HF diet in the aortas of WT and ApoE−/− mice. In addition, the expression of pro-IL-1β was also induced (Figure 1E). Taken together, the results demonstrated that early hyperlipidemia induces the upregulation of caspase-1/IL-1β expression and caspase-1/IL-1β activation in mouse aorta. Since Dr Ross and his colleagues pointed out that significant monocyte recruitment into ApoE−/− mouse aorta does not happen until 6 weeks after HF diet feeding,13 our results suggest that caspase-1 is activated in aortic residential cells at the early stage of atherosclerosis.

Figure 1. Early hyperlipidemia induces caspase-1 (casp-1) expression and activation in mouse aorta. A, Plasma levels of cholesterol and triglycerides in wild-type mice (WT) and lipoprotein E gene-deficient mice (ApoE−/−) after 0 week (ND), 3 weeks (HF3w), or 6 weeks (HF6w) of high fat (HF) diet (n=5 for each group). B, The protein expression of pro-caspase-1 and active caspase-1 p20 subunit in mouse aorta lysate of WT and ApoE−/− mice after 0, 3, or 6 weeks of HF diet (n=2 for each group). C, Correlation of caps-1 activity and plasma lipid levels (of A and B). D, Casp-1 mRNA expression in aortas of WT and ApoE−/− after 0, 3, or 6 weeks of HF diet (n=3 for each group). E, The protein expression of pro-IL-1β and active IL-1β in mouse aorta lysate of WT and ApoE−/− mice with or without HF diet for 3 weeks. Data are expressed as mean±SE. *P<0.05, changes with the statistical significance.
Deficiency of Caspase-1 in ApoE−/− Background Results in Decreased Recruitment of Monocytes into the Aorta

Because recruitment of monocytes and other inflammatory cells into the mouse aorta and other arteries is essential for atherogenesis, based on our above results of decreased expression of inflammatory cytokines and chemokines in double KO aorta, we hypothesized that caspase-1 deficiency may result in reduced monocyte recruitment into the mouse aorta. We performed single cell analysis of mouse aortic cells with fluorescence-conjugated antibody staining for F4/80 and CD11b followed by flow cytometric analysis as reported previously. The results (Figure 3A and 3B) showed that caspase-1 deficiency in ApoE−/− background decreased F4/80+/CD11b+ macrophage recruitment into the aorta, but the reduction did not have statistical significance (P=0.0621). In contrast, the results also showed that caspase-1 deficiency significantly decreased F4/80+/CD11b+ monocyte recruitment into the aorta (P=0.0045) and F4/80−CD11b+ monocyte recruitment into the aorta (P=0.0194), respectively. In addition, we further determined whether aortic monocyte composition changes resulted from the changes in the peripheral blood. The results in Figure 3C showed that total mononuclear cells and CD11b+ monocytes in ApoE−/−/Caspase-1−/− mouse blood had no statistical differences to that of ApoE−/− mice. Moreover, we determined whether aortic monocyte composition changes as a result of alterations in the proliferation of recruited monocytes in mouse aorta. Because cell size of cell populations detected by the forward scatter with flow cytometry could be an estimate of cell proliferation status, the results in Figure III in the online-only Data Supplement showed that the 3 cell size fractions (large, middle, and small) in 3 cell subsets, including F4/80+CD11b+ macrophages, F4/80−CD11b− macrophages, F4/80−CD11b+ monocytes, and F4/80−CD11b− monocytes, in ApoE−/−/Caspase-1−/− mouse aortas had no statistical differences in comparison to that of ApoE−/− mice. Taken together, our results demonstrated that first, caspase-1 deficiency in ApoE−/− background decreased the recruitment of monocytes into the mouse aorta in early atherosclerosis; second, caspase-1 deficiency in ApoE−/− background did not significantly decrease F4/80+/CD11b+ macrophage recruitment into the aorta in early atherosclerosis, suggesting that caspase-1 deficiency did not result in a defect of monocyte-to-macrophage differentiation in the early atherosclerosis; and third, the aortic data of caspase-1 deficiency in ApoE−/− background was a result of aortic recruitment of monocytes but not as a result of the percentage changes of mononuclear cell and CD11b+ monocyte populations in the peripheral blood in early atherosclerosis.

Deficiency of Caspase-1 in ApoE−/− Background Results in Decreased Endothelial Activation, Including Reduced Cell Adhesion Molecule Expression and Attenuated Cytokine and Chemokine Secretion

A significant decrease in the recruitment of monocytes into the mouse aorta without changes in the peripheral blood monocyte
compositions leads to our hypothesis that caspase-1 deficiency in early atherosclerosis decreases endothelial activation rather than reducing the potency of monocyte infiltration into the mouse aorta. Endothelial activation can be examined from 2 perspectives. First, we reasoned that decreased endothelial activation would result in decreased secretion of cytokines and chemokines. To examine this possibility, mouse aortic ECs (MAECs) from WT mice and caspase-1−/− mice were cultured and primed with 50 ng/mL lipopolysaccharide and treated with 200 μg/mL of oxidized low-density lipoprotein (oxLDL; first signals for the inflammasome activation) for 24 hours followed with adenosine-5′-triphosphate (5 mmol/L) spike (second signal for the inflammasome activation) for 20 minutes. The antibody array results (Figure IV in the online-only Data Supplement) showed that caspase-1 deficiency significantly attenuated the secretion of C-X-C motif chemokine 10 (CXCL10), CCL3, CXCL2 (MIP-2) and granulocyte-macrophage colony stimulation factor levels from MAECs.

Second, we further reasoned that decreased endothelial activation in caspase-1-deficient mice would result in decreased upregulation of endothelial adhesion molecules, including ICAM-1, vascular cell adhesion molecule (VCAM)-1, and E-selectin. To examine this possibility, we first examined the adhesion molecule expression in aortas from ApoE−/− mice and ApoE−/−/caspase-1−/− mice. The results (Figure 4A) showed that 3 weeks of HF feeding induced upregulation of ICAM-1 (17.8-folds) and VCAM-1 (3.5-fold) protein expressions in ApoE−/− mouse aorta, respectively. On the contrary, HF diet feeding upregulated ICAM-1 and VCAM-1 expressions only by 2-folds and 1.5-folds, respectively, in the ApoE−/−/caspase−/− aorta. Of note, we did not find a difference in E-selectin expression between ApoE−/− and ApoE−/−/caspase−/− aortas. We then used RT-polymerase chain reaction to further examine the mRNA transcripts of ICAM-1, VCAM-1, and E-selectin in MAECs from WT mice and caspase-1−/− mice stimulated with oxLDL (100 μg/mL). The results (Figure 4B) showed that oxLDL stimulation induced mRNA upregulation of ICAM-1, VCAM-1, and E-selectin in WT MAECs by 10-, 8-, and 17-folds, respectively. In contrast, oxLDL stimulation induced no mRNA upregulation of...
ICAM-1, VCAM-1, and E-selectin in caspase-1−/− MAECs. The differences between the protein expression of adhesion molecules in mouse aortas and their mRNA expressions in MAECs may be because in addition to ECs, some adhesion molecules are also expressed in other vascular cells, including smooth muscle cells in mouse aorta. Regardless of the differences between the 2 experimental systems, caspase-1 deficiency resulted in decreased induction of EC adhesion molecules ICAM-1 and VCAM-1 in mouse aorta and MAECs in response to hyperlipidemic stimulations. Because attenuation of hyperlipidemia-induced ICAM-1 upregulation by caspase-1 deficiency was most dramatic among adhesion molecules examined, we looked into the possibility that caspase-1 activity–positive ECs may have higher ICAM-1 expression than caspase-1-inactive ECs. The results (Figure 4C) showed that ECs with active caspase-1 have higher ICAM-1 expression than caspase-1-inactive ECs, suggesting that caspase-1 activation promotes ICAM-1 upregulation and endothelial activation. Furthermore, we wanted to determine whether caspase-1 activation functionally promotes human aortic ECs (HAECs) to be more adhesive to unstimulated monocytes. Indeed, we found that oxLDL increased adhesiveness of ECs to monocytes (Figure 4D), which were inhibited by caspase-1 inhibitors, suggesting that caspase-1 activation increases upregulation of adhesion molecules, promotes endothelial activation, and makes ECs more adhesive to monocytes.

**Deficiency of Caspase-1 in the Aorta of ApoE−/− Mice Results in Decreased Recruitment of Transplanted Caspase-1+/+ Bone Marrow–Derived Inflammatory Ly6Cmiddle/high Monocytes into the Aorta**

To further consolidate our finding on the role of caspase-1 in promoting aortic endothelial activation and monocyte recruitment into the aorta, we performed chimeric bone marrow (BM) transplantation with enhanced green fluorescence protein transgenic mouse BM as the donor group and ApoE−/− mice and ApoE−/−/caspase-1−/− mice as the 2 recipient groups (Figure 5A and 5B). We reasoned that if caspase-1 activation promotes endothelial activation and monocyte recruitment, then more caspase-1 activity–enhanced green fluorescence protein− BM-derived Ly6Cmiddle/high inflammatory monocytes should migrate into the ApoE−/− aorta than the ApoE−/−/caspase-1−/− aorta. Indeed, we found that significantly more GFP+CD11b+Ly6C middle cells and GFP+CD11b+Ly6C high BM-derived monocytes migrated into the ApoE−/− aorta than the ApoE−/−/caspase-1−/− aorta (Figure 5C and 5D; P<0.05). As control experiments, we examined the peripheral blood monocyte subsets in the 2 recipient mouse groups. In contrast, we did not find any significant difference in peripheral blood monocyte subsets between the 2 recipient groups (Figure 5E and 5F). In addition, after caspase-1+/+ (WT) GFP transgenic BM cell transplantation into either ApoE−/− recipient mice or caspase-1−/−/ApoE−/− double gene KO recipient mice, caspase-1−/−/ApoE−/− double gene KO recipient mice had significantly less atherosclerotic lesions than ApoE−/− recipient mice (Figure 5G). Although ECs are not the only vascular resident cells that have caspase-1 activation in response to inflammatory stimuli and that EC-specific role of caspase-1 may ultimately require the model of EC-specific deficient mice of caspase-1, the results correlated well with our previous findings and suggested that caspase-1 activation in aortic ECs promotes monocyte recruitment into the aorta.
Atherogenic Lipid Products Induce Caspase-1 Activation and Endothelial Inflammation via a Reactive Oxygen Species–Dependent Pathway

Our data demonstrated that caspase-1 plays a critical role in promoting EC activation and monocyte recruitment into the mouse aorta exposed to hyperlipidemia. To further determine whether atherogenic lipid products induce caspase-1 activation in ECs and whether reactive oxygen species (ROS) plays any role in caspase-1 activation in ECs, we used oxLDL and 2 oxLDL derivatives, lysophosphatidic acid (lysoPA), and lysophosphatidylcholine (lysoPC) to stimulate HAECs. Because plasma membrane rupture and caspase-1 activation are 2 key features of the newly characterized inflammatory cell death (pyroptosis), in addition to using a flow
Yin et al Early Hyperlipidemia Activates Endothelial Cells

cytometry–based fluorescence-labeled caspase-1 enzymatic activity assay to detect caspase-1 activation, we also used fluorescence dye 7-AAD to measure plasma membrane integrity. We classified caspase-1 enzymatically active (caspase-1+) and 7-AAD− (caspase-1+/7-AAD−) cells as inflammatory ECs, caspase-1+/7-AAD+ cells as pyroptotic cells, and caspase-1−/7-AAD+ cells as necrotic cells. We found that oxLDL, lysoPA, and lysoPC induced inflammation, inflammatory cell death (pyroptosis), and necrosis after 6-hour stimulation in HAECs (Figure 6A). We then examined whether ROS plays any role in oxidized lipids–induced caspase-1 activation by containing inflammatory, pyroptotic, and necrotic ECs with ROS probe dihydroethidium. Our results showed that the mean fluorescence intensities of dihydroethidium stain in ruptured cells (either pyroptotic cells or necrotic cells) were higher than that of the inflammatory cells (Figure 6B), suggesting that oxidized lipids increased ROS-mediated caspase-1 activation and that cell death requires higher ROS levels to trigger than inflammation. We further verified the results with ROS inhibitors allopurinol (xanthine oxidase inhibitor) and apocynin (NADPH oxidase inhibitor) for inhibition of caspase-1 activation (Figure 6C). Finally, we examined whether oxLDL induces upregulated caspase-1 and inflammasome component transcripts in ECs. The RT-polymerase chain reaction results (Figure 6D) showed that treatment of oxLDL for 24 hours upregulated significantly NLRP1, NLRP3, caspase-1, PYCARD, and IL-1β transcripts. Because inflammasome assembly for caspase-1 activation requires NLRP, PYCARD, and procaspase-1, and effective upregulation of transcription of inflammasome and caspase-1 occurs ≈24 hours after stimulation, these results suggest that post-translational caspase-1 activation is much earlier than upregulation of caspase-1 and inflammasome transcription in ECs.

Figure 6. Oxidized low-density lipoprotein (oxLDL) and its components induce caspase-1 (casp-1) activation in human aortic endothelial cells (HAECs) via a reactive oxygen species (ROS)–mediated pathway. A, Pyroptotic cell death in HAECs caused by activation of casp-1 induced by oxLDL and its components. HAECs were cultured and treated with low-density lipoprotein (LDL; 100 μg/mL), oxLDL(100 μg/mL), oxLDL-derivatives lysophosphatic acid (LPA, 100 μM), or lysophosphatidylcholine (LPC, 15 μM) as indicated for 6 hours. Casp-1 activity was determined by a commercial kit, and 7-aminoactinomycin D (7-AAD) fluorescence dye was used to determine the cell membrane integrity. Casp-1+/7-AAD+ cells were gated as pyroptotic cells (Q3), casp-1+ single positive cells (Q2) were gated as inflammatory cells, and 7-AAD+ single positive cells (Q4) were gated as necrotic cells. B, ROS levels in pyroptotic cells. ROS levels were determined by dihydroethidium (DHE) fluorescence dye staining, and the mean fluorescence intensity (MFI) of DHE+ cell fraction was determined. C, Attenuation of oxLDL-induced caspase-1 activation in HAECs with ROS inhibitors Allopurinol (xanthine oxidase inhibitor) and Apocynin (NADPH oxidase inhibitor). Allopurinol (1 mmol/L) and Apocynin (100 μM) were added 1 hour before oxLDL treatment. HAECs were then treated with oxLDL (100 μg/mL) for 6 hours and stained for caspase-1 activity. D, mRNA upregulation of inflammasome components, including NLRP1 (Nod-like receptor protein 1), NLRP3 (Nod-like receptor 3), PYCARD (or ASC, inflammasome adaptor apoptosis-associated speck-like protein containing a CARD), caspase-1, and IL-1β (interleukin-1β) in HAECs treated with oxLDL. Data are expressed as mean±SE. *P<0.05, changes with statistical significance.
HF diet, we hypothesized that the effect of caspase-1 activation on endothelial activation is probably contributed more by other pathways rather than the IL-1β pathway. Thus, we attempted to search for novel substrate of caspase-1 that could modulate inflammation and endothelial activation. Among 24 experimentally verified caspase-1 substrates that we found in a literature search, Sirt1 has recently been suggested to be cleaved by caspase-1. Because Sirt1 has previously been reported to regulate endothelial activation and has antiatherogenic function, we then hypothesized that caspase-1-deficient mouse aorta has accumulation of noncleaved Sirt1. To test this hypothesis, we examined Sirt1 expression by Western blot with Sirt1 antibody in the following 4 groups of mice (2 mice/group): (1) ApoE−/− mice fed a normal chow diet; (2) ApoE−/− mice fed a HF diet; (3) ApoE−/−/caspase-1−/− mice fed a normal chow diet; and (4) ApoE−/−/caspase-1−/− mice fed a HF diet. Our results showed that compared with ApoE−/−/caspase-1−/− mice fed a normal chow diet, ApoE−/−/caspase-1−/− aorta expressed significantly higher amount of Sirt1 (Figure 7A). HF-fed ApoE−/−/caspase-1−/− aorta had decreased Sirt1 accumulation to 1/3 of the level of ApoE−/−/caspase-1−/− mice fed a normal chow diet. These results suggest that a HF diet induces other proteinase(s) activities, which participate in Sirt1 cleavage in the absence of caspase-1. Of note, plasma cholesterol levels in ApoE−/− mice and ApoE−/−/caspase-1−/− mice (Figure IC in the online-only Data Supplement) were in the range of 220 to 320 mg/dL, which were a few folds higher than those in WT mice (average 109 mg/dL; Figure 1A). Our results suggest that caspase-1 activation induced by moderate hyperlipidemia is responsible for cleaving Sirt1 and hyperlipidemia induced by HF feeding further triggers additional uncharacterized proteinase(s) to cleave/degrade Sirt1. Then, we examined whether oxLDL decreases Sirt1 expression in HAECs by caspase-1 cleavage mechanism. The results (Figure 7B) showed that oxLDL induced the expression of cleaved-Sirt1 by 2.4-folds in HAECs. To examine whether the induced cleavage form of Sirt1 was the result from the specific enzyme activity of Casp1, we designed a new cell permeable noncleavable Sirt1 (NC-Sirt1) by replacing the aspartate (D) in the amino acid position 150 of human Sirt1 with alanine (A), the specific cleavage site of Sirt1 recognized by Casp1 (Figure V in the online-only Data Supplement). Our results showed that NC-Sirt1 dose-dependently decreases the cleavage of Sirt1 induced by oxLDL. In addition, 2 different ROS scavengers (PEG-SOD and PEG-catalase) independently and synergistically inhibit oxLDL-induced, Casp1-mediated Sirt1 cleavage. Furthermore, the proteasome inhibitor MG-132 inhibited oxLDL-induced Sirt1 cleavage, suggesting that the cleaved Sirt1 may be further subjected to a putative proteolysis by an uncharacterized proteasome-controlled proteinase. Thus, when MG-132 inhibits proteasome, the expression of this uncharacterized proteasome-controlled proteinase is increased, which leads to decreased expression of caspase-1 cleaved Sirt1. These results suggest that oxLDL first increases ROS, which promotes caspase-1 activation for cleaving Sirt1. We then used the PeptideCutter database of the Swiss Institute of Bioinformatics to analyze the potential enzymes that can cleave the human Sirt1 protein sequence. The results (Table
I in the online-only Data Supplement) showed that caspase-1 and caspase-3 are among the enzymes that can cleave Sirt1 and are regulated by ROS, although the predicted cleavage site on Sirt1 for caspase-1 is not the same one as experimentally determined.\textsuperscript{23} Taken together, our results suggest that caspase-1 in the mouse aorta and HAECs cleaves Sirt1 protein in response to hyperlipidemic stimuli.

Caspase-1 Activation Induces Expression of Cytokines, Chemokines, and Adhesion Molecules via an Sirt1-AP-1–Mediated Pathway

Our data showed that caspase-1 activation induces the upregulation of several EC activation-associated cytokines, chemokines, and endothelial adhesion molecules. To further explore the mechanism underlying this caspase-1 function, we hypothesized that caspase-1 activation leads to a Sirt1-controlled transcription factor pathway to regulate these genes. Among the transcription factors that are modulated by Sirt1 are AP-1\textsuperscript{34} and NF-κB.\textsuperscript{35} We examined whether lysoPC-activated AP-1 activity and NF-κB binding can be inhibited by caspase-1 inhibitors by performing electrophoretic mobility shift assay. The results (Figure 7C) showed that lysoPC-induced AP-1 binding to AP-1 consensus nucleotides were inhibited by caspase-1 inhibitor, whereas lysoPC-activated NF-κB binding to NF-κB consensus probe was not significantly affected by caspase-1 inhibitors. We then searched for published experimental evidence that caspase-1-induced cytokines, chemokines, and adhesion molecules (Figures II and IV in the online-only Data Supplement; Figure 4) are AP-1 targeted genes. The results (Table IIA in the online-only Data Supplement) showed that 11 out of 14 caspase-1-induced genes are experimentally verified AP-1 pathway-induced genes.

In addition, analysis from the microarray experimental results of Sirt1 gene–deficient mice in comparison to WT mice showed that the expression of these AP-1-targeted genes are increased in Sirt1-deficient mice (Table IIA in the online-only Data Supplement), suggesting that Sirt1 inhibits the expression of AP-1 targets. Moreover, the data-mining results (Table IIB in the online-only Data Supplement) showed that the expressions of AP-1 genes themselves, including Jun and Fos, are increased in Sirt1-deficient mice. Taken together, the results suggest that caspase-1-cleavable Sirt1 inhibits the expression of caspase-1-induced cytokines, chemokines, and adhesion molecules by suppressing AP-1 gene transcription and AP-1-targeted gene transcription, which further suggest that caspase-1 induces the expression of cytokines, chemokines, and adhesion molecules in ECs by cleavage and inhibition of Sirt1.

Discussion

Although the role of caspase-1 in atherogenesis remains controversial,\textsuperscript{36} the prevailing concept is that caspase-1 plays a proatherogenic role, which is supported by results collected from ApoE\textsuperscript{−/−}/caspase-1\textsuperscript{−/−} mice.\textsuperscript{15,37} Inflammusosome sensor NLRP3 KO BM cells in LDL receptor (LDLR)\textsuperscript{−/−} mice,\textsuperscript{12} ApoE\textsuperscript{−/−}/IL-1β\textsuperscript{−/−} mice,\textsuperscript{38} and ApoE\textsuperscript{−/−}/IL-18\textsuperscript{−/−} mice.\textsuperscript{39} Of note, Gage et al\textsuperscript{17} and Usui et al\textsuperscript{17} studied the role of caspase-1 deficiency in full-blown atherosclerosis in ApoE\textsuperscript{−/−} mice after HF feeding for 8 weeks\textsuperscript{17} and 12 weeks.\textsuperscript{37} In addition, it has been reported that NLRP3 mediates hemodynamic-induced EC activation\textsuperscript{40} and that the IL-1β mRNA/protein as well as NLRP3 mRNA are upregulated in 30 week HF feeding–induced atherosclerotic lesion and endothelium of diabetic pigs.\textsuperscript{41} Along the line, we further asked whether in the early atherogenesis associated with early hyperlipidemia induced by only 3 week HF feeding, caspase-1 activation, as metabolic stress-related danger signal–associated molecular pattern–sensing pathway,\textsuperscript{13} could be involved in endothelial activation. Using biochemical, immunologic, and pathological approaches and our newly generated ApoE\textsuperscript{−/−}/caspase-1\textsuperscript{−/−} mice, we addressed this question and have the following results: (1) early hyperlipidemia induces the upregulation of caspase-1 expression and caspase-1 activation in ApoE\textsuperscript{−/−} aorta, which supports our previously proposed 3-tier/inflammation privilege model for determining tissue readiness to caspase-1 activation and inflammation initiation;\textsuperscript{30} (2) caspase-1 deficiency in ApoE\textsuperscript{−/−} background results in decreased early atherosclerotic lesion formation, suggesting that caspase-1 activation in ECs promotes early atherogenesis; and (3) caspase-1 deficiency in ApoE\textsuperscript{−/−} background results in decreased expression of proinflammatory cytokines and chemokines in the aorta. Of note, the expression of 2 anti-inflammatory cytokines IL-10 and IL-1ra were also decreased in caspase-1-deficient aorta. However, the decreased expressions of as many as 15 proinflammatory cytokines in caspase-1-deficient aorta outweigh concomitant reduction of 2 anti-inflammatory cytokines, suggesting that caspase-1 activation promotes an inflammatory environment and a chemokine gradient more than anti-inflammatory environment for the recruitment of monocytes and other inflammatory cells into the aorta; (4) caspase-1 deficiency in ApoE\textsuperscript{−/−} background results in decreased recruitment of monocytes into the aorta but has no significant role in monocyte composition in the peripheral blood in the early stage of atherosclerosis, suggesting that caspase-1 activation promotes monocyte recruitment into the aorta presumably via promoting endothelial activation and not via increasing monocyte compositions in the peripheral blood; (5) caspase-1 deficiency in ApoE\textsuperscript{−/−} background results in decreased endothelial activation, including reduced cell adhesion molecule expression and attenuated cytokine and chemokine secretion, suggesting that increased caspase-1 activities promote endothelial activation; (6) caspase-1 deficiency in ApoE\textsuperscript{−/−} mice results in decreased recruitment of transplanted caspase-1\textsuperscript{−/−} BM-derived inflammatory Ly6C\textsuperscript{middle/high} monocytes into the caspase-1\textsuperscript{−/−} aorta, suggesting that caspase-1 activation can lead to endothelial activation, which subsequently recruits more monocyte into the aorta. Decreased recruitment of caspase-1\textsuperscript{−/−} BM-derived inflammatory Ly6C\textsuperscript{middle/high} monocytes into the caspase-1\textsuperscript{−/−} aorta results in less atherosclerosis than caspase-1\textsuperscript{−/−} aorta. To further determine the underlying molecular signaling mechanisms, we found (7) atherogenic lipid products induce caspase-1 activation and endothelial inflammation via a ROS-dependent pathway; (8) caspase-1 deficiency in ApoE\textsuperscript{−/−}/caspase-1\textsuperscript{−/−} aorta and inhibition of caspase-1 in ECs result in accumulation of anti-inflammatory protein/histone deacetylase Sirt1, which is a substrate of caspase-1, suggesting that caspase-1 activation in early atherogenesis promotes endothelial activation via a Sirt1 pathway; and (9) caspase-1...
activation induces the upregulation of cytokines, chemokines, and adhesion molecules in ECs via a Sirt1-AP-1–mediated pathway.

Although our previous report showed that caspase-1 can cleave numerous protein substrates,20 it is generally considered that caspase-1 fulfills its proinflammatory functions predominately by cleaving pro-IL-1β and pro-IL-18 into mature IL-1β and IL-18, respectively. Although the role of proinflammatory cytokines IL-1β38 and IL-1839, as the classical substrates of caspase-1, in the promotion of atherosclerosis has been reported, the role of proinflammatory cytokines IL-1β and IL-18 in promoting EC activation in the early stage of atherogenesis remained unknown. As defined by Ross’ laboratory in ApoE−/− mice,15 early atherosclerosis is the initiative stage precedent the occurrence of a large number of monocyte recruitment before 6 weeks of HF feeding in ApoE−/− mice. Our results were well correlated with a previous report that IL-1β secretion from human ECs are 70.6-folds lower than that secreted from human monocytes,16 suggesting that IL-1β role in ECs may not be as significant as that in monocytes. These results indicate that caspase-1 may not only act through an IL-1β- or IL-18-dependent pathway to promote endothelial activation. Instead, we found that caspase-1 activation in mouse aorta in early atherogenesis and in human aortic ECs stimulated by oxLDL promotes endothelial activation via a Sirt1-inhibitable pathway. It was reported that Sirt1 reduces endothelial activation,32 and overexpression of Sirt1 in ECs inhibits atherosclerosis.33 Mechanistically, adenovirus-mediated overexpression of Sirt1 significantly inhibits PMA (phorbol 12-myristate 13-acetate)/ionomycin-induced ICAM-1 expression in human umbilical vein ECs, whereas knockdown of Sirt1 by RNA interference results in increased expression of ICAM-1 and increases NF-kB p65 binding ability to the ICAM-1 promoter by ChIP assays in human umbilical vein endothelial cells.43 However, the issue of whether caspase-1 in aortic ECs senses hyperlipidemia to initiate vascular inflammation via inhibiting Sirt1 was not examined until this report. Taken together, our results demonstrate a novel mechanism in early atherosclerosis: caspase-1 promotes EC activation and monocyte recruitment via decreasing Sirt1 expression and activating AP-1 pathway. The novel caspase-1-Sirt1-AP-1 pathway and the classical caspase-1-IL-1β-IL-18 are not mutually exclusive.

HF diet feeding for >6 weeks promoted monocyte recruitment into the aorta,15 thus, the classical caspase-1-IL-1β and IL-18 pathway in recruited monocytes and macrophages may interplay with caspase-1-Sirt1-AP-1 pathway in ECs during later stages of atherosclerosis.

Endothelial activation is the first and essential step for atherogenesis, which includes 2 molecular events—upregulation of cell surface adhesion molecules to make ECs more adhesive and increased secretion of proinflammatory cytokines and chemokines to attract monocytes and other inflammatory cells for transendothelial recruitment.9 Monocytes and macrophages play an essential role in promoting atherogenesis; however, we reason that if ECs are not activated during the initiation of atherogenesis, then no monocytes in the peripheral blood can be recruited into the aorta. Our results showed that caspase-1 deficiency did not alter the composition of peripheral
blood monocytes and macrophages in early hyperlipidemia but instead significantly decreased aortic monocyte recruitment, suggesting that caspase-1-deficient ECs are less activated for recruitment of monocytes into the aorta. These findings were further supported by our BM transplantation results, as well as the decreased ICAM-1 and VCAM-1 and proinflammatory cytokine and chemokine expressions/secretion in HAECs and in caspase-1-deficient mouse aorta. A recent report showed that suppression of monocyte recruitment results in removal of macrophage from atherosclerotic plaques of ApoE−/− mice,44 which echoes the importance of our finding. It has been reported that chemokine CXCL16 and its receptor CXCR6 play a critical role in mediating T cell migration into aorta during atherogenesis.45 To determine whether CXCL16 and CXCR6 expressions and a role in mediating T cell migration into aorta during atherogenesis. Thus, T cell migration into caspase-1−/−/ApoE−/− mouse aortas may be decreased. In our newly proposed working model, we summarize our findings and highlight current understanding (Figure 8): (1) hyperlipidemia induces elevation of ROS via NADPH oxidase–dependent pathway; (2) increased ROS levels induce caspase-1 activation, EC inflammation, and endothelial pyroptosis (inflammatory cell death); (3) activated caspase-1 decreases anti-inflammatory protein/histone deacetylase Sirt1 expression by cleaving Sirt1; (4) Sirt1 is a high hierarchy gene that can deacetylate and inhibit proinflammatory transcription factors, including AP-1; and (5) the caspase-1–Sirt1–AP-1 pathway can promote endothelial activation, inflammation, and atherogenesis. Our results have demonstrated for the first time how hyperlipidemia, one of the most important metabolic risk factors, induces endothelial activation, which provides an important insight for future development of novel therapeutics for early intervention of cardiovascular diseases and other inflammatory diseases.

Acknowledgments

We are grateful to Dr Richard Flavell in Yale University School of Medicine for generously providing us caspase-1 gene knock-out mice and Dr Wenchao Song in University of Pennsylvania, Drs Michael Autieri, Muniswamy Madesh, Salim Merali, and Barrie Ashby in Temple University for the insightful suggestions.

Sources of Funding

This work was partially supported by the National Institutes of Health Grants to X.F. Yang and H. Wang.

Disclosures

None.

References


5. Combadère C, Potteaux S, Rodero M, Simon T, Pezard A, Esposito B, Merval R, Proudfoot A, Tedgui A, Mallat Z. Combined inhibition of CCL2, CX3CR1, and CCR5 abrogates Ly6c(bi) and Ly6c(Lo) monocyteycy and almost abolishes atherosclerosis in hypercholes-
The role of receptors for endogenous metabolic danger signals—associated molecular patterns has been characterized recently as bridging innate immune sensory systems for danger signals–associated molecular patterns to initiation of inflammation in bone marrow–derived cells, such as macrophages. However, an important question remained unknown whether endothelial cells, the cell type with the largest numbers such as macrophages. However, an important question remained unknown whether endothelial cells, the cell type with the largest numbers.


Supplemental Materials

Supplemental Methods:

Reagents

Dulbecco’s modified Eagle’s medium, M199, penicillin, streptomycin, L-glutamate, and heparin were purchased from Invitrogen (Carlsbad, CA). Dihydroethidium (DHE) was purchased from Molecular Probes (Eugene, OR). Caspase-1 peptide inhibitor (Ac-YVAD-CHO) was from ALEXIS Biochemicals (San Diego, CA), and caspase-1 small molecular inhibitor was generously provided by Dr. Craig Thomas of the NIH Chemical Genomics Center. Antibodies against β-actin were purchased from Sigma-Aldrich (St. Louis, MO). Caspase-1 antibody (Cat #14-9832-80) was purchased from eBioscience (San Diego, CA). Low-density lipoprotein (LDL), oxidized LDL (oxLDL), and acetylated LDL labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (Dil-Ac-LDL) were purchased from Biomedical Technologies (Stoughton, MA). Lysophosphatidylcholine (LysoPC, 1-hexadecanoyl-sn-glycerol-3-phosphorylcholine) and lysophosphatidic acid (LysoPA; mono-acylsn-glycerol-3-phosphate) were purchased from Avanti Polar Lipids (Alabaster, AL). Antibodies to intercellular adhesion molecule-1 (ICAM-1) (sc-18853), vascular cell adhesion molecule-1 (VCAM-1) (sc-8304), and E-selectin [CD62 antigen-like family member E (CD62E)] (sc-14011) were purchased from Santa Cruz (Santa Cruz, CA). All other reagents were purchased from Sigma-Aldrich, unless indicated otherwise.

Mice and Diets

C57BL/6J mice, chicken β-actin promoter possessing cytomegalovirus (CMV) enhancer driven enhanced green fluorescence protein (EGFP)-transgenic mice, and apolipoprotein E gene deficient (ApoE−/−) mice in a C57BL/6J background were obtained from the Jackson Laboratory (Bar Harbor, Me). Caspase-1−/− (Casp-1−/−) mice in a C57BL/6J background were generously provided by Dr. Richard Flavell’s laboratory (Yale University School of Medicine, CT). ApoE−/− mice and Casp-1−/− mice were crossed to establish ApoE−/−/Casp-1−/− mice. All mice were kept under specific pathogen-free conditions.
conditions in a temperature controlled environment. Age-matched male mice were used for all experiments. At the age of 8 weeks, mice were maintained on a normal chow diet (5% fat, Labdiet 5001) or fed a diet supplemented with 0.2% (w/w) cholesterol and 21.2% (w/w) fat (HF diet) (TD. 88137, Harlan Teklad, WI) for designated periods. For tissue collection, mouse heart, aorta, and spleen were collected under a dissecting microscope and weighed from euthanized mice. All procedures in animals were performed in accordance with the approvals of Temple University Institutional Animal Care and Use Committee (IACUC).

**Genotyping**

Murine genomic deoxyribonucleic acid (DNA) was collected using the following method. Briefly, a small piece of mouse tail tissue was collected from the mouse and digested with 500µl lysate buffer [100 mM Tris-HCl (pH 8.5), 5 mM EDTA, 0.2% SDS, 200 mM NaCl] supplemented with 200 mg/mL proteinase K (EMD Millipore, Billerica, MA) overnight at 55°C with shaking in a Thermomixer. The DNA was separated from the wrapping protein by occasional gentle taps of the digestion tube during the digestion process. The tissue debris was separated by centrifugation. The digestion supernatant (450µL) was transferred into a new tube and mixed with 700µL of isopropanol. The DNA was then precipitated and transferred into a new tube containing 200µL of H₂O, and dissolved by overnight incubation at 55°C. The genomic DNA was used for polymerase chain reaction (PCR) and the PCR product was separated on an agarose gel. Specific primers were used to determine the genotype of the mice. For ApoE, primer 180 (5'-GCCTAGCCGAGGAGAGGCCG-3'), 181 (5'-TGTGACTTGGGAGCTCTGCAGC-3'), and 182 (5'GCCGCCCGACTGCATCT-3') were used. The PCR cycle was 94°C for 30 seconds (sec), 68°C for 40 sec, 72°C for 1 minute (min), and repeated for 35 cycles. The DNA fragment for ApoE⁻/⁻ mice was 245 base pairs (bp), and the DNA fragment for WT mice was 150bp in length. For detection of caspase-1 KO mice, primer ICE 3(5'-ATGGCACACCACAGATATCGG-3'), ICEKO (5'-TGCTAAAGCGCATGCTCCAGACTG-3'), and ICE5 (5'-GAGACATATAAGGGAGAAGGG-3') were used. The PCR cycle was 94°C for 30 sec, 60°C for 1 min, 72°C
for 1 min, and repeat for 40 cycles. The DNA fragment for Casp-1<sup>-/-</sup> mice was 300bp and the DNA fragment for WT mice was 500bp in length.

**Lipid and Lipoprotein Analysis**

Blood was collected in 5% EDTA coated tubes from the inferior vena cava of anesthetized animals. Plasma was separated by low speed centrifugation for 20 min at 4°C. Plasma concentrations of total cholesterol (TC) and triglyceride (TG) in each sample were measured at the National Mouse Metabolic Phenotyping Center in Vanderbilt University (Nashville, TN).

**Aortic Sinus Cross-section and Atherosclerotic Lesion Characterization**

Mouse hearts were harvested, weighed, and fixed overnight with 4% paraformaldehyde (PFA). Fixed tissues were then impregnated with 20% (v/v) sucrose in PBS [1mM Na<sub>2</sub>HPO<sub>4</sub>, 137mM NaCl, 1.5mM KH<sub>2</sub>PO<sub>4</sub>, 2.7mM KCl at pH 7.4] embedded with optimal cutting temperature compound (OCT) (Tissue Tek, Sakura Finetek, DK), and quickly frozen on dry ice. Serial cross sections of the aortic root were collected on slides; 10μm cryostat sections were taken from the level where the 3 aortic valves first appeared to the level where the aortic valves disappeared. A total of 80 sections were collected on 10 slides. Sections of the aortic sinus were stained with Oil Red O and alum hematoxylin. Briefly, fixed sections were rinsed with 60% isopropanol and stained with freshly prepared Oil Red O working solution (0.3% Oil Red O in 60% isopropanol) for 18 min. Followed with another rinse with 60% isopropanol, the sections were then stained with alum hematoxylin and washed with distilled water. The stained sections were then mounted in aqueous mounting medium and stored in room temperature until imaging. Images were captured with a Zeiss Axioscope microscope (Carl Zeiss Inc., Thornwood, NY). Atherosclerotic lesion area was defined as the red area staining with Oil red O and measured with ImageJ (NIH, Bethesda, MD). The percentage of lesion area was calculated by dividing lesion area by the total sinus area, and the average value of eight sections on each slide was presented.

**Mouse Peripheral Blood Cell Isolation**
Peripheral blood was drawn from the inferior vena cava or by tail bleeding from anesthetized animals. Red blood cells were lysed with Ammonium-Chloride-Potassium (ACK) lysing buffer [0.15M ammonium chloride (NH₄Cl), 10mM potassium bicarbonate (KHCO₃), 0.1mM EDTA] for 8 min at room temperature. The remaining cells were washed with PBS supplemented with 2% (v/v) fetal bovine serum (FBS) (GIBCO Laboratory, Grand Island, NY) and stained as described below for flow cytometry analysis.

**Aortic Cell Isolation and Staining**

ApoE⁻/⁻ mice and ApoE⁻/⁻/Casp-1⁻/⁻ mice were fed with a HF diet for 3 weeks and sacrificed at the end of the feeding period. Their vasculatures were perfused by cardiac puncture with PBS containing 20U/mL of heparin to remove blood cells from all vessels. The aortas were collected and digested as previously described with slight modification. Briefly, the entire mouse thoracic and abdominal aortas were isolated from the surrounding fat, minced with scissors, and digested with 125U/mL collagenase type XI, 60U/mL hyaluronidase type I, 60U/ml DNase1, and 450U/mL collagenase type I in PBS containing 20mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (GIBCO Laboratory, Grand Island, NY) at 37°C for 45 min. Aortic cell suspensions were then washed with Hank’s balanced salt solution (HBSS) (Cellgro Mediatech, Washington, DC) supplemented with 2% FBS and filtered through a 70μM cell strainer (BD Falcon, San Jose, CA). The passed fluid containing the suspended cells was then ready for antibody staining.

Both cells from blood samples and from aortic tissues were first stained with LIVE/DEAD® Fixable Violet dye (Invitrogen, USA) for 20 min at 4°C to exclude dead cells, washed, and were co-incubated with three monoclonal antibodies, CD11b-phoerythrin (PE) (BD Biosciences, monocyte marker), Ly-6C-fluorescein isothiocyanate (FITC) (BD Biosciences, an inflammatory monocyte marker) and F4/80-phoerythrin-cyanine (PE-Cy7) (eBioscience, macrophage marker) for 30 min at 4 °C. The stained cells were then fixed with 2% PFA for at least 1 hour at 4°C and analyzed on the LSR II flow cytometer (BD Biosciences, San Jose, CA).
**Bone Marrow Transplantation**

Eight week-old ApoE\(^{-/-}\) mice and ApoE\(^{-/-}\)/Casp-1\(^{-/-}\) mice were irradiated with a single dose of nine Gy using the model 30-1 Irradiator (J.L. Shepherd & Associates, San Fernando, CA). The donor bone marrow (BM) cells were harvested from 8 week-old enhanced green fluorescent protein (EGFP)-transgenic mice. Briefly, BM cells were flushed out from tibia and femur with HBSS supplemented with 2% FBS and filtered through a 70μM cell strainer. The un-fractionated EGFP\(^+\) BM cells (5 x 10\(^6\) cells) were administered by retro-orbital injection into the irradiated mice 2 hours after irradiation. To assess the irradiation efficiency, a group of mice without receiving BM transplantation after irradiation was used as controls. More than 80% of the non-BM recipient control mice died after irradiation while all BM transplanted mice survived. To assess hematopoietic chimerism, peripheral blood cells were collected from the recipient mice 6 weeks after BM transplantation, and the frequency of EGFP\(^+\) cells among peripheral nucleated blood cells was determined by flow cytometry after hemolysis with ACK lysis buffer. The chimeric mice were then fed a HF diet for 3 weeks, and the rates of EGFP\(^+\) monocyte migration into the aorta were accessed by the single cell suspension method as described previously (reference).

**Human Aortic Endothelial Cell Culture**

Human aortic endothelial cells (HAECs) (Clonetics Corporation, San Diego, CA) were cultured as we previously described. The cells were maintained on a 0.2% gelatin-coated 75-cm\(^2\) flask in M199 (Hyclone Labs., Logan, UT) supplemented with 20% FBS, 1% Penicillin/Streptomycin (Invitrogen, Carlsbad, CA), 3ng/mL EC growth supplement (ECGS) (BD Biosciences, San Jose, CA), and 5U/ml heparin at 37°C under 5% CO\(_2\), 95% air until passage 8. For our experiments, HAECs (≤ passage 9) were used and treated with desired stimuli for indicated time.

**Mouse Endothelial Cell Isolation and Primary Culture**

Mouse aorta ECs (MAECs) were isolated and cultured as previous described with modifications. Briefly, the entire mouse thoracic aorta was exposed, perfused with
PBS containing 1,000U/mL heparin, and filled with Dulbecco's modified eagle medium (DMEM) (Hyclone Labs., Logan, UT) plus 300U/mL collagenase type 2 (Worthington Biochemical Corp., Freehold, NJ) with ligation at both ends. The aorta was then isolated and incubated in 20% FBS/DMEM at 37°C for 1 hour. The fluid inside the aorta was flushed out with 20% FBS/DMEM and drained into a 15-mL tube containing 10 mL of endothelial growth medium (EGM) [50% DMEM; 40% F-12 (Invitrogen, Carlsbad, CA), 10% FBS; 0.3% ECGS; 10U/mL Heparin; 1% Penicillin/Streptomycin]. After centrifugation, all cells were collected and re-suspended with fresh EGM. The cells were then transferred into collagen-coated 35-mm dishes (2 aortas/dish) and incubated for 1 hr. The non-adhered cells were then washed away with sterile Dulbecco's phosphate-buffered saline (DPBS) (Hyclone Labs., Logan, UT), and the MAECs were cultured with EGM until 80% confluence was achieved.

The specificity of ECs was determined by Dil-Ac-LDL uptake and CD31 staining. Briefly, Dil-Ac-LDL was added to the culture medium at the final concentration of 10µg/mL. MAECs were incubated with the dye-labeled lipoprotein for 4 hours at 37°C. The cells were then washed twice with PBS, fixed with 4% PFA for 20 min, and stained with 4′, 6-diamidino-2-phenylindole (DAPI) (1µg/mL) for 5 min. For CD31 (platelet endothelial cell adhesion molecule-1, EC marker) staining, MAECs were cultured on a sterile cover slide and fixed with 4% PFA for 20 min before staining. Rat anti-mouse CD31 antibody (BD Pharmingen, San Diego, CA) and FITC conjugated rabbit anti-rat secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) were used. All images were captured with a Zeiss Axioscope microscope.

**Caspase-1 Activity Assay**

Active caspase-1 level was determined with APO LOGIX kit (Cell Tech., Mountain View, CA). The kit contains a carboxyfluorescein (FAM) [Excitation/Emission (nm):490/520]-labeled peptide fluoromethyl ketone (FMK) caspase-1 inhibitor (FAM-YVAD-FMK), which irreversibly binds to active caspase-1. All procedures were performed according to the manufacturer's instruction. Briefly, HAECs (≤ passage 9) were cultured in 6-well plates and serum starved overnight to quiescent the cells before treatment. Next day, HAECs were treated with indicated stimuli for 6 hr. Cells were
then digested by trypsin-EDTA and suspended at $1 \times 10^6$ cells/ml. 150µl of cell suspension were incubated at 37°C with 1 x FAM-YVAD-FMK for 1 hour then washed with 1x washing buffer. Unfixed caspase-1 stained cells were then incubated with 7-aminoactinomycin D (7-AAD; cell membrane integrity marker) (BD Pharmingen, San Diego, CA) for no more than 10 min before analysis. The Calibur flow cytometer (BD Biosciences, San Jose, CA) was used to determine caspase-1 active (Caspase-1+) and 7-AAD+ cells. Data were analyzed with the FlowJo software (Tree Star, Ashland, OR).

**Reactive Oxygen Species Level Detection**

Dihydroethidium (DHE) was applied to detect reactive oxygen species (ROS) levels in HAECs. DHE can be oxidized by superoxide anion, which then bind with the cell’s DNA and stain its nucleus a bright red fluorescent [Excitation/Emission (nm):518/605]. For staining, suspended HAECs were incubated in 150µL culture medium containing 3 µM DHE for 40 min at 37°C in the dark. The samples were washed with flow cytometry washing buffer (PBS/2% FBS) and immediately analyzed by flow cytometry.

**Data Analysis using the FlowJo Software**

All flow cytometric data were analyzed with the FlowJo software. The uncompensated data was collected from the flow cytometer (either Calibur flow cytometer or LSRII flow cytometer). Forward and side scatter gates were used to select live cell population from clumps and debris. The positive gate was determined by its matched IgG control, and single staining was used to determine the compensation parameter.

**Protein Extraction and Western Blot Analysis**

Cell pellets from HAECs were collected and lysed with protein lysing buffer [0.75% SDS, 0.03M Tris-HCl stock (pH 6.8), 5.6% glycerol, 1mM EDTA, 0.04mg/ml phenylmethanesulfonylfluoride (PMSF), 1 x protease inhibitor tablet (Roche Applied Science, Indianapolis, IN)]. For aorta, the fat-free aortic tissues were collected and dissected with scissors before lysing. The cells/tissues were further lysed by sonication,
and the debris was centrifuged down. The supernatant was transferred into a new tube and the protein concentration was determined by the bicinchoninic acid assay (Pierce/Thermo, Rockford, IL). Forty µg to 100µg protein was loaded into a 10% sodium dodecyl sulfate polyacrylamide gels and transferred onto nitrocellulose membranes (Whatman, Clifton, NJ), and the membranes were then stained with 1% Ponceau S for loading controls. Then the blots were blocked with 5% non-fat milk in PBST (PBS + 0.1% Tween 20) for 1 hr at room temperature and probed with diluted primary antibodies overnight at 4°C. Blots were then washed 4 times with PBST and incubated with horseradish peroxidase (HRP) conjugated anti-mouse, anti-rabbit, or anti-goat secondary antibodies (Santa Cruz) for 1 hr at room temperature. After another 4 washes with PBST, the blots were then incubated with enhanced chemiluminescence (ECL) substrate for horseradish peroxidase (Pierce/Thermo, Rockford, IL), and the ECL intensity was detected by X-ray film exposure in a dark room. The X-ray films were developed by the SRX-101A medical film processor. The expression levels of proteins as indicated by the ECL intensity were measured with ImageJ (NIH, Bethesda, MD, USA).

**RNA Extraction and Real-Time PCR**

Messenger RNA (mRNA) was extracted from cultured cells or tissues using TRIzol® Reagent (Invitrogen, Carlsbad, CA) following the manufacturer’s instruction. Briefly, the cells or tissue samples were lysed with enough TRIzol® Reagent for 5 min at room temperature and then phase separated with chloroform. After high speed centrifugation, the upper aqueous phase was collected and transferred into a new tube with an equal volume of isopropanol. The mRNA was centrifuged down at high speed for 15 min, washed twice with 70% ethanol in RNAase free water (Qiagen, Valencia, CA), and dissolved in RNAase free water. The mRNA concentration was determined on a Nanodrop 2000 (Thermo Fisher Scientific, San Jose, CA). Two µg of mRNA was then reverse transcribed into complementary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). For real-time PCR (RT-PCR), a SYBR-green PCR system (SABiosciences, Frederick, MD) was used, and
the real-time PCR was performed on the StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA).

The primers used for the real-time PCR are listed below. Human NLRP1-Forward: 5'-AAGTGACTGCTCCATTCCGAA-3'; Human NLRP-Reverse: 5'-CTCCGAGAACAGCTGGTCTTCT-3'; Human NLRP3-Forward: 5'-TGAAGAGGATGGGTT-3'; Human NLRP3-Reverse: 5'-TTCAATGCACTGGAA TCTGC-3'; Human PYCARD-Forward: 5'-ATGGACGCCTTGGACCTCACC-3'; Human PYCARD-Reverse: 5'-TGGCTTGGCTGCCGACTGAGG-3'; Human CASPASE-1-Forward: 5'-AGCTCCTCAGGCAGTGCAGTT-3'; Human CASPASE-1-Reverse: 5'-AGAGCAAGACGTGTGCGGCT-3'; Human IL1β-Forward: 5'-ACAGATGAGTGCTCCTCCA-3'; Human IL1β-Reverse: 5'-GTCGGAGATTTCGTAG GCAT-3'; Human β-actin-Forward: 5'-ACCTTCTACAAT GAGCTGCG-3'; Human β-actin-Reverse: 5'-CCTGGATAGCAAGTACATGG-3'; Mouse Caspase-1-Forward: 5'-CCCTCAAGTTTTGCCTTTTTAG-3'; Mouse Caspase-1-Reverse: 5'-CCCTCGGAG AAAGATGTTGAAA -3'; Mouse ICAM-1-Forward: 5'-GTTCTCTAATGTGCTCCGAG GC-3'; Mouse ICAM-1-Reverse: 5'-C TTCAACCTTTTGCCTTTTAGA-3'; Mouse ICAM-1-Forward: 5'-GAAGAGAACACTGGAA AAGAG-3'; Mouse ICAM-1-Reverse: 5'-GCAAAGGACACTGGAAAAGAG-3'; Mouse VCAM-1-Forward: 5'-GCTGGAGAACTTGC GTTAAAG-3'; Mouse VCAM-1-Reverse: 5'-GCAAAGGACACTGGAA AAGAG-3'; Mouse GAPDH-Forward: 5'-GAAGGCCGCTGTGAGTATGTCG TGGA; Mouse GAPDH-Reverse: 5'-CACACCACATCACAAACTGGG GGCAT-3'.

**Cytokine Array**

A mouse cytokine array (R&D Systems, Minneapolis, MN) was used to determine the cytokine and chemokine expression in mouse aorta as well as in stimulated MAECs following the manufacturer's instruction. Briefly, the nitrocellulose membranes pre-spotted with 40 cytokine and chemokine antibodies were first blocked with 1× blocking buffer for 1 hr. Meanwhile, protein lysates or supernatant were incubated with the detection antibody cocktail for 1 hr. The blocked membranes were incubated with the premixed protein/antibody solutions overnight at 4°C. Then
membranes were washed with 1x wash buffer three times and incubated with HRP-conjugated Streptavidin for 30 min at room temperature, and followed with another three washes with 1x wash buffer. The membranes were then incubated with the chemiluminescent reagents and exposed to X-ray films for 1-10 min. The expression levels of the cytokines and chemokines were determined by the intensity of the spots measured with ImageJ software. The variations of the manufacture’s designate positive control (PC) spots between each array were used to determine the confidence interval of non-specific variations between samples.

**Static Adhesion Assay**

HAECs were cultured and seeded on 24-well plates. THP-1 human monocyctic cells were maintained in RPMI 1640 with 10% FBS and 2mM L-Glutamine (GIBCO Laboratory, Grand Island, NY). THP-1 cells were stained with 2 µM calcein green AM for 30 min at 37ºC. Then 1×10⁶/mL THP-1 cells were suspended in 1 mL of 1640 and M199 medium (1:1) and added to HAEC monolayer. After 1 hr incubation at 37ºC, unattached cells were removed by PBS washes and then the plates were read in a fluorescence microplate reader.

**Preparation of Nuclear Extracts**

HAECs cultured in 100-mm dishes were collected and homogenized in a low-salt buffer [10mM HEPES (pH 7.9), 1.5mM magnesium chloride (MgCl₂), 10mM KCl, 0.2mM phenylmethylsulfonyl fluoride (PMSF), 0.5M dithiothreitol (DTT)]. The cytoplasmic fraction was removed, and the isolated nuclei were resuspended in a high-salt buffer [20mM HEPES (pH 7.9), 25% glycerol, 1.5mM MgCl₂, 420mM NaCl, 0.2mM EDTA, 0.2mM PMSF, 0.5M DTT] to release soluble proteins. The nuclear protein preparation was then collected and stored at -80ºC. Protein concentration was determined by the bovine serum albumin (BCA) assay (Pierce) using BSA as a standard.
**Generation of Cell Permeable Human Non-Casp1 Cleavable Sirt1 Polypeptide**

The specific cleavage site of Human Sirt1 by Casp1 was identified by running NIH-NCBI Blast homology search between mouse Sirt1 (NIH-NCBI protein ID: NP_062786) and human Sirt1 protein sequence (NIH-NCBI protein ID: NP_036370) (Suppl. Fig. VA). After matching mouse Casp1 cleavage site of Sirt1 (D142, Asp at the amino acid 142) identified previously¹, human Casp1 cleavage site of Sirt1 (D150) was predicted and confirmed using our previously published method². Human Non-Casp1 cleavable Sirt1 (NC-Sirt1) was then generated, as shown for the single site mutated sequence (D150A, replacing the caspase-1 cleavage essential amino acid Asp with the amino acid Ala) of human Sirt1 sequence rendering the sequence non-Casp1 cleavable (Suppl. Fig. VB).

**Electrophoretic Mobility Shift Assay**

Transcription factor interactions with DNA response elements were assessed using electrophoretic mobility shift assay (EMSA). Activator protein-1 (AP-1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) consensus oligonucleotides end-labeled with IR700 were purchased from LI-COR (Lincoln, Nebraska). The sequences of the probes are as follows: AP-1, 5’-CGCTTGATGACTCAGCCGGAA-3’; and NF-κB, 5’-AGTTGAGGGGACTTTCCCAGGC-3’. Unlabeled probes were purchased from Santa Cruz and were used at a 30-fold excess of labeled probe. EMSA were carried out using an Odyssey Infrared EMSA kit (LI-COR) according to the manufacturer’s instructions. Three μg of nuclear extracts were added to each binding reaction. The probe and nuclear proteins were incubated for 30 min at room temperature and DNA-protein complexes were resolved on a 5% non-denaturing polyacrylamide gels afterwards. Images of gel were then obtained in an Odyssey scanner (LI-COR).

**Data Analysis**

All experiments were performed at least three times, and results were expressed as the mean ± standard error (S.E.). Statistical comparison of single parameters between 2 groups was performed by paired Student t test. The Kruskal-Wallis 1-way
ANOVA was used to compare the means of multiple groups and were followed by Dunn’s test. Data were considered statistically significant if $p$ was $<0.05$.

References:

Figure I. Genotyping and characterization of ApoE<sup>−/−</sup>Casp-1<sup>−/−</sup> mice. A. Polymerase chain reaction (PCR) analysis of ApoE and caspase-1 (casp-1) gene expressions in ApoE<sup>−/−</sup> mice and ApoE<sup>−/−</sup>/Casp-1<sup>−/−</sup> mice (left panel). Western blot analysis of pro-casp-1 expression in the aortas of ApoE<sup>−/−</sup> mice and ApoE<sup>−/−</sup>/Casp-1<sup>−/−</sup> mice (right panel) (n=2). B. General phenotype of Casp-1 deficiency in ApoE<sup>−/−</sup> mice after 0 or 3 weeks of HF diet: body weight (BW), ratio of heart weight (HW) to BW, and ratio of spleen weight (SW) to BW. C. Plasma levels of cholesterol and triglycerides in ApoE<sup>−/−</sup> mice and ApoE<sup>−/−</sup>/Casp-1<sup>−/−</sup> mice after 3 weeks of HF diet (HF3w).
A.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
<th>16</th>
<th>17</th>
<th>18</th>
<th>19</th>
<th>20</th>
<th>21</th>
<th>22</th>
<th>23</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ApoE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PC</td>
</tr>
<tr>
<td>B</td>
<td>CXCL13</td>
<td>C5a</td>
<td>G-CSF</td>
<td>GM-CSF</td>
<td>CCL1</td>
<td>CCL11</td>
<td>sICAM-1</td>
<td>IFN-γ</td>
<td>IL-1α</td>
<td>IL-1β</td>
<td>IL-1ra</td>
<td>IL-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>IL-3</td>
<td>IL-4</td>
<td>IL-5</td>
<td>IL-6</td>
<td>IL-7</td>
<td>IL-10</td>
<td>IL-13</td>
<td>IL-12p70</td>
<td>IL-16</td>
<td>IL-17</td>
<td>IL-23</td>
<td>IL-27</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>CXCL10</td>
<td>CXCL11</td>
<td>CXCL1</td>
<td>M-CSF</td>
<td>CCL2</td>
<td>CCL12</td>
<td>CXCL9</td>
<td>CCL3</td>
<td>CCL4</td>
<td>MIP-2</td>
<td>CCL5</td>
<td>CXCL12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>CCL17</td>
<td>TIMP-1</td>
<td>TNF-α</td>
<td>TREM-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>PC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B.

ApoE<sup>−/−</sup>

ApoE<sup>−/−</sup>/Casp-1<sup>−/−</sup>

C.

Figure II. Caspase-1 Deficiency Attenuates Cytokine and Chemokine Expression in ApoE<sup>−/−</sup>/Casp-1<sup>−/−</sup> Mouse Aorta. A. Layout of the cytokine and chemokine array (R&D system). B. The representative array images of the aortic lysates from ApoE<sup>−/−</sup> mice or ApoE<sup>−/−</sup>/Casp-1<sup>−/−</sup> mice. Two aortas were pooled together for blotting each array. The signal areas of a caspase-1 substrate, IL-1β, in two arrays were selectively highlighted with red boxes. C. The quantification of cytokine and chemokine expressions. The variations of the manufacture’s designate positive control (PC) spots between each array were used to determine the confidence interval of non-specific variations between samples (n=4 for each group). *, p<0.05 indicates the expression changes with statistical significance.
Figure III. No differences are found between the proliferation of macrophages/monocytes in ApoE<sup>−/−</sup> Casp-1<sup>−/−</sup> mouse aortas and that of ApoE<sup>−/−</sup> mice as reflected by the cell size. Cell size is determined with the scales of forward scatter by flow cytometry as an estimate of cell proliferation status.
Figure IV. Caspase-1 Promotes Secretome of Pro-inflammatory Cytokines and Chemokines in MAECs. A. Layout of the cytokines and chemokine array purchased from R&D systems. B. Array images of the culture supernatant from WT MAECs or Casp-1/− MAECs cultured and primed with 50ng/ml LPS and treated with 200 µg/ml oxLDL for 24 hours followed with ATP (5mM) spike for 20 min. The array spots of IL-1β were highlighted with the red boxes where the array spots of GM-CSF, CCL3, CCL5 and CXCL12 were indicated with the black ovals, respectively. C. The quantification of cytokine and chemokine expressions. The variations of the manufacturer’s designate positive control (PC) spots between each array were used to determine the confidence interval of non-specific variations between samples (n=2 for each group). *, p<0.05 change with significance.
A. NIH-NCBI Blast homology


Figure V. Generation of Cell-permeable non-Casp1 cleavable Sirt1 polypeptide. A. NIH-NCBI Blast homology search between mouse SIRT1 (upper) and human SIRT1 (lower). Caspase-1 cleavage site is highlighted in the red box. B. The non-cleavable Sirt1 polypeptide was generated with a single amino acid replacement in the sequence from 140-160 position of human SIRT1. Capase-1 cleavage site D150 from human SIRT1 was mutated to A150, rendering the peptide sequence non-casp1 cleavable.
Table I. Predicted proteinases for human Sirt1 protein cleavage. The analysis with the PeptideCutter database predicted potential proteinases for human Sirt1 protein cleavages. Data are expressed as mean ± SE. *, p<0.05, changes with statistical significance.
### Table II. Caspase-1 induces upregulation of proinflammatory cytokines, chemokines and adhesion molecules via sirtuin 1 (Sirt1)-AP-1 pathway.

A. Caspase-1-induced molecules have the AP-1 binding site in their promoters and Sirt1 gene deficiency increases the expression of caspase-1-induced molecules. The list of PubMed IDs showed that published papers experimentally identified AP-1 binding site in their promoters of caspase-1 induced genes except CCL-12 and IL-7. The database mining analysis of published microarray data of Sirt1 gene deficient (/-) mice versus wild-type control mice demonstrated that Sirt1 deficiency increases the expression of caspase-1-induced genes. The symbols indicate the figures, in which the results showed that caspase-1 induced the gene upregulation/secretion: *Figure S2, †Figure S4, ‡Fig.4). B. Sirtuin 1 deficiency increases the expression of AP-1 gene expression. The AP-1 subunits expression was retrieved from Sirt1 KO (knockout) microarray dataset GSE30247.