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Critical role of caspase-1 in vascular inflammation and development of atherosclerosis in Western diet-fed apolipoprotein E-deficient mice

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ABSTRACT

Objective: Recent investigations have suggested that the inflammasome plays a role in the development of vascular inflammation and atherosclerosis; however, its precise role remains controversial. We produced double-deficient mice for apolipoprotien E (*Apoe*) and caspase-1 (*Casp1*), a key component molecule of the inflammasome, and investigated the effect of caspase-1 deficiency on vascular inflammation and atherosclerosis.

Methods and results: Atherosclerotic plaque areas in whole aortas and aortic root of Western diet (WD)-fed $Apoe^{-/-}Casp1^{-/-}$ mice were significantly reduced compared to those in $Apoe^{-/-}$ mice. The amount of macrophages and vascular smooth muscle cells in the plaques was also reduced in $Apoe^{-/-}Casp1^{-/-}$ mice. No significant differences in plasma lipid profiles and body weight change were observed between these mice. Expression of interleukin (IL)-1β in the plaques as well as plasma levels of IL-1β, IL-1α, IL-6, CCL2, and TNF-α, in $Apoe^{-/-}Casp1^{-/-}$ mice were lower than those in $Apoe^{-/-}$ mice. *In vitro* experiments showed that calcium phosphate crystals induced caspase-1 activation and secretion of IL-1β and IL-1α in macrophages.

Conclusion: Our findings suggest that caspase-1 plays a critical role in vascular inflammation and atherosclerosis, and that modulation of caspase-1 could be a potential target for prevention and treatment of atherosclerosis.

1. Introduction

Atherosclerosis is a chronic inflammatory disease characterized by lipid deposition, leukocyte infiltration, and vascular smooth muscle cell (VSMC) proliferation in the vascular walls [1]. The inflammatory nature of atherosclerosis is evidenced by many findings. For instance, the inflammatory cells, mainly macrophages, infiltrate into the atherosclerotic plaques and the number of these cells is linked to the severity of the disease. Inflammatory cytokines and chemokines are involved in all stages of the process of atherosclerosis [2]. Furthermore, the association between cardiovascular events and serum inflammatory markers, particularly C-reactive protein, has been demonstrated [3]. However, the molecular basis by which an inflammatory response can occur in the process of atherosclerosis is not known.

Inflammation in the atherosclerotic process is considered sterile inflammation because it mostly occurs in the absence of microbial infection [4]. A number of inflammatory cytokines are involved in the inflammatory response of atherosclerosis. Of these, clinical and experimental studies have shown the importance of IL-1β in the pathogenesis of atherosclerosis [2,5]. A growing body of evidence suggests that some types of sterile inflammation are mediated through a newly discovered innate immune pathway known as the inflammasome, which is a large multiprotein complex in the cytosol and regulates IL-1β production [4,6,7]. The inflammasome contains Nod-like receptors (NLRs) associated with an apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), which recruits caspase-1 and induces its activation. Since caspase-1 is an IL-1β -converting enzyme (ICE), it processes pro-IL-1β into mature IL-1β. These findings suggest the involvement of the inflammasome in the development of atherosclerosis. We have previously reported that ASC deficiency attenuates inflammatory responses and neointimal formation in the vascular walls in a murine model for vascular injury [8]. Furthermore, recent studies have shown that cholesterol crystals activate the inflammasome in macrophages, which may promote the development of atherosclerosis [7,9]. In contrast, Menu et al. [10] have reported that atherosclerosis progresses independent of the inflammasome. Although the reason for this discrepancy is unclear,

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the distinct role of the inflammasome in atherosclerosis remains controversial. In the present study, we evaluated the role of the inflammasome in atherosclerosis in apolipoprotein E-deficient (*Apoe*$^{-/-}$) mice that developed spontaneous atherosclerotic lesions in the vascular walls due to hypercholesterolemia – a pattern similar to that observed in humans [11]. We produced the double deficient mice for *Apoe* and caspase-1 (*Apoe*$^{-/-}$*Casp1*$^{-/-}$). The male age-matched *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Casp1*$^{-/-}$ mice were fed a high-cholesterol diet (Western diet, WD) and were analyzed for atherosclerotic plaque formation and vascular inflammation. Since vascular calcification actively influences macrophages and promotes the plaque progression [12], we further investigated whether calcium phosphate crystals can activate caspase-1 and induce secretion of IL-1β and IL-1α in macrophages. The findings from this study demonstrate the critical role of caspase-1 in the development of atherosclerosis and provide a new insight into the role of the inflammasome in the pathogenesis of atherosclerosis.

2. Materials and methods

2.1. Animals and the development of atherosclerotic plaques

All experiments in this study were performed in accordance with the Jichi Medical University Guide for Laboratory Animals. C57BL/6J and *Apoe*$^{-/-}$ mice (male, 8-weeks-old; C57BL/6J background) were purchased from Clea Japan (Tokyo, Japan) and the Jackson Laboratory (Bar Harbor, ME), respectively. *Casp1*$^{-/-}$ mice (male, 8-weeks-old; C57BL/6 background) were kindly provided by Dr. Hiroko Tsutsui (Hyogo Medical College, Japan) [13,14]. *Apoe*$^{-/-}$*Casp1*$^{-/-}$ mice were produced by mating these mice. The mice were fed and watered and maintained on a 12-h light and dark cycle. Atherosclerotic plaques were developed when the mice were fed with WD (0.15% w/w cholesterol, 40 kcal% butter fat; D12079B, Research Diets, Inc., NJ) for 12 weeks.

2.2. Plasma lipid analyses

Plasma levels of total cholesterol (TC), triglycerides (TG), and nonesterified fatty acids (NEFA) after a 16-h fast were determined using colorimetric enzyme assay kits (Kyowa Medex Co. Ltd. for TC, and Wako Chemicals [Osaka, Japan] for TG and NEFA) according to the manufacturers' instructions.

2.3. Assessment of atherosclerosis in the whole aortas and aortic sinus

After the mice were euthanized, the aortas were excised and adventitial fat was removed. Aortas were then fixed in phosphate-buffered saline (PBS) containing 10% (w/v) formalin (Wako Chemicals). Whole aortas were opened longitudinally from the aortic arch to the iliac bifurcation, mounted *en face*, and stained for lipids with Sudan IV (Wako Chemicals) as previously described [15]. The extent of atherosclerotic areas was expressed as the percentage of lesion area in the entire aortic surface area. The hearts, perfused with PBS containing 10% (w/v) formalin, were embedded in Tissue-Tek O.C.T. compound (Sakura Finetechnical Co. Ltd., Tokyo, Japan), and 6-μm thick serial sections were cut using a cryostat (Leica CM1850; Leica Microsystems, Germany). The cross-sections of the aortic sinus were stained with Oil Red O (Sigma) and hematoxylin (Wako Chemicals). The average lesion area was measured using 10 sections from each mouse stained with Oil Red O. Images for atherosclerotic plaque areas were captured using the Leica Application Suite software (ver. 3.4.1, Leica Microsystems) and analyzed using the Adobe Photoshop CS4 software (Adobe Systems Inc., CA).

2.4. Immunohistochemistry

Immunohistochemical analysis was performed to detect IL-1β expression, macrophage infiltration, and VSMC composition in the plaques [15,16]. Briefly, the sections were incubated with primary antibody to IL-1β (R&D Systems), macrophage marker MOMA-2 (AbD Serotec, Raleigh, NC), and smooth muscle cell marker a-smooth muscle actin (α-SMA; clone 1A4, Sigma, St. Louis, MO). This was followed by incubation with biotin-conjugated secondary antibodies. The sections were washed and treated with avidin-peroxidase (ABC kit; Vector Laboratories, Burlingame, CA). The reaction was developed using a DAB substrate kit (Vector Laboratories). The sections were then counterstained with hematoxylin. No signals were detected when an irrelevant IgG (Vector Laboratories) was used instead of the primary antibody as a negative control. All measurements were conducted in a double-blind manner by 2 independent researchers.

2.5. Measurement of inflammatory cytokines

The levels of IL-1β, IL-1α, IL-6 CCL2 (MCP-1, monocyte chemoattractant protein-1), and tumor necrosis factor-a (TNF-a) were assessed using a mouse enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems) and cytometric bead array (CBA) kit (BD, Biosciences). For the CBA analysis, flow cytometry (FACS Calibur, BD Biosciences) was used.

2.6. Cell cultures and in vitro experiments

To generate murine primary bone marrow-derived macrophages (BMDMs), bone marrow cells were isolated from the femurs and tibias of mice and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Dainippon Pharmaceutical Co., Osaka, Japan) and 15% conditioned medium of L929 cells (ATCC, Rockville, MD) for 7 days. Murine J774 macrophage cells were obtained from RIKEN Gene Bank (Tsukuba, Japan) and cultured in 10%FCS/DMEM. After serum starvations and priming with a low-dose lipopolysaccharide (LPS, 10 ng/mL) for 16 h, the cells treated with monosodium urate monohydrate (MSU; InvivoGen, San Diego, CA) and tricalcium phosphate (TCP, Sigma) crystals in the presence or absence of bafilomycin (Sigma), CA-074 Me (Wako Chemicals), and Z-Tyr-Val-Ala-Asp-fluoromethylketone (YVAD-FMK) (MBL, Nagoya, Japan) for 6 h. All other reagents were obtained from Sigma unless otherwise specified.

2.7. Measurement of caspase-1 activity

Caspase-1 activity was analyzed using the carboxyfluoroescein FLICA Caspase-1 assay kit (Immunochemistry Technologies, Bloomington, MN) according to the manufacturer's instructions. Nuclei were co-stained with Hoechst33342. Fluorescence was detected by using confocal laser scanning microscopy (FV-10i, Olympus, Tokyo, Japan).

2.8. Statistical analyses

Data are expressed as mean ± standard error of the mean (SEM). An unpaired *t* test was used to compare 2 groups. For comparisons between multiple groups, the significance of differences between-group means was determined by one-way analysis of variance (ANOVA) combined with the Turkey–Kramer test. All analyses were performed using the GraphPad Prism Software (ver. 4, San Diego, CA). A p-value of <0.05 was considered statistically significant.

3. Results

3.1. Caspase-1 deficiency reduced the development of atherosclerosis

To investigate whether caspase-1 is critical for the development of atherosclerosis, we used *Apoe*$^{-/-}$ mice fed with WD for 12 weeks because they exhibit severe hypercholesterolemia and are an excellent model for human atherosclerosis [11]. Sudan IV staining revealed that atherosclerotic plaque areas in whole aortas of *Apoe*$^{-/-}$*Casp1*$^{-/-}$ mice were significantly decreased compared to those in *Apoe*$^{-/-}$ mice ($p < 0.01$) (Fig. 1A and B). Cross-sections of aortic sinuses stained with Oil Red O also showed that the plaques of aortic sinuses in *Apoe*$^{-/-}$*Casp1*$^{-/-}$ mice were significantly reduced compared to those in *Apoe*$^{-/-}$ mice ($p < 0.01$) (Fig. 1C and D). Since the progression of atherosclerosis is influenced by lipid metabolism, we determined the plasma levels of TC, NEFA, and TG. Although the concentration of plasma TC (but neither NEFA nor TG) was markedly elevated in WD-fed *Apoe*$^{-/-}$ mice compared to normal chow-fed *Apoe*$^{-/-}$ mice (data not shown), there were no significant differences in the plasma lipid levels of *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Casp1*$^{-/-}$ mice (Supplementary Table I). In addition, no difference in body weight between *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Casp1*$^{-/-}$ mice was observed.

3.2. Caspase-1 deficiency decreased macrophage infiltration and VSMC content

Next we examined whether caspase-1 deficiency can affect macrophage infiltration (MOMA-2) and VSMC content (α-SMA) in the plaques by immunohistochemical analysis. In the plaques of *Apoe*$^{-/-}$ mice, marked macrophage infiltration was detected, whereas its infiltration was significantly decreased in plaques of *Apoe*$^{-/-}$*Casp1*$^{-/-}$ mice ($p < 0.01$) (Fig. 2A and B). Furthermore, VSMC content of the plaques was also decreased in *Apoe*$^{-/-}$*Casp1*$^{-/-}$ mice compared to that in *Apoe*$^{-/-}$ mice ($p < 0.01$) (Fig. 2C and D).

3.3. Caspase-1 deficiency reduced IL-1β and other inflammatory cytokine expression

Since caspase-1 is a key proteolytic enzyme that regulates IL-1β maturation, we investigated whether IL-1β is involved in the progression of atherosclerosis. After feeding the WD for a 12 week period, IL-1β expression was clearly visible in the plaques of *Apoe*$^{-/-}$ mice, but showed decreased expression in those of *Apoe*$^{-/-}$*Casp1*$^{-/-}$ mice ($p < 0.05$) (Fig. 3A and B). Similarly, plasma levels of IL-1β were substantially increased in WD-fed *Apoe*$^{-/-}$ mice, and this increase in the level of plasma IL-1β was significantly low in *Apoe*$^{-/-}$*Casp1*$^{-/-}$ mice ($p < 0.05$) (Fig. 3C). The plasma levels of other inflammatory cytokines including IL-1α, IL-6, CCL2, and TNF-α, also decreased in *Apoe*$^{-/-}$*Casp1*$^{-/-}$ mice, compared to *Apoe*$^{-/-}$ mice, when mice were fed with WD (Fig. 3D–G).

3.4. Caspase-1 deficiency inhibited TCP crystal-induced inflammasome activation in macrophages

To further explore the role of caspase-1, we examined whether calcium phosphate crystals can activate the inflammasome in macrophages *in vitro* because it is now known that vascular calcification actively participates in plaque progression and instability via its action on macrophages [12]. Similar to a substantial release of IL-1α and IL-1β in response to MSU crystals, TCP crystals also stimulated a dose-dependent release of IL-1α and IL-1β by J774 macrophages (Fig. 4A and B). Because lysosomal destabilization and cathepsin B have been shown to mediate the inflammasome activation in response to cholesterol crystals [9,17], we tested the effects of bafilomycin, an inhibitor of lysosomal acidification, and CA-074 Me, a specific cathepsin B inhibitor. Treatment with bafilomycin or CA-074 Me significantly decreased TCP crystal-induced IL-1α and IL-1β release (Fig. 4C and D). In addition, a caspase-1 inhibitor YVAD-FMK inhibited TCP crystal-induced release of IL-1β but not of IL-1α. Caspase-1 activation by TCP and MSU

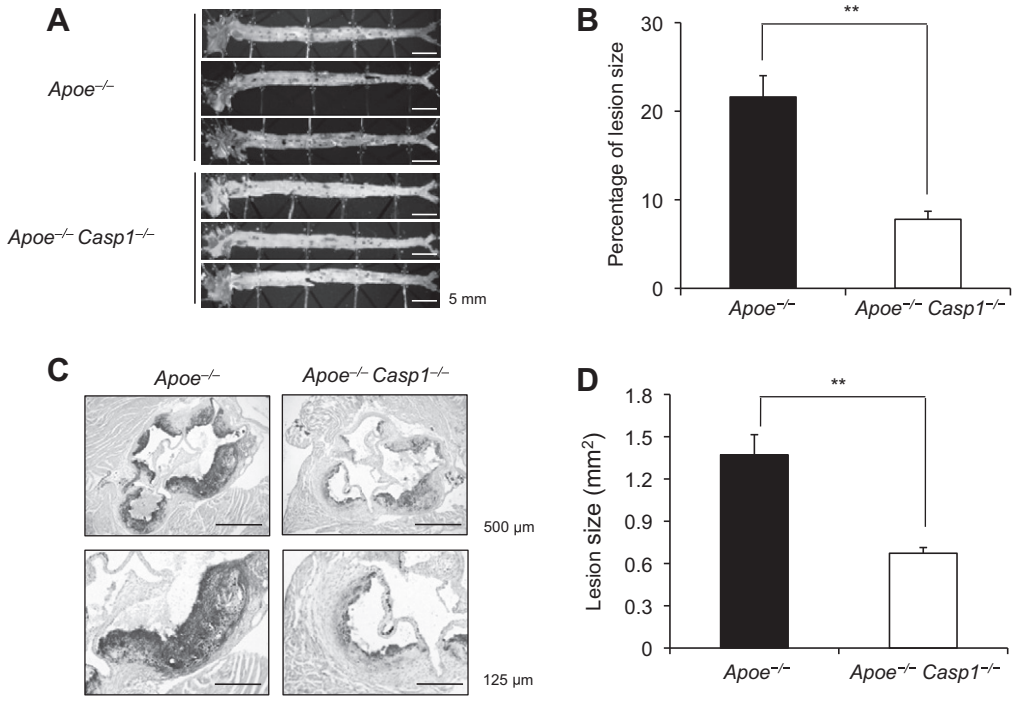

Fig. 1. Caspase-1 deficiency decreased the development of atherosclerosis *Apoe*$^{-/-}$ and *ApoE*$^{-/-}$*Casp1*$^{-/-}$ mice were fed with WD for 12 weeks. (A and B) Whole aortas were stained with Sudan IV. Representative photographs (A) and percentage (B) of atherosclerotic plaque areas are shown. Data are expressed as mean ± SEM ($n = 13$ for each). (C and D) Cross-sections of aortic sinuses were stained with Oil Red O. Representative photographs (C) and quantitative plaque areas (D) are shown. Data are expressed as mean ± SEM ($n = 12$–13 for each). $^{**}p < 0.01$.

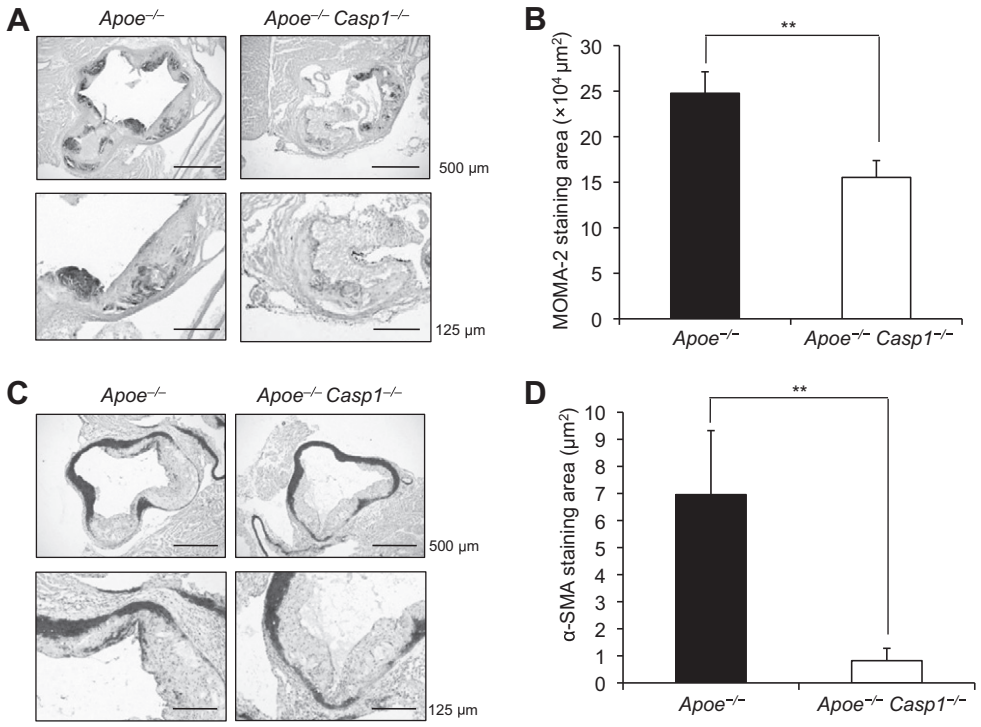

Fig. 2. Caspase-1 deficiency decreased macrophage infiltration and VSMC content *Apoe*$^{-/-}$ and *ApoE*$^{-/-}$*Casp1*$^{-/-}$ mice were fed with WD for 12 weeks. Cross-sections of aortic sinuses were immunochemically stained to identify macrophages (MOMA-2) and VSMCs (α-SMA). Representative photographs of infiltrated macrophages (A) and VSMC content (C) are shown. Quantitative analyses were performed (B for macrophages, D for VSMCs). Data are expressed as mean ± SEM (n = 6–8 for each). $^{**}p < 0.01$.

crystals was also confirmed by detection of the fluorescent cell-permeable probe that specifically binds to activated caspase-1 (Fig. 4E and F). Consistent with the data on J774 macrophages, YVAD-FMK inhibited TCP crystal-induced release of IL-1β but not of IL-1α in *Apoe*$^{-/-}$ BMDMs (Fig. 4 G and H). IL-1α release in response to TCP crystals tended to be reduced in *Apoe*$^{-/-}$*Casp1*$^{-/-}$ BMDMs.

4. Discussion

The major findings of this study are as follows: (1) caspase-1 deficiency significantly decreased the atherosclerotic lesions of the whole aortas and aortic sinuses in *Apoe*$^{-/-}$ mice fed the WD; (2) no significant differences in plasma lipid levels and body weight change were observed between *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Casp1*$^{-/-}$ mice; (3) caspase-1 deficiency reduced macrophage infiltration and VSMC content in the plaques; (4) caspase-1 deficiency decreased IL-1β expression in the plaques and plasma levels of inflammatory cytokines including IL-1β and IL-1α; (5) *in vitro* experiments showed that calcium phosphate crystals induced caspase-1 activation and release of IL-1β and IL-1α via lysosomal destabilization and cathepsin B in macrophages; and (6) calcium phosphate crystal-induced IL-1β release was caspase-1-dependent, but IL-1α release was caspase-1-independent. This study revealed a critical role of caspase-1 and IL-1β for vascular inflammation and atherosclerosis development induced by hypercholesterolemia. Furthermore, IL-1α may also be involved in the development of atherosclerosis; however, calcium phosphate crystals stimulate IL-1α release independent of caspase-1. Despite the fact that several reports have described that the inflammasome plays a role in the pathogenesis of atherosclerosis [9,10,17], its precise role remains unclear. Therefore, the findings of this study provide new insight into the role of inflammasome in vascular inflammation and atherosclerosis.

Many clinical and experimental studies have reported the importance of IL-1β in the pathogenesis of atherosclerosis and shown that IL-1β acts as a pro-atherogenic cytokine [2,5]. These findings suggest that the inflammasome is involved in the development of atherosclerosis. Moreover, Duewell et al. [17] have reported that cholesterol crystals activate the NLRP3 inflammasome, thereby resulting in cleavage and secretion of IL-1β in macrophages. In addition, they produced several types of bone marrow transplanted *Ldlr*$^{-/-}$ mice which had *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, and *Il1*a/b$^{-/-}$ bone marrow cells, and showed the reduction of atherosclerotic plaque size in the mice deficient for these inflammasome-related molecules specifically in bone marrow cells. Conversely, more recently, Menu et al. [10] have created *Apoe*$^{-/-}$*Nlrp3*$^{-/-}$, *Apoe*$^{-/-}$*Asc*$^{-/-}$, and *Apoe*$^{-/-}$*Casp1*$^{-/-}$ double-deficient mice and observed no significant differences in the plaque size or macrophage infiltration in these mice compared to *Apoe*$^{-/-}$mice. The authors concluded that atherosclerosis in *Apoe*$^{-/-}$ mice can progress independent of the inflammasome. In the present study, however, we showed that the plaque size and macrophage infiltration were clearly inhibited in *Apoe*$^{-/-}$*Casp1*$^{-/-}$ mice compared to those in *Apoe*$^{-/-}$ mice despite a similarly used experimental model. Although the reason for the discrepancy is unclear, the difference between our study and Menu's study is the diet: we used a WD that contained 0.15% cholesterol, which is widely used with *Apoe*$^{-/-}$ mice, whereas Menu et al. [10] used a highly atherogenic containing 1.25% cholesterol. Therefore, we hypothesized that the diet used in each study may have influenced the effect of caspase-1 deficiency. Supporting this is Duewell's study, which showed reduction of atherosclerosis by inflammasome deficiency using the same atherogenic diet as our study [17]. Thus, we speculate that the inflammasome contributes to the development of atherosclerosis and vascular inflammation, but its effect depends on the status of immune and inflammatory responses as well as specific disease models.

Vascular calcification influences progression and instability of atherosclerosis [12]. In fact, basic calcium phosphate crystals, including octacalcifum phosphate (OCP), TCP, and hydroxyapatite (HA), directly induce inflammatory response in macrophages and

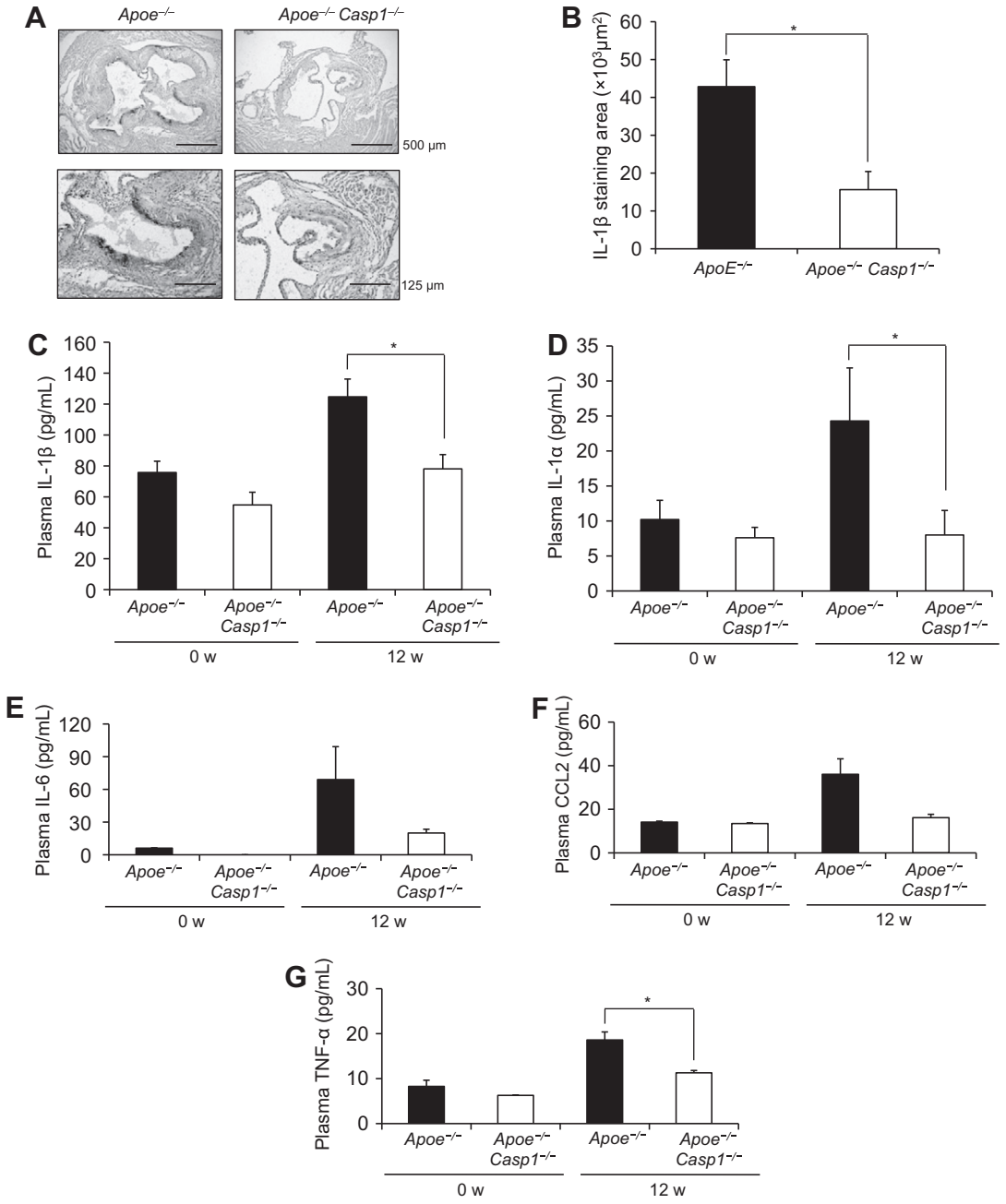

Fig. 3. Caspase-1 deficiency decreased IL-1β and other inflammatory cytokine expression *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Casp1*$^{-/-}$ mice were fed with WD for 12 weeks. (A and B) Cross-sections of aortic sinuses were immunochemically stained to identify IL-1β. Representative photographs are shown (A). Quantitative analyses of IL-1β staining areas were performed (B; $n = 5$). (C–G) Plasma levels of IL-1β, IL-1α, IL-6, CCL2, and TNF-α were determined by using ELISA and CBA. Data are expressed as mean ± SEM ($n = 15$ for IL-1β; $n = 8$ [0 w] and 12 [12 w] for IL-1α; $n = 4$ for IL-6, CCL2, and TNF-α). $^{*}p < 0.05$.

participate in the process of atherosclerosis [18]. On the other hand, several types of crystals including MSU and cholesterol crystals are reported to activate the inflammasome and induce IL-1β release [9,19]. In particular, recent studies have reported that the inflammasome is activated in response to OCP and HA crystals in both human and murine macrophages [20,21]. In the present study, we have shown that TCP crystals clearly stimulate caspase-1 activation and subsequent IL-1β release in macrophages. Thus, in addition to cholesterol crystals, our data suggest that calcium phosphate crystals can contribute to the progression and instability of atherosclerotic plaques through activating the inflammasome.

We have shown that TCP crystal-induced inflammasome activation was mediated through lysosomal destabilization and release of cathepsin B. This finding was consistent with the reports describing inflammasome activation in response to cholesterol crystals [9,17]. However, several recent reports suggest a possible redundancy of cathepsin B and the closely related cathepsin L. In particular, Dostert et al. [22] have reported that cathepsin B-deficient macrophages were not impaired in caspase-1 activation and IL-1β release in response to MSU crystals. Further, Duewell et al. [17] analyzed the mice deficient in single cathepsins (B or L) and suggested the functional redundancy of cathepsin B and L in inflammasome activation in response to cholesterol crystals. In

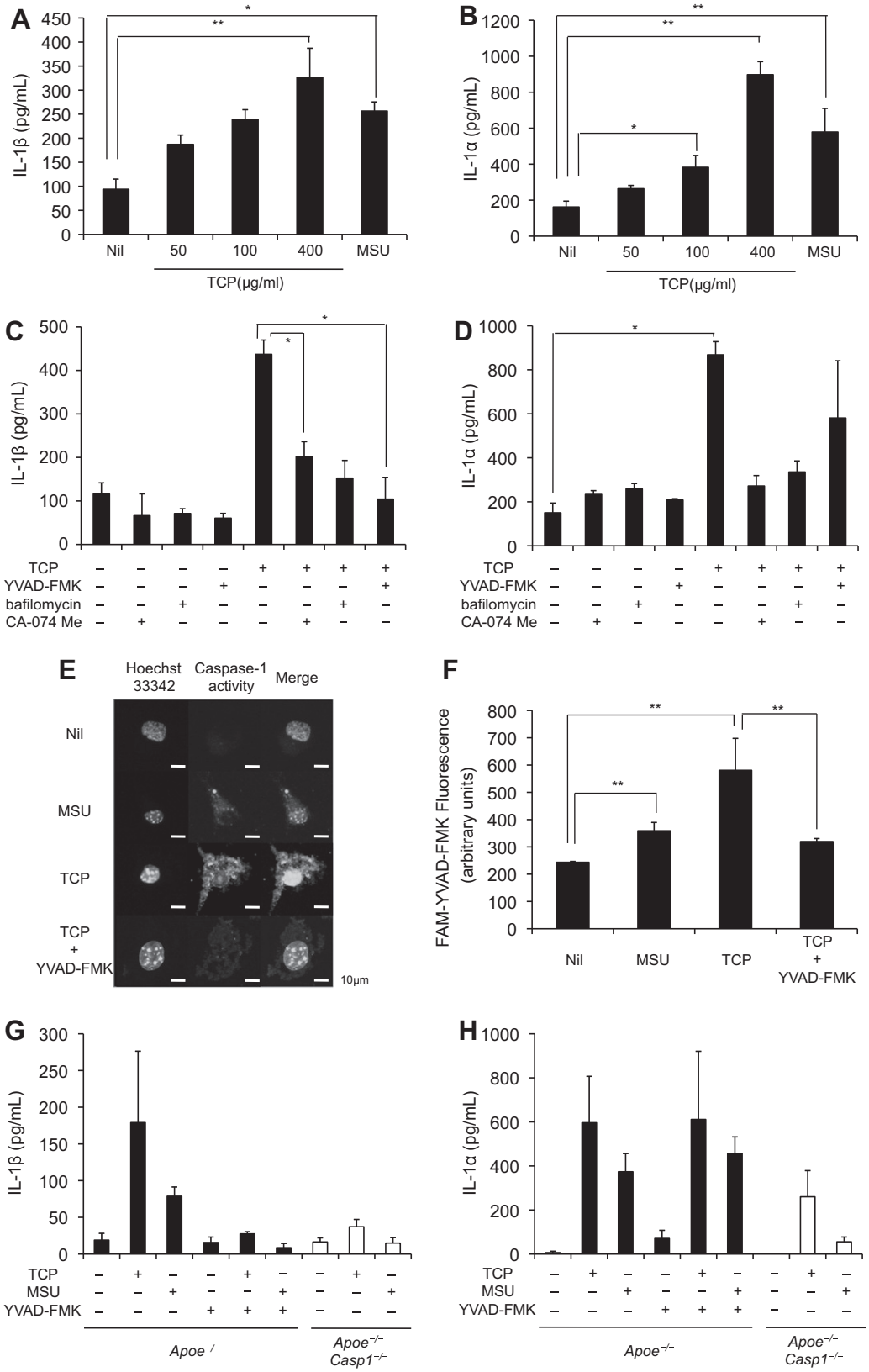

Fig. 4. Caspase-1 deficiency reduced calcium phosphate crystal-induced IL-1β release in macrophages (A–D) LPS-primed J774 macrophages were treated with TCP (50–400 μg/mL) or MSU (100 μg/mL) crystals in the absence or presence of bafilomycin (50 nM), CA-074 Me (10 μM), or YVAD-FMK (20 μM). The levels of IL-1β and IL-1α in the supernatants were assessed (n = 7 for A and B; n = 4 for C and D). (E and F) LPS-primed J774 macrophages were treated with TCP or MSU crystals (400 μg/mL) in the absence or presence of YVAD-FMK. Caspase-1 activity was visualized by incubation with a fluorescent cell-permeable probe that binds only to activated caspase-1. Nuclei were co-stained with Hoechst 33342. Representative photographs are shown (E). Quantitative analyses of caspase-1 activation were performed (F; n = 25). (G and H) BMDMs were prepared from *Apoe*$^{-/-}$ and *ApoE*$^{-/-}$*Casp1*$^{-/-}$ mice. After BMDMs were primed with LPS, cells were treated with TCP or MSU crystals in the absence or presence of YVAD-FMK. The levels of IL-1α and IL-1α in the supernatants were assessed. Data are expressed as ± SEM ($n = 4$ for each). $^{*}p < 0.05$; $^{**}p < 0.01$.

contrast to IL-1β release in response to TCP crystals, IL-1α release in response to TCP crystals was independent of the caspase-1 protease activity as shown by YVAD-FMK experiments. Supporting this finding, Gross et al. [23] recently reported that IL-1α release could be differently regulated by various stimuli as part of an inflammasome-dependent or independent pathway, and that MSU and alum-induced IL-1α release was not influenced by inhibiting caspase-1 protease activity.

In conclusion, we have shown that caspase-1 deficiency reduced macrophage infiltration, IL-1β and other inflammatory cytokine expression, and progression of atherosclerosis in WD-fed *Apoe*$^{-/-}$ mice. Although there are currently conflicting data on whether the inflammasome contributes to the development of atherosclerosis [10,17], our findings indicate that its contribution could be considerably influenced by diet. Further, these findings suggest the importance of the inflammasome in the pathogenesis of atherosclerosis and that modulation of the inflammasome could possibly be a potential target in the prevention and treatment of atheroslcerosis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.07.058.

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