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1 **Caspase-1 Deficiency Promotes High-Fat Diet-Induced Adipose Tissue**
2 **Inflammation and the Development of Obesity**
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29 **ABSTRACT**

30 Caspase-1 is a cysteine protease responsible for the processing of the proinflammatory cytokine
31 interleukin-1β and activated by the formation of inflammasome complexes. Although several
32 investigations have found a link between diet-induced obesity and caspase-1, the relationship of
33 remains controversial. Here, we found that mice deficient in caspase-1 were susceptible to
34 high-fat diet-induced obesity with increased adiposity as well as normal lipid and glucose
35 metabolism. Caspase-1 deficiency clearly promoted the infiltration of inflammatory
36 macrophages and increased the production of C-C motif chemokine ligand 2 (CCL2) in the
37 adipose tissue. The dominant cellular source of CCL2 was stromal vascular fraction rather than
38 adipocytes in the adipose tissue. These findings demonstrate a critical role of caspase-1 in
39 macrophage-driven inflammation in the adipose tissue and the development of obesity. These
40 data provide novel insights into the mechanisms underlying inflammation in the
41 pathophysiology of obesity.

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44 Key words: adipocytes; cytokines: inflammasome; macrophages; stromal vascular fraction

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46

47 **INTRODUCTION**

48 Obesity is a major risk factor for insulin resistance, hyperglycemia, dyslipidemia,
49 and hypertension, which are collectively known as metabolic syndrome, and is a growing global
50 public health concern. These metabolic disorders increase the risk for cardiovascular disease
51 and type 2 diabetes and contribute to increased mortality and morbidity. Although the
52 pathogenesis of obesity is complex and involves various factors, recent studies have revealed
53 that sterile inflammation in the adipose tissue is involved in the development of obesity (3, 5).
54 Indeed, the infiltration of macrophages into the adipose tissue is observed in animal models of
55 obesity as well as in obese human subjects with metabolic syndrome. These cells are the major
56 source of inflammatory cytokines and chemokines including tumor necrosis factor-α (TNF-α),
57 interleukin (IL)-1β, and C-C motif chemokine ligand 2 (CCL2, also known as monocyte
58 chemoattractant protein-1 [MCP-1]). These factors in turn recruit inflammatory cells and further
59 promote adipose tissue inflammation. However, the mechanisms of adipose tissue and sterile
60 inflammation in obesity have not been fully understood.

61 Caspase-1, also known as an IL-1β-converting enzyme (ICE), is a cysteine protease
62 responsible for the proteolytic cleavage of the proinflammatory cytokine IL-1β (4, 17). The role
63 of caspase-1 is well characterized in the context of inflammasomes. Inflammasomes are
64 intracellular large multiprotein complexes that regulate the release of IL-1β release and
65 subsequent inflammation (3, 16). We have recently shown that the nucleotide-binding
66 oligomerization domain-like receptor family pyrin domain containing 3 (NLRP3, also known as
67 NALP3 and cryopyrin) inflammasome-mediated sterile inflammation is involved in various
68 cardiovascular and renal diseases (9, 12, 13, 23, 26, 27, 29). Our findings are further supported
69 by recent works showing that NLRP3 inflammasomes are key mediators of other diseases,
70 including gout, pseudogout, asbestosis, silicosis, Alzheimer's disease, metabolic syndrome, and
71 type 2 diabetes (3, 16). However, inconsistent results have been reported with respect to the link
72 between diet-induced obesity and caspase-1 (14, 22, 28). Furthermore, several studies have
73 identified numerous proteins as potential substrates for caspase-1, suggesting that caspase-1

74 exerts inflammasome-independent functions. Thus, the function of caspase-1 remains under
75 debate. Therefore, its role in the development of obesity is currently controversial.
76 In the present study, we investigated the effect of caspase-1 deficiency on the
77 development of obesity and found that caspase-1-knockout (Casp1-KO) mice are susceptible to
78 the development of high-fat diet (HFD)-induced obesity with increased adiposity and
79 inflammation. Furthermore, we found that elevated of CCL2 mediate increased infiltration of
80 macrophages in the adipose tissue by caspase-1 deficiency. These findings indicate that
81 caspase-1 contributes to the development of obesity via a CCL2/C-C chemokine receptor 2
82 (CCR2) axis in the adipose tissue and provide insights into the mechanism underlying
83 inflammation in the pathophysiology of obesity.
84
85 **MATERIALS AND METHODS**
86 *Animals*
87 All animal experiments were approved by the Use and Care of Experimental
88 Animals Committee of the Jichi Medical University Guide for Laboratory Animals, and were
89 carried out in accordance with the Jichi Medical University guidelines. C57BL/6J wild-type
90 (WT) mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Casp1-KO mice were
91 generated as described previously (15, 25). IL-1β-KO mice were kindly provided by Dr.
92 Yoichiro Iwakura (Tokyo University of Science, Chiba, Japan) (6). The male mice (9–10
93 week-old) were fed with either 60 kcal% HFD (D12492: Research Diets, Japan LSG, Tokyo) or
94 a normal chow diet (CE-2; CLEA Japan Inc., Osaka). Each mouse was weighed every 2 weeks
95 and at terminal points, the mice were fasted for 6 h, weighed again, and then blood was
96 collected to obtain serum. After perfusion, the tissues were carefully excised and weighed.
97
98 *Pair-feeding experiments*
99 The results of the food intake were obtained from the mice individually housed in
100 cages; the weight of the freely available HFD in a jar was measured every 3 or 4 days, and the

101 daily food intake was calculated. Based on these results, we set up a pair-feeding experiment to
102 compare the body weight changes of mice fed with the same amount (2.6 g) of HFD every day
103 for 56 days.
104
105 *Micro-CT analysis*
106 A micro-CT analysis was performed using LaTheta LCT-200 (Hitachi Aloka
107 Medical, Tokyo, Japan). The mass of muscle, visceral, and subcutaneous fat was analyzed using
108 the LaTheta software (Hitachi Aloka Medical).
109
110 *Biochemical test*
111 Blood was collected from the tail vein of 6 h-fasted mice. The blood glucose levels
112 were measured using a Terumo MEDISAFE™ Blood Glucose Meter (Terumo Co., Tokyo,
113 Japan). Serum levels of the total cholesterol (TCHO) and triglycerides (TG) were measured
114 using a FUJI DRI-CHEM system (Fujifilm, Tokyo, Japan).
115
116 *Glucose tolerance test (GTT)*
117 A GTT was performed in mice fed a normal chow or HFD for 8 weeks. Mice were
118 fasted for 6 h before the test with free access to water. Blood glucose was then measured just
119 before the intraperitoneal glucose injection (1 g/kg body weight in saline) and subsequently at
120 15, 30, 60, 90, and 120 min post-administration.
121
122 *Flow cytometric analysis*
123 Infiltrating leukocytes in the epididymal white adipose tissue (WAT) were analyzed
124 by flow cytometry as described previously (11). The data on flow cytometry was obtained using
125 FACSVerse (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (Treestar,
126 Inc., San Carlos, CA).
127

128 *Histology*

129 Hematoxylin and eosin (HE) staining was performed as described previously (18).
130 Digital images of the HE-stained histopathology of WAT were captured using a light
131 microscope FSX-100 (Olympus, Tokyo, Japan). The size (relative area) of adipocytes was
132 measured by NIH imaging software (Image J 1.44p, National Institute of Health).

133

134 *Respiratory gas and locomotor analyses*

135 Mice were housed individually in a metabolic chamber for 48 h to allow them to
136 adapt to the environment and to attain a constant respiratory exchange ratio (RER). Respiratory
137 gas (O_2 and CO_2) analysis was performed using an open-circuit metabolic gas analysis system
138 connected directly to a mass spectrometer (Arco2000; ArcoSystem, Chiba, Japan). Oxygen
139 consumption (VO_2), and carbon dioxide production (VCO_2) of each mouse were measured
140 every 5 min in each cage. RER was calculated as the ratio VCO_2/VO_2. Locomotor activity of
141 each mouse was determined using an activity monitoring system (ACTIMO-100; Shinfactory,
142 Fukuoka, Japan) combined with individual metabolic chambers.

143

144 *Measurement of inflammatory cytokines*

145 Levels of CCL2, TNF-α, IL-6, IL-12 (p70), interferon (IFN)-γ, IL-1β, IL-18, leptin,
146 adiponectin, and insulin were assessed using a mouse enzyme-linked immunosorbent assay
147 (ELISA) kit (R&D Systems, BioVendor, and Shibayagi Co., Ltd.) or cytometric bead array
148 (CBA) kit (BD Biosciences). For the CBA analysis, flow cytometry (FACSVerse, BD
149 Biosciences) was used. WAT protein was extracted in RIPA buffer with protease inhibitor
150 cocktail and $CHCl_3$. Total protein concentration was masured using Pierce BCA protein assay
151 kit (Thermo Scientific).

152

153 *Migration assay*

154 Resident peritoneal macrophages from the mice were harvested, and macrophage

155 migration in response to CCL2 was assessed using 5-μm pore size transwell plates.

156

157 *Real-time RT-PCR analysis*

158 Total RNA was prepared from the adipose tissues using ISOGEN (Nippon Gene Co.,

159 Ltd., Toyama, Japan) following the manufacturer's instructions. Real-time RT-PCR analysis

160 was performed using the Takara TP960 PCR Thermal Cycler Dice Detection System (Takara

161 Bio Inc, Shiga, Japan) to detect mRNA expression. Gene expression was normalized using *Actb*

162 (β-actin) expression using the software provided with the system. Sequences of primers used for

163 real-time RT-PCR analysis have been described elsewhere (11).

164

165 *Cell and adipose tissue cultures*

166 Adipose tissues (epididymal and subcutaneous WAT tissues) were carefully excised

167 from WT or Casp1-KO mice on normal chow diet and divided into pieces of equivalent size.

168 The pieces were weighed and cultured overnight in 1 mL of Dulbecco's modified Eagle's

169 medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (FBS), and 1%

170 antibiotic-antimycotic solution (Sigma). The next day, culture media were replaced with new

171 media in the presence or absence of 10 ng/mL LPS, and the tissues were cultured for 24 h.

172 To obtain the adipocytes and stromal vascular fraction (SVF) cells, freshly isolated

173 mouse adipose tissue from WT or Casp1-KO mice on normal chow diet was cut into small

174 pieces and digested in 0.5 mg/mL collagenase type II (Calbiochem, San Diego, CA) in

175 Krebs-Ringer bicarbonate-HEPES (KRBH) buffer at 37°C for 40 min. After washing twice in

176 DMEM, tissue cells were filtrated through series of nylon mesh, and the single cell suspension

177 was centrifuged at 750 x *g* at 4°C for 10 min. The floating cells were used as adipocytes, and

178 the precipitated cells were used as SVF cells. The cells (adipocytes: 2 x10^5 cells/mL; SVF cells:

179 5 x10^5 cells/mL) were seeded and cultured overnight, the culture media were replaced with new

180 media in the presence or absence of 10 ng/mL LPS, and the cells were cultured for 24 h.

181

182 <u>*Statistical analysis*</u>

183 Outcomes with a normal distribution (*e.g.*, flow cytometry results) were analyzed by
184 parametric unpaired *t*-tests. Outcomes without a normal distribution were analyzed by the
185 following: comparisons of unpaired outcomes were assessed using the Mann–Whitney test;
186 differences among more than three groups were assessed using a nonparametric Kruskal–Wallis
187 test; and longitudinal analyses were performed for body weight and GTT. All analyses were
188 performed using Stata software, release 13 (Stata Corp., College Station, TX).

189

190 **RESULTS**

191 <u>*Caspase-1 deficiency promoted HFD-induced WAT accumulation and obesity*</u>

192 We first examined whether a caspase-1 deficiency could influence the development
193 of obesity in mice fed with a normal chow diet or HFD for 8 weeks. In WT mice, the HFD
194 increased the body weight gain from that in mice fed a normal chow diet (**Fig. 1A**).
195 Interestingly, Casp1-KO mice gained significantly more body weight than the WT mice did on
196 the HFD. On a normal chow diet, the Casp1-KO mice tended to have greater body weight gain
197 than the WT mice did, but this difference did not reach a statistical significance. Therefore, the
198 following experiments were primarily conducted on HFD feeding. An abdominal CT analysis
199 revealed that visceral and subcutaneous fat contents were also significantly increased in
200 Casp1-KO mice, whereas the muscle content was decreased (**Fig. 1B and C**). Furthermore, the
201 relative weight (tissue weight/body weight) of the epididymal and subcutaneous WATs in the
202 Casp1-KO mice was significantly higher than that of the WT mice (**Fig. 1D–F**). Consistent with
203 this finding, the increased size of the adipocytes was ascertained histologically in both types of
204 WAT in the Casp1-KO mice (**Fig. 1G, Supporting Figure 1 for reviewers only**). These
205 findings indicate that Casp1-KO mice are susceptible to HFD-induced obesity with increased
206 adiposity.

207 Because caspase-1 is an ICE and induces IL-1β maturation, we next assessed the
208 effect of HFD feeding on the development of obesity using IL-1β-KO mice. IL-1β-KO mice

209 significantly gained less body weight than WT mice on HFD feeding (Fig. 1H). In addition, the
210 relative weight (tissue weight/body weight) of the epididymal and subcutaneous WATs in
211 IL-1β-KO mice tended to be less than that of WT mice (Fig. 1I and J). These results indicate
212 that Casp1-KO mice are susceptible to HFD-induced obesity, independently of IL-1β.
213
214 *Caspase-1 deficiency had no effects on lipid profiles, glucose intolerance, energy expenditure*
215 *and locomotor activity*
216 Since obesity leads to metabolic disorders (*e.g*., dyslipidemia and hyperglycemia),
217 we next determined the levels of serum TCHO and TG, blood glucose, and plasma insulin. The
218 levels of these parameters were significantly increased in both WT and Casp1-KO mice by HFD
219 feeding (**Fig. 2A–D**). The levels of blood glucose and plasma insulin tended to be higher in
220 Casp1-KO mice than that those in WT mice, but they did not reach the statistical significance.
221 Furthermore, the GTT on a normal and HFD feeding exhibited no differences in glucose
222 intolerance between the WT and Casp1-KO mice (**Fig. 2E and F**). We also assessed energy
223 expenditure and locomotor activity in both strains on HFD feeding and found no significant
224 differences in VO_2, RER, and locomotor activity between WT and Casp-1 KO mice (**Fig. 2G-I**).
225 These findings suggest that caspase-1 deficiency had no effects on lipid, glucose metabolism,
226 energy expenditure, or locomotor activity.
227
228 *Caspase-1 deficiency enhanced WAT accumulation and obesity in pair-fed mice*
229 We observed that the food intake tended to be slightly higher in Casp1-KO mice than
230 in WT mice fed a chow and HFD feeding (**Fig. 3A**). To ascertain whether the HFD-induced
231 obesity in Casp1-KO mice is attributed to increased food intake, we performed pair-feeding
232 experiments. Similar to the free-feeding conditions, the body weight gain was significantly
233 increased in the Casp1-KO mice under pair-feeding conditions from that in the WT mice (**Fig.**
234 **3B**). An abdominal CT analysis revealed that the subcutaneous fat contents were significantly
235 increased in the Casp1-KO mice (**Fig. 3C and D**). Epididymal fat weight was also increased in

236 the Casp1-KO mice from that in the WT mice, but this difference did not reach statistical

237 significance ($p = 0.1161$, **Fig. 3E**). However, there was no difference in the liver weight

238 between these mice. These findings indicate that caspase-1 deficiency enhanced WAT

239 accumulation and obesity independent of food intake.

240

241 *Caspase-1 deficiency influenced serum levels of IL-18, leptin, and adiponectin*

242 Because caspase-1 also cleaves pro-IL-18 into its mature form (4), and IL-18-KO

243 mice spontaneously develop obesity (19), we assessed serum IL-18 levels, and found that IL-18

244 was detectable in HFD-fed WT mice, whereas IL-18 was not detected in HFD-fed Casp1-KO

245 mice (**Fig. 3F**). We also assessed serum levels of leptin and adiponectin, and found that leptin

246 and adiponectin were significantly increased and decreased, respectively, in HFD-fed

247 Casp1-KO mice, compared to those in HFD-fed WT mice (**Fig. 3G and H**). These findings

248 suggest that IL-18, leptin, and adiponectin are involved in the development of HFD-induced

249 obesity in Casp1-KO mice.

250

251 *Caspase-1 deficiency promoted macrophage infiltration in WAT*

252 Since infiltration of macrophages in WAT is the hallmark of obesity, we assessed

253 this infiltration in WT and Casp1-KO mice fed with HFD using flow cytometry analysis. The

254 number of infiltrated macrophages ($CD11b^{+}F4/80^{+}$) was significantly increased in the

255 epididymal and subcutaneous WAT of the Casp1-KO mice from that in the respective tissue of

256 the WT mice (**Fig. 4A–B**). The number of inflammatory macrophages ($CCR2^{+}F4/80^{+}$) was also

257 increased in the WAT of Casp1-KO mice (epididymal WAT, p = 0.0532; subcutaneous WAT, p

258 < 0.05). Furthermore, we performed real-time RT-PCR analysis to assess gene expression of

259 M1 and M2 macrophage markers (M1: *Cd86* and *Nos2*; M2: *Cd206*, *Cd163*, and *Chil3*) in the

260 subcutaneous WAT of HFD-fed WT and Casp1-KO mice. *Cd206* expression was significantly

261 decreased in Casp1-KO mice. Expression of other M2 markers, *Cd163*, and *Chil3*, also tended

262 to be decreased in Casp1-KO mice (**Fig. 4C**). On the other hand, no difference of M1 marker

263 expression was observed (**Fig. 4D**). These findings suggest that caspase-1 deficiency promoted
264 the inflammatory macrophage infiltration and influenced M1 and M2 macrophage polarization
265 in the adipose tissue.
266
267 *Caspase-1 deficiency enhanced CCL2 production in WAT*
268 There are two possibilities to explain the findings that macrophage infiltration was
269 promoted in the WAT of Casp1-KO mice: 1) the increased migration activity of Casp1-KO
270 macrophages; and 2) the increased production of macrophage-tropic chemokines such as CCL2.
271 To test the first possibility, we assessed macrophage migration using a transwell migration
272 assay and found that there were no significant differences in the migration activity in response
273 to CCL2 between macrophages from the WT and Casp1-KO mice (**Fig. 4E**). To test the second
274 possibility, we assessed the inflammatory cytokine levels in the subcutaneous WAT because
275 increased adiposity and macrophage infiltration were found to be more prominent in the
276 subcutaneous WAT of the Casp1-KO mice (**Fig. 3C and 4**). In the subcutaneous WAT, the
277 CCL2 levels were significantly increased the Casp1-KO mice from those in the WT mice (**Fig.
278 5**). The levels of TNF-α, IL-6, IL-12p70, IFN-γ, and IL-1β were elevated in the Casp1-KO mice,
279 but it did not reach statistical significance. This result is in accordance with the findings that the
280 infiltration of inflammatory macrophages was increased in the WAT of Casp1-KO mice.
281 To investigate the tissue and cellular sources of CCL2, the epididymal and
282 subcutaneous WATs were isolated and cultured in the presence or absence of the prototypical
283 inflammatory stimulus, lipopolysaccharide (LPS). LPS clearly induced CCL2 production in
284 both types of WAT from the WT and Casp1-KO mice, but CCL2 production in response to LPS
285 was significantly higher in the WATs of the Casp1-KO mice than in those of the WT mice (**Fig.
286 6A and B**). The WAT is composed of different cell populations including adipocytes and
287 non-adipocytes. The non-adipocyte cells were fractionated from the WAT after collagenase
288 digestion, known as SVF. Therefore, we cultured SVF and adipocytes, and assessed the
289 production of CCL2 in response to LPS. A marked CCL2 production was observed in the

290 subcutaneous WAT-derived adipocytes and SVF (**Fig. 7A and B**). Furthermore, the SVF
291 culture from the epididymal and subcutaneous WATs produced more CCL2 than the adipocyte
292 culture did. Additionally, CCL2 production in response to LPS was more apparent in the cell
293 fractions isolated from Casp1-KO mice. These findings suggest that SVF is the dominant
294 cellular source of CCL2 in the WAT.
295
296 **DISCUSSION**
297 Inflammation plays a pivotal role in the pathophysiology of obesity. Since caspase-1
298 participates in the inflammasome assembly and is an inflammatory caspase, the link between
299 diet-induced obesity and caspase-1 was demonstrated (14, 22, 28). However, the role of
300 caspase-1 in obesity is still unclear. In this study, we found that mice deficient in caspase-1
301 were susceptible to HFD-induced obesity with increased adiposity, normal lipid profiles and
302 glucose metabolism. The caspase-1 deficiency clearly promoted the infiltration of inflammatory
303 macrophages and increased the production of CCL2 in the adipose tissue. The dominant cellular
304 source of CCL2 was stromal vascular fraction rather than adipocytes in the adipose tissue.
305 These findings demonstrate a critical role of caspase-1 in macrophage-driven inflammation in
306 the adipose tissues and also provide insights into the mechanisms underlying inflammation in
307 the pathophysiology of obesity.
308 Increasing evidence indicates that NLRP3 inflammasomes are implicated in sterile
309 inflammatory diseases. Indeed, the link between NLRP3 inflammasomes and obesity has been
310 reported, yet the role of caspase-1 in obesity remains controversial. Stienstra et al. (22)
311 demonstrated that Casp1-KO mice were resistant to HFD-induced obesity and insulin resistance
312 through enhanced energy expenditure. Conversely, two studies by Kotas et al. (14) and Wang et
313 al. (28) reported that Casp1-KO mice were susceptible to HFD-induced obesity. Although the
314 latter studies are consistent with our results, they did not evaluate any macrophage-driven
315 inflammation. Thus, our results provide additional information regarding increased obesity in
316 Casp1-KO mice.

317 Another important point to be noted is that different HFD was used in each study. In
318 the studies by Stienstra et al. (22) and Wang et al. (28), HFD containing 45 kcal% fat was used,
319 whereas HFD containing 60 kcal% fat was used in the study by Kotas et al. (14). Therefore, we
320 postulate that the diet used in each study may have influenced the effect of caspase-1 deficiency
321 on the development of obesity. Supporting this, we used the same HFD (60 kcal% fat) as in the
322 Kotas's study and found consistent results with their findings: increased body weight and fat
323 mass, and no difference of glucose metabolism and energy expenditure. The differences
324 between our study and Kotas's study are serum levels of TG and TCHO on normal chow diet
325 (Kotas et al. did not provide the data on HFD feeding). In this regard, recent studies
326 demonstrated that gut flora can significantly influence lipid metabolism (1). Therefore, we
327 speculate that the discrepancy may be partially explained by the changes of gut flora.
328 In this study, we showed that a caspase-1 deficiency promoted the development of
329 obesity in an inflammasome- and IL-1β-independent manner; this finding is based on the
330 following observations: First, the IL-1β levels in the adipose tissue were not decreased in the
331 Casp1-KO mice. Second, IL-1β-KO mice significantly gained less body weight than WT mice
332 on HFD feeding. In this regard, inflammasome-independent functions of NLRP3 or ASC have
333 recently received increasing attention. For instance, Ippagunta et al. (8) reported that an
334 inflammasome-independent role of ASC, which regulates the migration of lymphocytes, and
335 contributes to the generation of adaptive immune responses. Shigeoka et al. (21) demonstrated
336 that renal I/R injury was attenuated in Nlrp3-KO mice, but not in Asc-KO and Casp1-KO mice,
337 and concluded that NLPR3 contributes to renal I/R injury through an
338 inflammasome-independent pathway. Similarly, we have recently demonstrated that NLRP3
339 regulates neutrophil migration and contributes to the pathophysiology of hepatic I/R injury and
340 hyperoxia-induced lung injury independent of the inflammasomes or IL-1β (7, 18). More
341 recently, Bruchard et al. (2) reported that NLRP3 in $CD4^+$ T cells was required for the
342 differentiation of T helper type 2 cells in an inflammasome-independent manner. These findings
343 imply that caspase-1 can function independently of the inflammasomes. Indeed previous studies

344 using a proteomic-based approach indicate that in addition to IL-1β, numerous proteins act as
345 candidates for caspase-1 substrates (4, 17). Of these, IL-18 has shown to be implicated in the
346 progression of obesity (19). In addition, IL-18 is processed in an inflammasome-dependent and
347 independent manner (20). Therefore, we assessed serum IL-18 levels and showed that no IL-18
348 was detected in HFD-fed Casp1-KO mice, suggesting the contribution of IL-18 to this process.
349 Further studies are required to elucidate the precise role of caspase-1 in adipose tissue
350 inflammation and the development of obesity.

351 We found that in Casp1-KO mice, subcutaneous WAT was prone to inflammatory
352 responses (*e.g*., inflammatory macrophage infiltration and CCL2 production), rather than the
353 epididymal WAT. Additionally, we showed that the primary cellular source of CCL2 was SVF,
354 rather than the adipocytes in the adipose tissue. The dominant cells in the SVF are leukocytes
355 (*e.g*., macrophages and lymphocytes) and stromal cells (*e.g*., pre-adipocytes and fibroblasts),
356 and macrophages produce a large amount of CCL2 (24). Thus, we assumed that CCL2 was
357 produced by the infiltrating macrophages in the adipose tissue. Taken together, under HFD
358 conditions, inflammatory macrophage infiltration is amplified via the CCL2/CCR2 axis in the
359 adipose tissue of Casp1-KO mice. This in turn enhances adipose tissue inflammation and
360 promotes the development of obesity.

361 There are several limitations of this study that should be noted: 1) we found that a
362 caspase-1 deficiency increased the production of CCL2. However, the molecular mechanism
363 underlying caspase-1 regulation of CCL2 production in the adipose tissue remains to be
364 addressed; 2) since we observed that the IL-1β levels tended to be elevated in Casp1-KO mice,
365 we postulated that there was little contribution of the inflammasomes and IL-1β to the
366 development of obesity in these mice. Furthermore, the Casp1-KO mice used in this study and
367 in other studies (14, 22, 28) lack both caspase-1 and caspase-11 (10, 15). Therefore, the precise
368 contributions of the inflammasomes and caspase-1 remain to be determined in mice deficient in
369 the inflammasome-related molecules, such as NLRP3, ASC, IL-18, or caspase-1 alone. Thus,
370 further investigations are necessary to elucidate the precise role of caspase-1 in the development

371 of obesity.

372 In conclusion, we clearly demonstrate that caspase-1 deficiency promotes the
373 development of obesity with increased adiposity, as well as normal lipid profiles and glucose
374 metabolism. Caspase-1 deficiency also promotes the infiltration of inflammatory macrophages
375 via the CCL2/CCR2 axis in the WAT, which accelerates the development of obesity. These
376 findings demonstrate a critical role for caspase-1 in macrophage-driven inflammation in the
377 adipose tissue and provide novel insights into the mechanism underlying inflammation in the
378 pathophysiology of obesity.

379

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385

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392

393 **DISCLOSURES**

394 The authors have no conflict of interest to declare relating this work.

395

396 **AUTHOR CONTRIBUTIONS**

397 H.K. and M.T. designed the study and wrote the manuscript; H.K., T.K., F.U., A.K., Y.E. M.K.,

398 A.S., J.N. and Y.I. performed experiments; T.K., T.Y. and H.T. discussed the data and provided
399 scientific advice.
400

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508

509 **FIGURE LEGENDS**

510 *Figure 1. Caspse-1 deficiency promotes HFD-induced WAT accumulation and obesity*

511 (**A**) Body weight curves of WT and Casp1-KO mice on a normal chow (n = 7 for each) or HFD
512 (n = 8–9 for each). (**B**) Representative abdominal images of WT and Casp1-KO mice fed with
513 HFD for 4 weeks by CT analysis (blue: muscle; pink: visceral fat; yellow: subcutaneous fat).
514 (**C**) Quantitative analysis of muscle, visceral fat, and subcutaneous fat contents (n = 8–9 for
515 each). (**D**) Body weight, epididymal WAT, (**E**) subcutaneous WAT, and (**F**) relative WAT
516 weights (WAT weight/body weight) of WT and Casp1-KO mice fed with HFD for 8 weeks (n =
517 10–12). (**G**) Representative images of HE staining in epididymal and subcutaneous WATs in
518 WT and Casp1-KO mice fed with HFD for 8 weeks. (**H–J**) Body weight (**H**), epididymal and
519 subcutaneous WAT weight (**I**), and relative weight of epididymal and subcutaneous WAT
520 (WAT weight/body weight) of WT and IL-1β-KO mice fed with HFD for 8 weeks (n = 3–5).
521 Data are expressed as mean ± SEM. *$p < 0.05$; **$p < 0.01$.

522

523 *Figure 2. Effect of Caspase-1 deficiency on lipid and glucose metabolism, energy expenditure,*
524 *and locomotor activity*

525 (**A–C**) Levels of serum TCHO (**A**), TG (**B**), blood glucose (**C**), and plasma insulin (**D**) in WT
526 and Casp1-KO mice before and 8 weeks after HFD feeding (n = 7–10 for each). (**E, F**) Results
527 of GTT in WT and Casp1-KO mice on a normal chow (**E**) and HFD (**F**) (n = 6 for each). (**G-I**)
528 Data on energy expenditure. VO_2 (**G**) and RER (**H**) in WT and Casp1-KO mice fed with HFD
529 (n = 5 for each). (**I**) Locomotor activity in WT and Casp1-KO mice fed with HFD (n = 5 for
530 each). Data are expressed as mean ± SEM. *$p < 0.05$; **$p < 0.01$.

531

532 *Figure 3. Caspase-1 deficiency enhances WAT accumulation and obesity in pair-fed mice*

533 (**A**) Food intake in WT and Casp1-KO mice on a normal chow and HFD (chow, n = 3; HFD, n
534 = 8–9). Average values of each type of food intake were shown above the bars. (**B**) Body
535 weight curves of pair-fed WT and Casp1-KO mice on HFD (n = 6–7 for each). (**C**)

536 Representative abdominal images of pair-fed WT and Casp1-KO mice fed HFD for 8 weeks by

537 CT analysis (blue: muscle; pink: visceral fat; yellow: subcutaneous fat). (**D**) Quantitative

538 analysis of muscle, visceral fat, and subcutaneous fat contents of pair-fed WT and Casp1-KO

539 mice on HFD for 8 weeks (n = 3–4 for each). (**E**) Relative tissue weight (liver or epididymal

540 WAT weight/body weight) of pair-fed WT and Casp1-KO mice on HFD for 8 weeks (n =6–7).

541 (**F–H**) Levels of serum IL-18 (**F**), leptin (**G**), and adiponectin (**H**) in WT and Casp1-KO mice

542 on HFD for 8 weeks (n = 5–6 for each). Data are expressed as mean ± SEM. $^{*}p < 0.05$; $^{**}p <$

543 0.01.

544

545 *Figure 4. Caspase-1 deficiency promoted macrophage infiltration in WAT*

546 (**A** and **B**) Representative images of infiltrated macrophages ($CD11b^{+}F4/80^{+}$ and $F4/80^{+}CCR2^{+}$)

547 in epididymal (**A**) and subcutaneous (**B**) WATs of WT and Casp1-KO mice fed with HFD for 8

548 weeks, by flow cytometry analysis. Quantitative analysis of infiltrated macrophages

549 ($CD11b^{+}F4/80^{+}$ and $F4/80^{+}CCR2^{+}$) in epididymal WAT (**A**) (n = 5–6) and subcutaneous WAT

550 (**B**) (n = 6–7). Data are expressed as mean ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$. (**C** and **D**) Expression

551 of macrophage M2 markers (**C**: *Cd206*, *Cd163*, and *Chil3*) and M1 markers (**D**: *Cd86*, and

552 *Nos2*) in subcutaneous WAT of HFD-fed WT and Casp1-KO mice (n = 4 for each). (**E**)

553 Migration activity in response to CCL2 (100 ng/mL) of primary WT and Casp1-KO

554 macrophages (n = 4 for each). Data are expressed as mean ± SEM. $^{*}p < 0.05$.

555

556 *Figure 5. Caspase-1 deficiency enhances inflammatory cytokine production*

557 Tissue levels of CCL2, IL-1β, TNF-α, IL-6, IL-12p70, and IFN-γ, in the subcutaneous WAT of

558 WT and Casp1-KO mice fed HFD for 8 weeks (n = 6 for each). Data are expressed as mean ±

559 SEM. $^{*}p < 0.05$.

560

561 *Figure 6. Caspase-1 deficiency increases CCL2 production in WAT*

562 Tissue levels of CCL2 in the culture of epididymal (**A**) and subcutaneous (**B**) WAT of WT and

563 Casp1-KO mice in the presence or absence of LPS (100 ng/mL) for 24 h (n = 4 for each). Data

564 are expressed as mean ± SEM. *$p < 0.05$.

565

566 *Figure 7. Caspase-1 deficiency increases CCL2 production in SVF and adipocytes*

567 CCL2 levels in the culture supernatants of SVF (**A**: 5 x 10^5 cells/mL) and adipocytes (**B**: 2 x 10^5

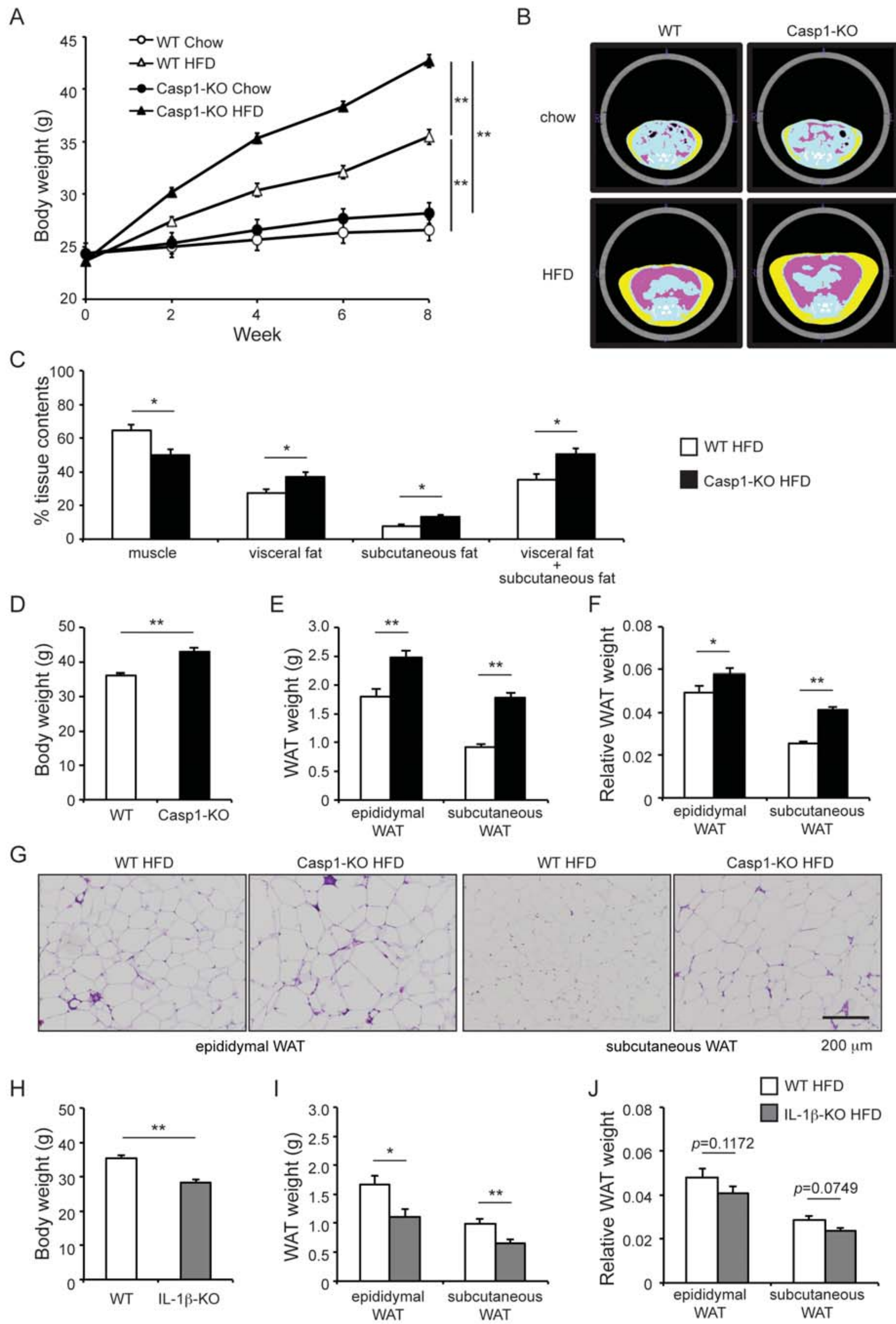
568 cells/mL) isolated from epididymal and subcutaneous WATs of WT and Casp1-KO mice in the

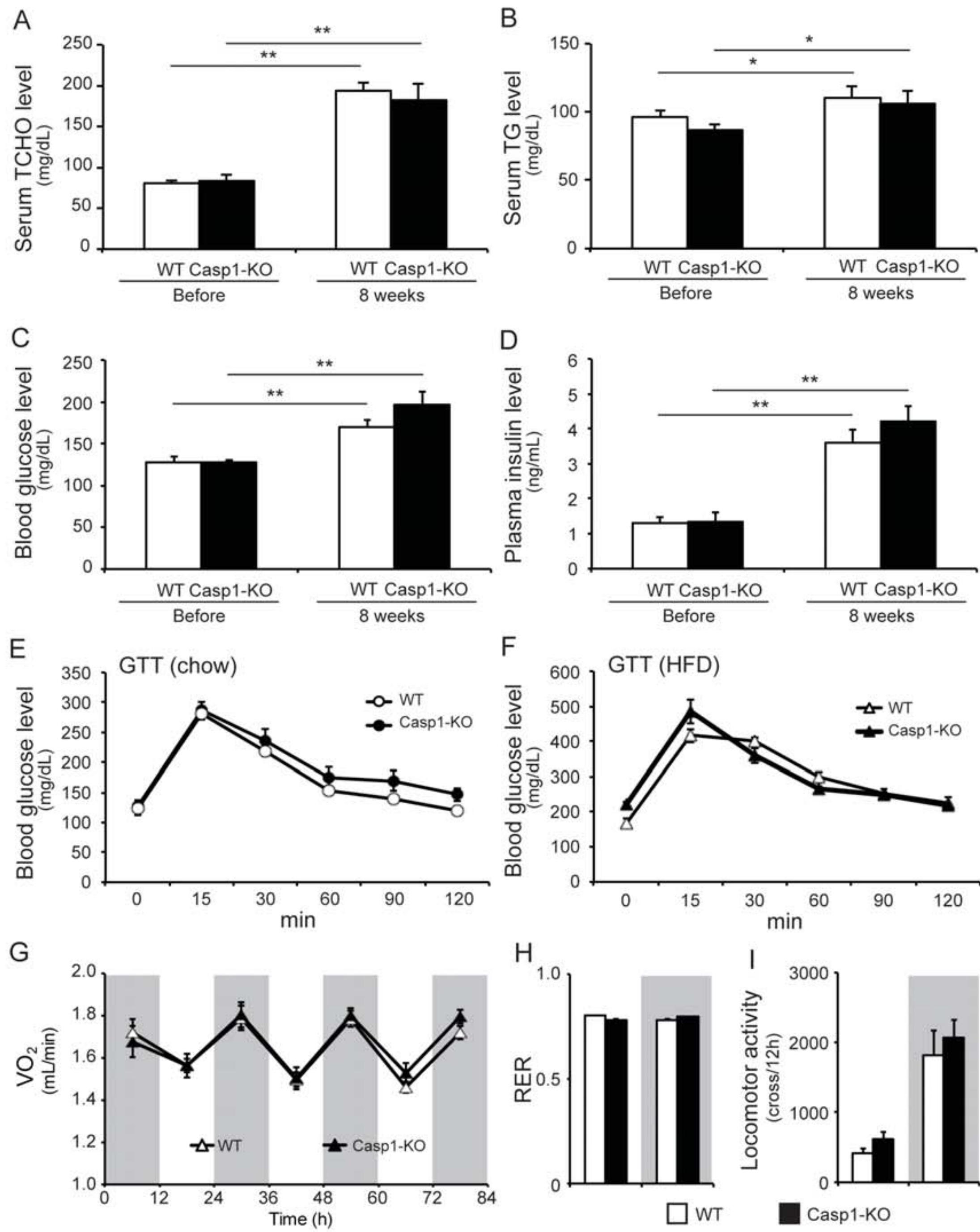
569 presence or absence of LPS (100 ng/mL) for 24 h (n = 4 for each). Data are expressed as mean ±

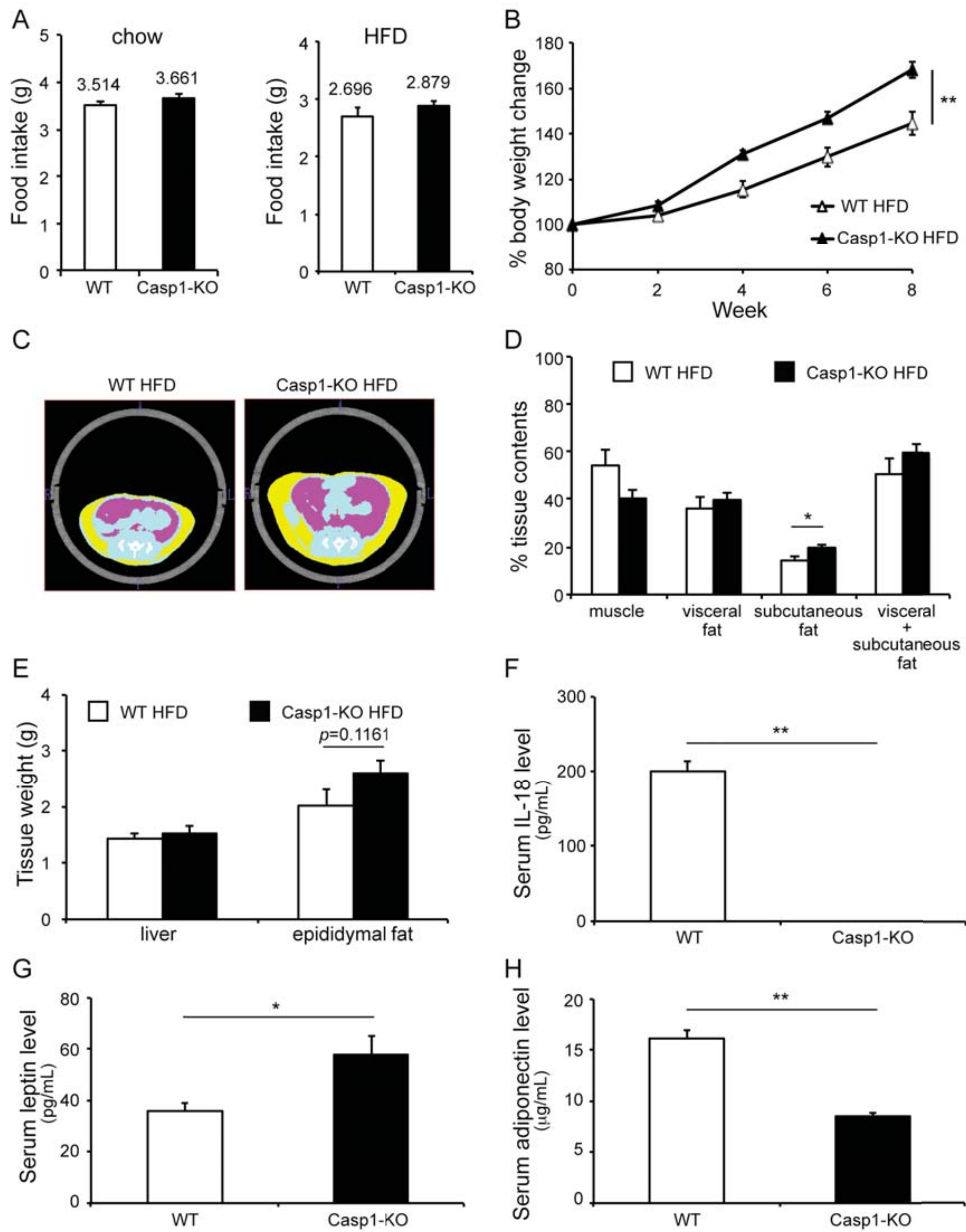
570 SEM. *$p < 0.05$.

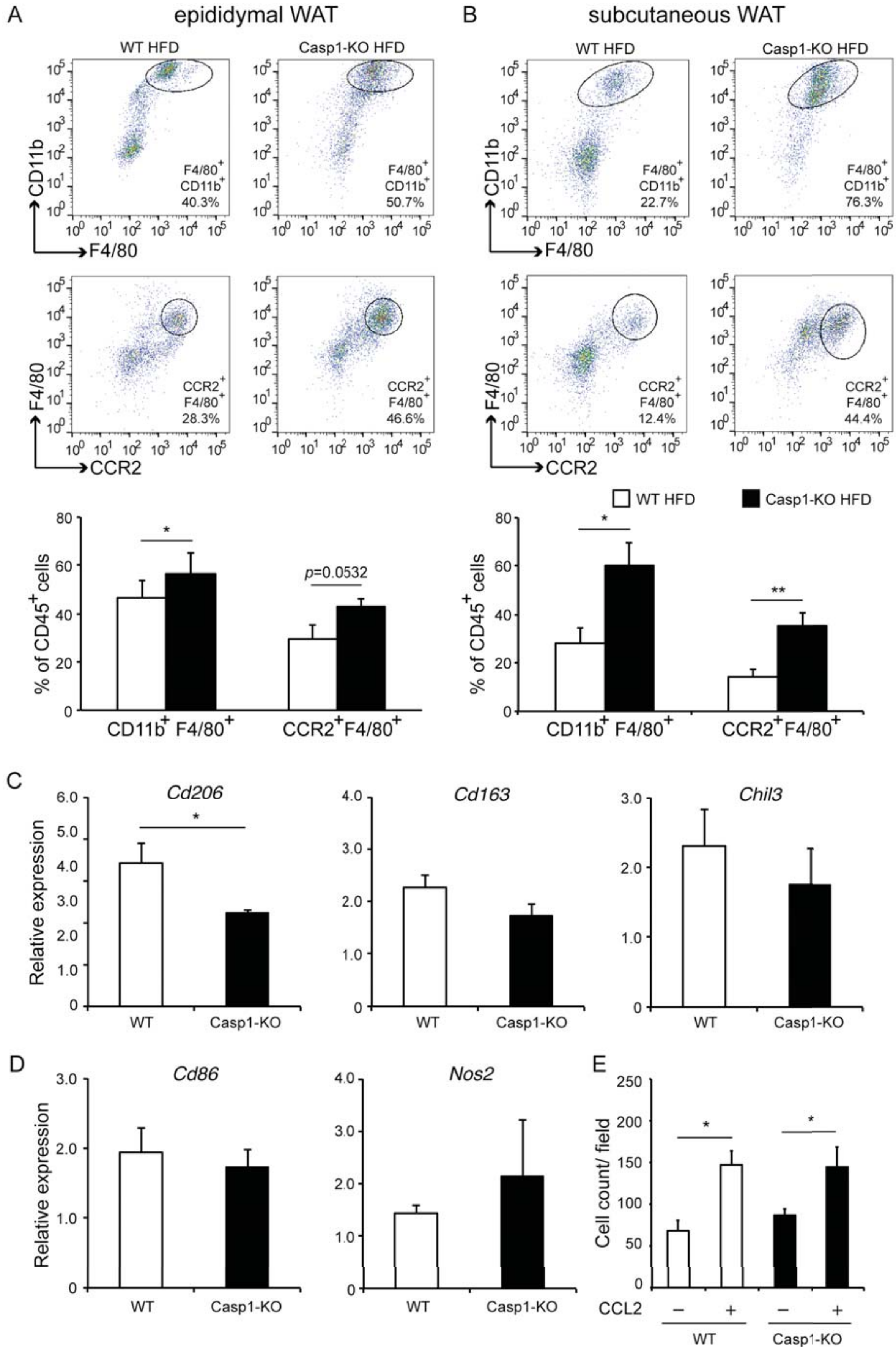
571

572


A
WT Chow
WT HFD
Casp1-KO Chow
Casp1-KO HFD
Body weight (g)
Week
B
WT
Casp1-KO
chow
HFD
C
% tissue contents
muscle
visceral fat
subcutaneous fat
visceral fat + subcutaneous fat
WT HFD
Casp1-KO HFD
D
Body weight (g)
WT
Casp1-KO
E
WAT weight (g)
epididymal WAT
subcutaneous WAT
F
Relative WAT weight
epididymal WAT
subcutaneous WAT
G
WT HFD
Casp1-KO HFD
WT HFD
Casp1-KO HFD
epididymal WAT
subcutaneous WAT
200 μm
H
Body weight (g)
WT
IL-1β-KO
I
WAT weight (g)
epididymal WAT
subcutaneous WAT
J
WT HFD
IL-1β-KO HFD
Relative WAT weight
p=0.1172
p=0.0749
epididymal WAT
subcutaneous WAT


A
Serum TCHO level (mg/dL)
WT Casp1-KO
Before
8 weeks
B
Serum TG level (mg/dL)
C
Blood glucose level (mg/dL)
D
Plasma insulin level (ng/mL)
E
GTT (chow)
WT
Casp1-KO
min
F
GTT (HFD)
G
VO2 (mL/min)
Time (h)
H
RER
I
Locomotor activity (cross/12h)


A
chow
HFD
Food intake (g)
3.514
3.661
2.696
2.879
WT
Casp1-KO
B
% body weight change
Week
WT HFD
Casp1-KO HFD
**
C
WT HFD
Casp1-KO HFD
D
% tissue contents
muscle
visceral fat
subcutaneous fat
visceral + subcutaneous fat
E
Tissue weight (g)
p=0.1161
liver
epididymal fat
F
Serum IL-18 level (pg/mL)
G
Serum leptin level (pg/mL)
H
Serum adiponectin level (μg/mL)


A
epididymal WAT
B
subcutaneous WAT
WT HFD
Casp1-KO HFD
CD11b
F4/80
F4/80+ CD11b+ 40.3%
F4/80+ CD11b+ 50.7%
F4/80+ CD11b+ 22.7%
F4/80+ CD11b+ 76.3%
CCR2
CCR2+ F4/80+ 28.3%
CCR2+ F4/80+ 46.6%
CCR2+ F4/80+ 12.4%
CCR2+ F4/80+ 44.4%
% of CD45+ cells
p=0.0532
CD11b+ F4/80+
CCR2+F4/80+
C
Cd206
Cd163
Chil3
Relative expression
WT
Casp1-KO
D
Cd86
Nos2
E
Cell count/ field
CCL2

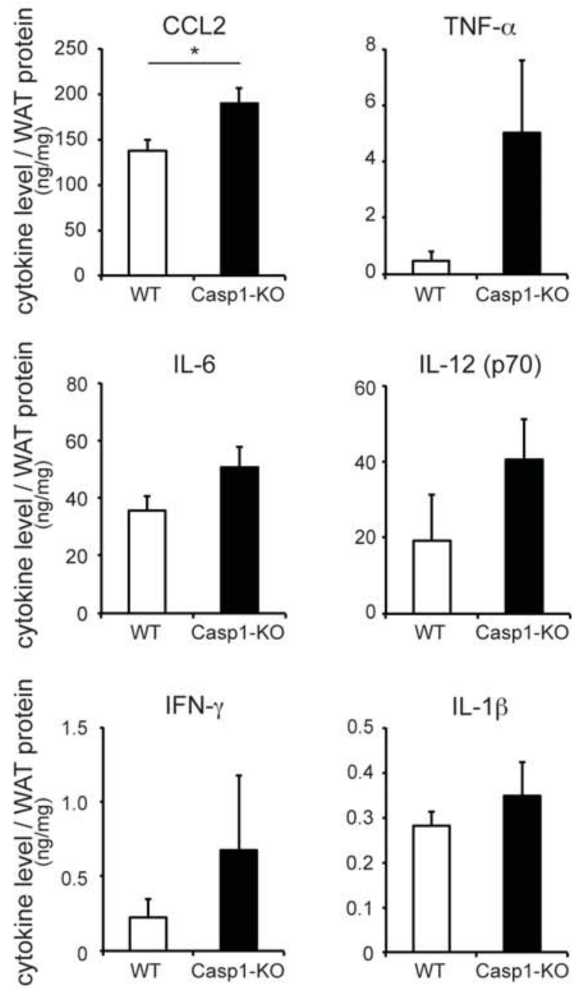
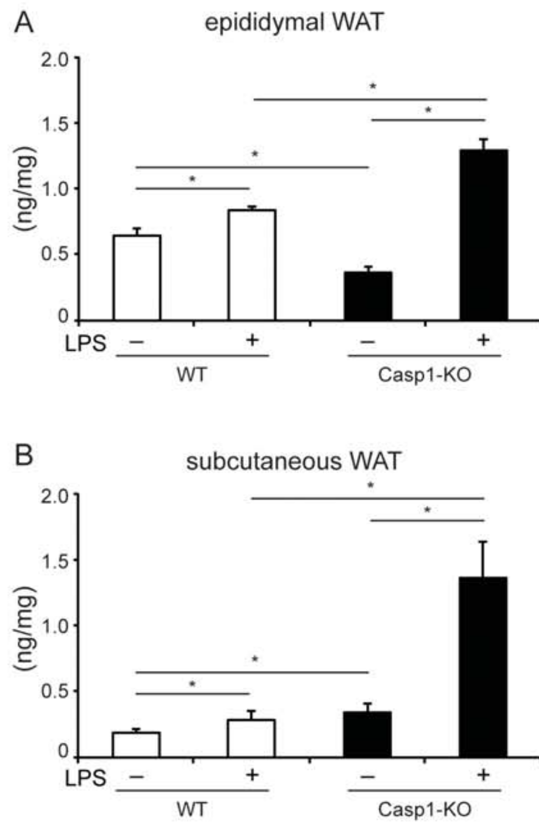
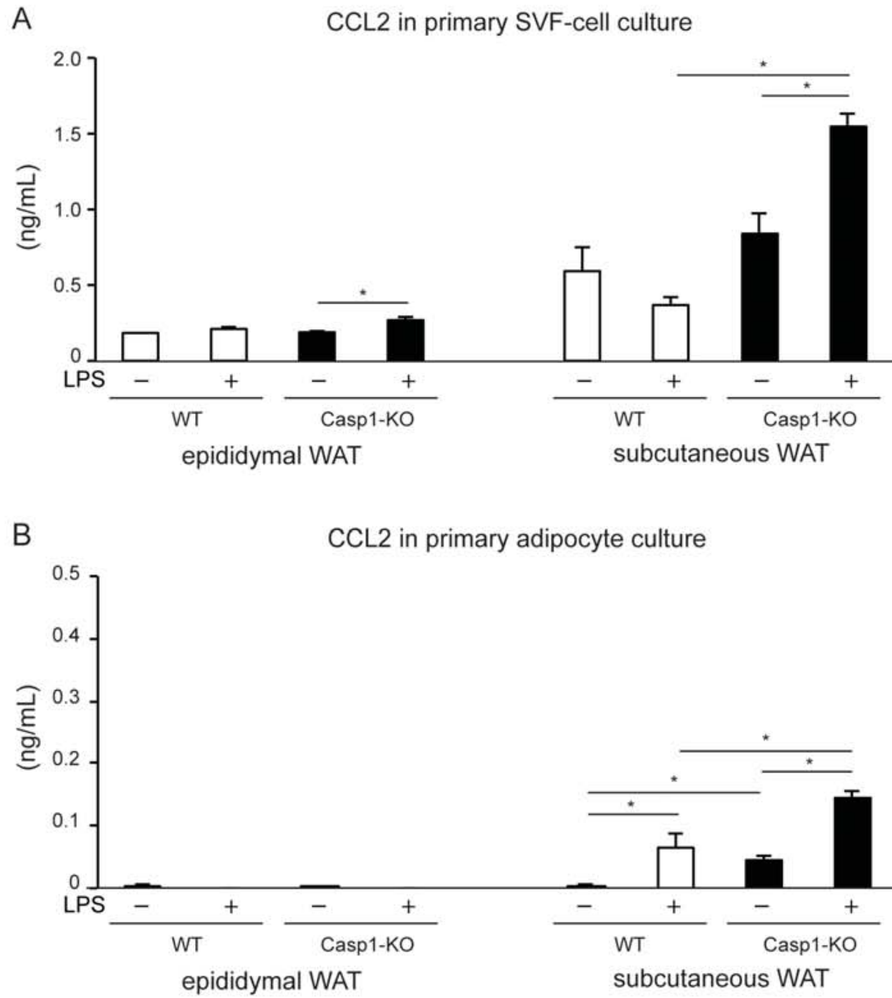

CCL2
TNF-α
IL-6
IL-12 (p70)
IFN-γ
IL-1β
cytokine level / WAT protein (ng/mg)
WT
Casp1-KO
*

Figure 6. Kimura H, *et al.*




A
CCL2 in primary SVF-cell culture
(ng/mL)
2.0
1.5
1.0
0.5
0
*
*
*
LPS
−
+
−
+
−
+
−
+
WT
Casp1-KO
WT
Casp1-KO
epididymal WAT
subcutaneous WAT
B
CCL2 in primary adipocyte culture
(ng/mL)
0.5
0.4
0.3
0.2
0.1
0
*
*
*
*
LPS
−
+
−
+
−
+
−
+
WT
Casp1-KO
WT
Casp1-KO
epididymal WAT
subcutaneous WAT