

1 **A long-chain fatty acid elongase Elovl6 regulates mechanical damage-induced**
2 **keratinocyte death and skin inflammation**

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20 This research was supported in part by grants provided by the Ministry of Education,
21 Culture, Sports, Science, and Technology of Japan (to A.S. [16H06387, 15H01365] and
22 C. N.-O. [16H05350]). Y.N. is a fellow of the Kibo Project of the Tadamitsu Kishimoto
23 Foundation of the Japanese Society of Immunology.

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28 **Abstract**

29 Mechanical damage on the skin not only affect the barrier function but also induce
30 various immune responses, which trigger or exacerbate the inflammation in healthy
31 individuals and patients with inflammatory skin diseases. However, how mechanical
32 damage-induced skin inflammation is regulated remains largely unknown. Here, we
33 show that mechanical damage due to tape stripping triggered keratinocyte death and
34 release of danger-associated molecular patterns (DAMPs) such as high-mobility group
35 box 1 protein (HMGB-1) and IL-1 α , which induced production of proinflammatory
36 cytokines and chemokines IL-1 β and CXCL-1 by keratinocytes in mice. We also show
37 that a long-chain fatty acid elongase Elovl6 is expressed in keratinocytes. Mice deficient
38 in Elovl6 had increased epidermal levels of cis-vaccenic acid (CVA); this accelerated
39 keratinocyte death triggered by tape stripping and release of DAMPs and exacerbated
40 skin inflammation. Our results demonstrate that Elovl6 regulates mechanical damage-
41 triggered keratinocyte death and skin inflammation.

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43

44 **Introduction**

45 The mechanical damage induced by physical forces—including stretching, compression,
46 and friction—on epithelial and endothelial cells plays a critical role in tissue
47 homeostasis (Abe and Berk 2014; Angelini *et al.* 2012; Hofmann *et al.* 2004; Reichelt
48 2007; Wyatt *et al.* 2016). Under physiologic conditions, keratinocytes are the epidermal
49 cell population most affected by mechanical damage (Reichelt 2007), which induces
50 them to proliferate and produce cytokines. Stretching of keratinocytes in vitro opens
51 Ca²⁺ channels, resulting in the phosphorylation of Akt (Yano *et al.* 2006). This process
52 also induces clustering and co-localization of β -integrins and epidermal growth factor
53 receptor, followed by activation of the downstream signaling molecule extracellular
54 signal-regulated kinase (ERK) 1/2 (Knies *et al.* 2006).

55 Mechanical damage not only affect the barrier function of the skin but also
56 induce various immune responses (Verhoeven *et al.* 2008), which trigger inflammation
57 at the site of the stress on the skin of healthy individuals. Moreover, mechanical damage
58 on the skin exacerbates the inflammation in patients with inflammatory skin diseases.
59 For example, scratching of itching lesions exacerbates the skin inflammation in atopic
60 dermatitis (AD), which is called the itch-scratch cycle (Verhoeven *et al.* 2008; Verhoeven
61 *et al.* 2009; Wahlgren 1999). In addition, scratching induces development of new skin

62 lesions in psoriasis, well known as the Koebner phenomenon (Köbner 1876). However,
63 how mechanical damage-induced skin inflammation is regulated remains largely
64 unknown.

65 Elongation of long-chain fatty acids family member 6 (Elovl6) is a
66 rate-limiting microsomal enzyme that catalyzes the elongation of saturated and
67 monounsaturated fatty acids (Saito *et al.* 2011). Elovl6 elongates palmitate (PA) (C16:0)
68 to stearate (SA) (C18:0) and palmitoleate (POA) (C16:1n-7) to cis-vaccenic acid (CVA)
69 (C18:1n-7) (Saito *et al.* 2011). Elovl6 is highly expressed in white adipose tissue and
70 liver (Matsuzaka *et al.* 2002). In previous studies, mice deficient in Elovl6 (*Elovl6*^{-/-}
71 mice) had increased levels of PA in the liver and lung (Matsuzaka *et al.* 2007; Sunaga *et*
72 *al.* 2013). Elovl6 is involved in metabolic diseases, such as insulin resistance
73 (Matsuzaka *et al.* 2007) and atherogenesis (Saito *et al.* 2011), as well as inflammatory
74 diseases, including attenuated high-fat-diet-induced hepatic inflammation (Matsuzaka
75 *et al.* 2012) and regulated bleomycin-induced pulmonary fibrosis (Sunaga *et al.* 2013) .
76 In addition, Elovl6 is highly expressed in skin (Matsuzaka *et al.* 2002), which is one of
77 the most lipid-enriched organs. Lipids in the skin play crucial roles in homeostasis; they
78 are involved in epidermal permeability and barrier function (Ishikawa *et al.* 2010), the

79 composition of microbiota (Nguyen *et al.* 2016), epithelialization (Liu *et al.* 2014), and
80 inflammation (Zhang *et al.* 2015).

81 In the current study, we examined how mechanical damage induces skin
82 inflammation and whether long-chain fatty-acid composition regulated by Elovl6 is
83 involved in mechanical damage onto the skin.

84

85 **Results**

86 ***Elovl6*^{-/-} mice show exacerbated mechanical damage–induced skin inflammation.**

87 Tape stripping, which mimics scratching, is a well-established method for inducing
88 mechanical stress or damage on the skin (Onoue *et al.* 2009; Takahashi *et al.* 2013). To
89 investigate the role of *Elovl6* in mechanical damage–induced skin inflammation, we
90 established a mouse model of dermatitis by using repeated tape stripping twenty times,
91 which induced the skin damage and barrier disruption. After this treatment, erythema
92 was more severe in *Elovl6*^{-/-} mice than in wild-type mice (**Figure 1A**). Moreover, the
93 epidermis was thicker, and neutrophil infiltration was significantly greater, in *Elovl6*^{-/-}
94 mice than in wild-type mice (**Figure 1B–D**). Since *Elovl6* expression was higher in the
95 epidermis than in the dermis (**Figure supplement 1A**), we speculated that *Elovl6* is
96 expressed in keratinocytes. Indeed, the epidermis in mice deficient in *Elovl6*
97 specifically in the keratinocytes (*Elovl6*^{fl/fl} *K14*-Cre mice) showed significantly
98 decreased *Elovl6* expression (**Figure supplement 1B**). As in *Elovl6*^{-/-} mice, *Elovl6*^{fl/fl}
99 *K14*-Cre mice also showed increased epidermal thickness and neutrophil infiltration
100 after tape stripping compared with control mice (**Figure 1E, F**).

101 To address how *Elovl6* suppressed mechanical damage-induced skin
102 inflammation, we compared skin barrier function of *Elovl6*^{-/-} mice with that of wild-type

103 mice. However, it was comparable between two genotypes of mice, as determined by
104 toluidine blue skin permeability assay and a transepidermal water loss test (Sassa *et al.*
105 2013) (**Figure supplement 2A, B**). These results suggested that Elov16 in the
106 keratinocytes suppressed mechanical damage-induced skin inflammation, in which a
107 novel mechanism might be involved.

108

109 ***Elov16*^{-/-} mice show increased IL-1 β and CXCL-1 production after mechanical**
110 **damage**

111 To investigate how Elov16 suppressed mechanical damage-induced skin inflammation,
112 we examined the expression levels of pro-inflammatory and anti-inflammatory
113 cytokines and chemokines potentially involved in dermatitis (Effendy *et al.* 2000).
114 Among them, transcript levels of *Il1b* and *Cxcl1* in epidermis were increased in both
115 wild-type and *Elov16*^{-/-} mice after tape stripping (**Figure 2A and Figure supplement 3**).
116 Moreover, *Elov16*^{-/-} mice showed higher expression of *Il1b* and *Cxcl1* than did wild-type
117 mice after tape stripping (**Figure 2A**). In accordance with these results, the
118 concentrations of IL-1 β and CXCL-1 were significantly higher in the culture
119 supernatants of *Elov16*^{-/-} epidermis harvested from mice after tape stripping than in those
120 from wild-type epidermis (**Figure 2B**). These cytokine levels in the epidermis from

121 mice deficient in an adaptor of Toll-like receptors (TLRs) MyD88, but not TRIF, were
122 lower than those in wild-type mice after tape stripping (**Figure 2C**). These results
123 suggest that Elov16 suppressed mechanical damage-induced IL-1 β and CXCL-1
124 productions that are dependent on MyD88.

125

126 ***Elov16*^{-/-} mice show increased keratinocyte death after mechanical damage**

127 Since histologic analysis of the skin after tape stripping revealed greater numbers of
128 degenerated keratinocytes in *Elov16*^{-/-} mice than in wild-type mice (**Figure 3A**), we
129 speculated that the number of dead cells were greater in the skin of *Elov16*^{-/-} mice than
130 in that of wild-type mice after tape stripping. Indeed, flow cytometry analysis
131 demonstrated that, although the proportion of dead keratinocytes in the epidermis of
132 *Elov16*^{-/-} mice was comparable with that in wild-type mice in the steady state, tape
133 stripping enhanced keratinocyte death in *Elov16*^{-/-} mice significantly more than in
134 wild-type mice (**Fig. 3B, C**). These results suggest that Elov16 suppressed mechanical
135 damage-induced keratinocyte death.

136

137 **Cis-vaccenic acid (CVA) is increased in *Elov16*^{-/-} mice.**

138 To investigate how keratinocytes death and the skin inflammation after tape stripping
139 were exacerbated in *Elovl6*^{-/-} mice, we analyzed the fatty acid composition of the
140 epidermis of wild-type and *Elovl6*^{-/-} mice. Unlike in our previous reports of increased
141 PA levels in the lung and liver of *Elovl6*^{-/-} mice (Matsuzaka *et al.* 2007; Sunaga *et al.*
142 2013), PA was not increased in the epidermis (**Figure 4A**). Instead, *Elovl6*^{-/-} mice had
143 significantly increased CVA levels (**Figure 4A**) and greater epidermal expression of the
144 long-chain fatty acid elongases *Elovl1*, *Elovl3*, and *Elovl5* and of the stearyl-CoA
145 desaturase *Scd3* than did wild-type mice (**Figure 4B**). Among these, *Elovl5* and *Scd3*
146 may influence CVA generation through the elongation of POA (C16:1n-7) (Burns *et al.*
147 2012) and by the conversion of PA to POA (Guillou *et al.* 2010), respectively (**Figure**
148 **supplement 4**). These results suggest that CVA might be involved in the keratinocytes
149 death and skin inflammation after tape stripping in *Elovl6*^{-/-} mice.

150

151 **CVA induces keratinocyte death.**

152 To address whether CVA is involved in keratinocyte death, we cultured a human
153 keratinocyte cell line HaCaT and primary keratinocytes derived from mice in the
154 presence of CVA. We found that CVA decreased the numbers of live cells of HaCaT
155 cells and primary keratinocytes in a dose-dependent manner (**Figure 5A, B**) and

156 increased the proportion of dead primary keratinocytes (**Figure 5C**). In contrast, neither
157 oleic acid (OA), PA, POA, SA, nor trans-vaccenic acid (TVA) influenced the number of
158 live primary keratinocytes after culture (**Figure 5A, B, D**). In addition, CVA decreased
159 the number of live peritoneal macrophages as well (**Figure supplement 5A**). CVA did
160 not affect the proliferation of HaCaT cells but instead increased the number of dead
161 cells compared with those after the addition of OA (**Figure 5E**), thus indicating that
162 treatment with CVA induced cell death of HaCaT cells. This cell death was not affected
163 by triacsin C, an inhibitor of long-chain acyl-CoA synthetases (Igal *et al.* 1997; Wang *et*
164 *al.* 2012) (**Figure supplement 5B**), suggesting that CVA itself, but not its metabolites,
165 induced death of HaCaT cells. Morphologic analyses under transmission electronic
166 microscopy demonstrated increased plasma membrane rupture without in the
167 keratinocytes after CVA treatment (**Figure supplement 5C**). In vivo, we found that
168 topical application of CVA, but not OA, at a dose of 45 mM to the dorsal skin of
169 wild-type mice increased the proportion of dead keratinocytes, as analyzed by flow
170 cytometry (**Figure 5F**). Anti-cleaved caspase-9 (CC9) antibody did not stain
171 CVA-treated dead keratinocytes (**Figure supplement 5D**). Together, these results
172 suggest that CVA induced non-apoptotic cell death. Pretreatment with necrostatin-1 or
173 necrosulfonamide, which are inhibitors of receptor-interacting protein 1 (RIP1) kinase

174 and mixed lineage kinase domain-like protein (MLKL), respectively, did not suppress
175 the CVA-induced death of keratinocytes (**Figure supplement 5E**), suggesting that the
176 cell death due to CVA likely was not necroptosis (Skrzeczynska-Moncznik *et al.* 2015;
177 Zhao *et al.* 2017). These combined results suggest that CVA induced necrosis rather
178 than programmed cell death of keratinocytes. In addition, treatment with inhibitors of
179 oxidative stress (IM-54) or cyclophilin D (cyclosporine A) did not influence the cell
180 death (**Figure supplement 5E**), suggesting that the CVA-induced necrosis of
181 keratinocytes was independent of oxidative stress (IM-54) or cyclophilin D-mediated
182 changes in mitochondrial permeability (Chen *et al.* 2013; Nakagawa *et al.* 2005; Zeng *et*
183 *al.* 2016).

184

185 **CVA increased IL-1 β and CXCL-1 production.**

186 Since CVA induced non-apoptotic cell death, we then examined whether CVA increased
187 the release of DAMPs from dead cells. The addition of CVA, but not OA, to cultures of
188 primary keratinocytes from wild-type mice increased the concentrations of HMGB-1
189 and IL-1 α in the supernatants (**Figure 6A**). We further examined whether these DAMPs
190 is involved in the increase in IL-1 β and CXCL-1 expression. Stimulation of primary
191 keratinocytes derived from wild type or *Elovl6*^{-/-} mice in vitro and of the epidermis from

192 the either genotype of mice in vivo with HMGB-1 or IL-1 α induced *Il1 β* and *Cxcl1*, and
193 the expression levels of these cytokines transcripts did not differ between both
194 genotypes of mice (**Figure supplementary 6A, B**). These results suggest that CVA
195 enhanced IL-1 β and CXCL-1 production by keratinocytes via HMGB-1 or
196 IL-1 α . Indeed, we found that topical application of CVA, but not OA, at a dose of 45
197 mM to the dorsal skin of wild-type mice increased the expressions of *Il1 β* and *Cxcl1* in
198 the epidermis (**Figure 6B**). Finally, treatment with either antagonist of IL-1 receptor or
199 CXCR-2 intradermally and intraperitoneally reduced epidermal thickness and the
200 number of neutrophils in the skin of *Elovl6*^{-/-} mice (**Figure 6C, D**). These results
201 suggest that the IL-1 β and CXCL-1 produced by keratinocytes played crucial roles in
202 the exacerbation of mechanical damage-induced skin inflammation in *Elovl6*^{-/-} mice.
203 Taken all together, these results suggest that tape stripping triggered keratinocyte death
204 and release of HMGB-1 and IL-1 α , which then stimulated the surrounding live
205 keratinocytes to produce IL-1 β and CXCL-1. *Elovl6* deficiency increased the proportion
206 of CVA in the skin, which accelerated keratinocyte death triggered by tape stripping and
207 the subsequent signaling cascade to the production of IL-1 β and CXCL-1, thus
208 exacerbating dermatitis (**Figure 6E**).

209

210

211 **Discussion**

212 Previous studies have revealed increased PA and decreased OA contents in the liver and
213 lung of *Elovl6*^{-/-} mice compared with wild-type mice (Matsuzaka *et al.* 2007; Sunaga *et*
214 *al.* 2013). In the current study, we noted that the OA level in the skin of *Elovl6*^{-/-} mice
215 was decreased compared with that in wild-type mice. However, PA content did not
216 differ between the two genotypes. Instead, epidermal levels of CVA were greater in
217 *Elovl6*^{-/-} mice than in wild-type mice, presumably owing to its efficient conversion to
218 CVA by the concomitant induction of SCD3 and Elovl5 productions (Burns *et al.* 2012;
219 Guillou *et al.* 2010). Although it remains unclear at present how Elovl6 regulates the
220 fatty acid composition and the enzyme alteration in the skin, these combined results
221 suggest that regulation of the elongation of saturated and monounsaturated fatty acids
222 is—in part—dependent on the organs or tissues.

223 The biologic function of CVA has been poorly understood. Here, we
224 demonstrated that CVA directly induced cell death in cultures of primary keratinocytes
225 from mice. Specifically, the cells killed by CVA lacked one of the hallmarks of
226 apoptosis, namely caspase-9 activation. In addition, morphologic analyses revealed
227 characteristics of necrosis, including plasma membrane rupture without blebbing
228 (Krysko *et al.* 2008). None of the inhibitors of necroptosis, oxidative stress, or

229 cyclophilin-D-associated cell death—including necrostatin-1, NSA, IM-54, and
230 cyclosporine A—inhibited the CVA-induced cell death. In addition, triacsin-C treatment
231 failed to suppress CVA-induced cell death. These results suggest that CVA induced
232 necrosis rather than programmed cell death of keratinocytes.

233 Long-chain fatty acids elicit a variety of biologic effects, including cell death.
234 For example, PA induces apoptosis in many cell types, and this response is abrogated by
235 OA (Gillet *et al.* 2015; Sunaga *et al.* 2013). In contrast, PA induces
236 RIP1/RIP3-dependent necroptosis in RAW 264.7 cells (Kim *et al.* 2017), and (although
237 the results are controversial) OA has been reported to induce the death of various cell
238 types (Brinkmann *et al.* 2013; Moravcova *et al.* 2015). The molecular mechanisms of
239 these cytotoxic effects remain poorly understood; features proposed to be involved in
240 these toxicities include loss of membrane integrity, changes in mitochondrial
241 transmembrane potential, activation of caspase-3, and the production of reactive oxygen
242 species (Brinkmann *et al.* 2013; Fontana *et al.* 2013). In the current study, we found that,
243 beginning soon after its addition to the cultures, CVA was severely cytotoxic to HaCaT
244 cells, mouse peritoneal macrophages, and primary keratinocytes; CVA can thus be
245 added to the list of possible lipotoxins.

246 Previous studies have demonstrated that, compared with OA and SA,
247 supplementation with both CVA and TVA (dose, 30 μ M) significantly suppress the
248 growth of HT-29 tumor cells after culture for 9 days (Awad *et al.* 1995). Moreover, CVA
249 leads to greater hydrolysis of phosphoinositides in the plasma membrane than does TVA
250 (Awad *et al.* 1995), suggesting that CVA is incorporated into the plasma membrane and
251 affects the phospholipids composition. In the present study, we showed that CVA at
252 concentrations of 200 μ M or greater induced the death, rather than the suppression of
253 growth, of keratinocytes, thus suggesting that the higher amount of CVA induces
254 damage of the plasma membrane sufficiently to induce necrosis. In addition, given that
255 TVA did not induce keratinocyte death, the cytotoxic effect of CVA may be structure
256 dependent. Whereas trans-unsaturated fatty acids have a linear structure and can be
257 packed regularly in the plasma membrane, cis-unsaturated fatty acids such as OA and
258 CVA, which have a characteristic angular kink, may distort the structure of the lipid
259 bilayer and thus destabilize the plasma membrane (Fontana *et al.* 2013). Therefore,
260 although further studies are required to determine the detailed mechanism of
261 CVA-induced cell death, we speculate that incorporation of CVA into the plasma
262 membrane creates a bulky 3-dimensional structure compared with those associated with

263 other cis-monounsaturated fatty acids and thus induces cell death by disrupting the
264 plasma membrane.

265 Fatty acids reportedly play important roles in modulating dermatitis. For
266 example, a high-fat diet enriched with oleic acid impairs contact hypersensitivity
267 responses to trinitrochlorobenzene and FITC (Katagiri *et al.* 2008). In addition, oral
268 administration of docosahexaenoic acid leads to the generation of regulatory T cells,
269 which thus attenuate dinitrochlorobenzene-induced dermatitis (Han *et al.* 2015).

270 Moreover, topical or oral application of linoleic acid and TVA, which are enriched in
271 milk fat, decreases the severity of OVA-induced atopic dermatitis (Sun *et al.* 2011).

272 Atopic dermatitis (AD) is one of the most common skin diseases and is
273 characterized by pruritic and eczematous skin lesions. The outermost layer of the
274 epidermis, the stratum corneum, contains decreased levels of ceramides (very
275 long-chain fatty acids), leading to impaired barrier function in patients with AD, and the
276 average chain length of ceramide fatty acids is negatively correlated with epidermal
277 permeability in these patients (Ishikawa *et al.* 2010). Moreover, *Elovl1*^{-/-} and *Elovl4*^{-/-}
278 mice, both of which demonstrate a global decrease in the chain length of ceramide fatty
279 acids of the stratum corneum, show impaired barrier function (Li *et al.* 2007; Sassa *et al.*
280 2013). Mechanical stress, such as scratching, increases the severity of AD by removing

281 the stratum corneum (thus diminishing the epidermal barrier function) and by inducing
282 the production of pro-inflammatory cytokines (Verhoeven *et al.* 2008; Wahlgren 1999).
283 On the other hand, psoriasis is characterized by well-demarcated scaly erythema and
284 plaque, which sometimes show itching, and histopathologically, these lesions reveal
285 hyperproliferation of keratinocytes and neutrophil infiltration (Hirotzu *et al.* 2012). The
286 skin lesions of psoriasis are well-known to be triggered or exacerbated, as the Koebner
287 phenomenon, by mechanical stress (Köbner 1876). Our current results suggest that
288 Elovl6 may regulates mechanical stress-induced exacerbation of skin inflammation due
289 to inhibition of keratinocyte death by CVA in patients with AD and psoriasis.

290

291

292 **Materials and Methods**

293 **Mice.** *Elovl6*^{-/-} mice on the C57BL/6J background were described previously
294 (Matsuzaka *et al.* 2007). C57BL/6J mice raised under specific pathogen-free conditions
295 were purchased from Clea Japan (Tokyo, Japan). Germ-free mice were bred and
296 maintained in isolators at Sankyo Laboratories (Tsukuba, Japan), and their germ-free
297 status was routinely confirmed by in-house aerobic and anaerobic culture of feces.
298 *K14-Cre*, *Ticam1*^{-/-}, and *Myd88*^{-/-} mice on the B57BL/6 background were purchased
299 from Jackson Laboratories (Bar Harbor, Maine, USA). *Elovl6*^{fl/fl} mice were crossed with
300 *K14-Cre* transgenic mice to generate *Elovl6*-knockout mice specifically in keratinocytes
301 (*Elovl6*^{fl/fl} *K14-Cre*). Mice between 8 and 10 weeks of age were used for the
302 experiments. All experiments were performed in accordance with the guidelines of the
303 animal ethics committee of the University of Tsukuba Animal Research Center.

304

305 **Tape stripping (Nakajima *et al.* 2012).** To generate mechanical damage-induced
306 dermatitis, a 2.5×2.5 cm area of the dorsal skin was shaved and tape-stripped 20 times
307 by using adhesive tape (Johnson and Johnson); a 1×1-cm piece of sterile gauze
308 moistened with 100 μl PBS was placed on the shaved skin and secured with transparent
309 bio-occlusive tape (Tegaderm Roll, 3M, Maplewood, Minnesota, USA) to prevent the

310 mice from licking the area. These procedures were repeated every other day until
311 analysis.
312
313 **Cytokine measurement of epidermis or cultured keratinocytes.** Dorsal skin
314 samples before and after tape stripping were resected from adult mice and incubated in
315 RPMI medium in the presence of dispase II (3 mg/ml) (Wako Pure Chemical, Osaka,
316 Japan) for 1 h at 37 °C under 5% CO₂. The epidermis was then separated from the
317 dermis under a stereomicroscope. Samples of epidermis (diameter, 4 mm) were cultured
318 in 50 µl of DMEM containing 10% FBS in a 96-well plate at 37 °C under 5% CO₂ for
319 24 h and the concentrations of IL-1β, CXCL-1, TNFα, and IL-10 in the culture
320 supernatants were measured by using cytometric bead arrays (CBA) (BD Biosciences)
321 according to the manufacturer's protocol. The skin of new born mice (younger than 3
322 days old) was incubated in CnT-07 medium (CELLnTEC Advanced Cell Systems) in
323 the presence of dispase II (1mg/ml) (CELLnTEC Advanced Cell Systems) at 4 °C for
324 16 h. The epidermis was isolated from the skin and incubated in Accutase (CellnTEC
325 Advanced Cell Systems) at room temperature for 20 min. The keratinocytes collected
326 were maintained in CnT-07 medium (CELLnTEC Advanced Cell Systems) according to
327 the manufacturer's protocol. Keratinocytes were then stimulated with long-chain fatty

328 acids at 37 °C under 5% CO₂ for 10 h and the culture supernatants were analyzed for
329 IL-1 α and HMBG-1 by flow cytometry using cytokine beads array (CBA) and for
330 HMGB-1 using an ELISA KIT II (Shino-test Corporation). Keratinocytes were also
331 stimulated with IL-1 α or HMGB-1 at 37 °C under 5% CO₂ for 3 h and analyzed for *Il1b*
332 and *Cxcl1* by quantitative real-time PCR analysis (qRT-PCR).
333
334 **qRT-PCR.** Total RNA was isolated by using ISOGEN (Wako Pure Chemical).
335 Quantitative real-time PCR analysis was performed on a 7500 Fast Real-Time PCR
336 System (Applied Biosystems, Foster City, California, USA) with Power SYBR Green
337 PCR Master Mix (Applied Biosystems). Results are presented relative to those of the
338 housekeeping gene encoding GAPDH (*gapdh*). Primers used were as follows: *Il1b* fwd,
339 GAAATGCCACCTTTTGACAGTG; *Il1b* rev, TGGATGCTCTCATCAGGACAG;
340 *Cxcl1* fwd, CTGGGATTCACCTCAAGAACATC; *Cxcl1* rev,
341 CAGGGTCAAGGCAAGCCTC; *Ccl2* fwd, CAGGTCCCTGTCATGCTTC; *Ccl2* rev
342 ATGAGTAGCAGCAGGTGAGTG; *Elovl6* fwd, ACAATGGACCTGTCAGCAAA;
343 *Elovl6* rev, GTACCAGTGCAGGAAGATCAGT; *Tnfa* fwd,
344 CCTGTAGCCCACGTCGTAG; *Tnfa* rev, GGGAGTAGACAAGGTACAACCC; *Il1a*
345 fwd, AGGGAGTCAACTCATTGGCG; *Il1a* rev, TGGCAGAACTGTAGTCTTCGT; *Il6*

346 rev, TCCACGATTTCCCAGAGAAC; *Il10* fwd, GCTGGACAACATACTGCTAACC;
347 *Il10* rev, ATTTCCGATAAGGCTTGGCAA; *Il33* fwd,
348 GGTGAACATGAGTCCCATCA; *Il33* rev, CGTCACCCCTTTGAAGCTC; *Tgfb* fwd,
349 TGACGTCACTGGAGTTGTACGG; *Tgfb* rev, GGTTTCATGTCATGGATGGTGC;
350 *Ccl2* rev ATGAGTAGCAGCAGGTGAGTG; *Ifng* fwd,
351 ACAGCAAGGCGAAAAAGGATG; *Ifng* rev, TGGTGGACCACTCGGATGA; *Il4*
352 fwd; ATCATCGGCATTTTGAACGAGG; *Il4* rev; TGCAGCTCCATGAGAACACTA;
353 *Il17* fwd, TTTAACTCCCTTGGCGCAAAA; *Il17* rev,
354 CTTTCCCTCCGCATTGACAC; *Elovl1* fwd, TCCAAAGCTACCCTCTGATGG;
355 *Elovl1* rev, AGGGAGAGTATCACCAGTGAGA; *Elovl2* fwd,
356 ACGCTGGTCATCCTGTTCTT; *Elovl2* rev, GCCACAATTAAGTGGGCTTT; *Elovl3*
357 fwd, TTCTCACGCGGGTTAAAAATGG; *Elovl3* rev,
358 GAGCAACAGATAGACGACCAC; *Elovl4* fwd, GCCCTGTGGTGGTATTTTGT;
359 *Elovl4* rev, TGGTGGTACACGTGAAGGAA; *Elovl5* fwd,
360 GGTGGCTGTTCTTCCAGATT , *Elovl5* rev, CCCTTCAGGTGGTCTTTCC, *Elovl6*
361 fwd, ACAATGGACCTGTCAGCAAA; *Elovl6* rev,
362 GTACCAGTGCAGGAAGATCAGT; *Elovl7* fwd, CATCGAGGACTGTGCGTTTTT;
363 *Elovl7* rev, CCAGGATGATGGTTTGTGGCA; *Scd1* fwd,

364 TCAACTTCACCACGTTCTTCA; *Scd1* rev, CTCCCGTCTCCAGTTCTCTT; *Scd2*
365 fwd, TGGTTTCCATGGGAGCTG; *Scd2* rev, TTGATGTGCCAGCGGTACT; *Scd3*
366 fwd, CTGACCTGAAAGCCGAGAAG; *Scd3* rev, GCAGAATGCCAGGCTTGTA;
367 *Gapdh* fwd, AGGTCGGTGTGAACGGATTTG; and *Gapdh* rev
368 TGTAGACCATGTAGTTGAGGTCA.

369

370 **Histology.** For histologic analysis, mouse skin was fixed in 10% formalin, embedded in
371 paraffin, sectioned, and stained with hematoxylin and eosin. For analysis of epidermal
372 thickness or cell number, 18 randomly selected sites were evaluated by using light
373 microscopy or fluorescent microscopy (MZ-X710, Keyence, Osaka, Japan) and its
374 associated software.

375

376 **Analysis of cleaved caspase-9.** Cells were treated or not with CVA or UV (180 mJ/
377 cm² for 3 min) and then fixed with 4% paraformaldehyde for 15 min and then with 5 %
378 BSA in PBS containing 0.3% Triton X-100 for 1 h. Subsequently, cells were incubated
379 with rabbit anti-cleaved caspase-9 antibody (1:200; Cell Signaling) for 1 h at room
380 temperature, followed by incubation with Alexa Fluor 594-conjugated donkey

381 anti-rabbit IgG (1:200; Thermo Fisher Scientific) secondary antibody for 30 min.
382 Finally, cells were counterstained with DAPI.
383
384 **Cell death and proliferation analyses.** Primary keratinocytes from neonatal epidermis,
385 prepared as described above, or human HaCaT cells were maintained in CnT-07
386 medium, as described above, and DMEM with 10% FBS, respectively, at 37 °C under
387 5% CO₂. Peritoneal macrophages were harvested by lavage of the peritoneal cavity and
388 suspended in DMEM containing 10% FBS. Primary keratinocytes and HaCaT cells and
389 peritoneal macrophages were seeded onto 48-well plates at a density of 1×10⁵ cells and
390 2×10⁵ cells/ well, respectively. After incubation at 37 °C for 2 h, the cells were washed
391 with PBS three times to remove unattached cells and stimulated with different
392 concentrations of free fatty acids dissolved in ethanol (final dose of 100 ~ 500 μM),
393 including oleic acid (OA), palmitic acid (PA), stearic acid (SA), palmitoleic acid (POA)
394 (Wako Pure Chemical), trans-vaccenic acid (TVA) or cis-vaccenic acid (CVA)
395 (Sigma-Aldrich). To block specific types of cell death or inhibit long-chain
396 acyl-coenzyme A synthetase, cells were pretreated with 1 mM of necrostatin-1 (Cayman
397 Chemicals), 1 mM of necrosulfonamide (Cellagen Technology), 2 mM of IM-54 (Sigma

398 Aldrich), 1 mM of cyclosporine A (Sigma Aldrich), or 10 μ M of triacsin C (Abcam) for
399 6 h before fatty acid stimulation.

400 For proliferation assay, these cells were stained or not with 10 μ M of CFSE
401 (Invitrogen) for 5min at 37°C before fatty acid stimulation, according to the
402 manufacturer instructions. Cells were then stained with propidium iodine (PI) and
403 analyzed for PI-positive and -negative cell populations and CFSE dilution by flow
404 cytometry. For the analysis of cell death in vivo, skin tissue was incubated in 0.5%
405 trypsin (Wako) in PBS, and separated into epidermis. Epidermal cells were stained with
406 CD45.2 (clone:104, BD pharmingen), CD49f (clone: GoH3, Miltenyi Biotec) and PI,
407 and then analyzed by flow cytometry.

408

409 **Transmission electron microscopy.** Cultured keratinocytes were fixed by incubating
410 2.5% glutaraldehyde in PBS (pH 7.4) at 4 °C overnight, postfixed in 1% osmium
411 tetraoxide at 4 °C for 30 min, and then dehydrated through graded concentrations of
412 ethanol. Cells were then transferred to propylene oxide and embedded (Poly/Bed 812,
413 Polysciences, Warrington, Pennsylvania, USA). The samples were analyzed by electron
414 microscopy (JEM-1400, JEOL, Peabody, Massachusetts, USA).

415

416 **Cytokine stimulation.** Primary mouse keratinocytes were stimulated with bovine
417 HMGB-1 (Chondrex, Redmond, Washington, USA) or mouse IL-1 α (Miltenyi Biotec,
418 Bergisch Gladbach, Germany). For stimulation of keratinocytes in vitro, primary
419 keratinocytes were stimulated 500ng/ml of bovine HMGB-1 or mouse IL-1 α . For
420 stimulation of keratinocytes in vivo, 200 ng of bovine HMGB-1 or mouse IL-1 α in 50
421 μ l PBS was injected intradermally.
422
423 **Fatty acid composition.** Lipids from mouse epidermis were extracted by using the
424 method of Bligh and Dyer (Breil *et al.* 2017). In brief, epidermis was extracted with
425 chloroform/ methanol (1:2, v/v) solution. One molar NaCl solution and chloroform were
426 added to break the monophasic and incubated on ice for 10 min. After centrifugation at
427 300 G for 5 min, aqueous solution was discarded and the phase of chloroform was
428 evaporated using nitrogen gas. Following the addition of acetonitrile/ 6N HCl (90/10,
429 v/v), samples were incubated at 100 °C for 45 min. Finally, liquid-liquid extraction
430 (Milne and Zhitomirsky 2018) with ethyl acetate was performed and the reconstituted
431 samples were injected into an optimized LC/ MS/MS system. The relative abundance of
432 each fatty acid was quantified by gas chromatography.
433

434 **Transepidermal water loss (TEWL) test.** Fetuses obtained at E13.5 and E17.5 were
435 incubated in methanol for 5 min and rinsed in PBS, followed by incubation with 0.1%
436 toluidine blue for 24 h. After staining, fetuses were rinsed with PBS and the staining
437 intensity was evaluated (Sassa *et al.* 2013). TEWL was also measured on the dorsal skin
438 of wild-type and *Elovl6*^{-/-} mice between 8 and 10 weeks of age by Tewameter[®] TM 300
439 (Integral) before and after tape stripping (Sassa *et al.* 2013). Measurements were
440 performed in triplicate for each mouse.

441

442 **Antagonist treatment.** To neutralize the IL-1 receptor, mice received 200 µl of the IL-1
443 receptor antagonist anakinra (10 mg/ml) (Kineret, Swedish Orphan Biovitrum)
444 intradermally and 300 µl of the same concentration of the antagonist intraperitoneally
445 daily during the induction of dermatitis by the mechanical stress or OVA treatment. To
446 block CXCR-2, mice received 200 µl of a CXCR-2 antagonist (0.25 mg/ml in PBS
447 containing 1% DMSO) (SB225002, Cayman Chemical, Ann Arbor, Michigan, USA)
448 intradermally and 150 µg of the same antagonist (0.5 mg/ml in PBS containing 1%
449 DMSO) intraperitoneally daily during the induction of dermatitis by the mechanical
450 stress.

451

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452 **Statistical analyses.** Statistical analyses were performed by using an unpaired,
453 two-tailed Student's *t*-test (GraphPad Prism 6, GraphPad Software, La Jolla, USA). A *P*
454 value less than 0.05 was considered to be statistically significant.

455 **Acknowledgments**

456 We thank S. Mitsuishi and Y. Nomura for their secretarial assistance.

457

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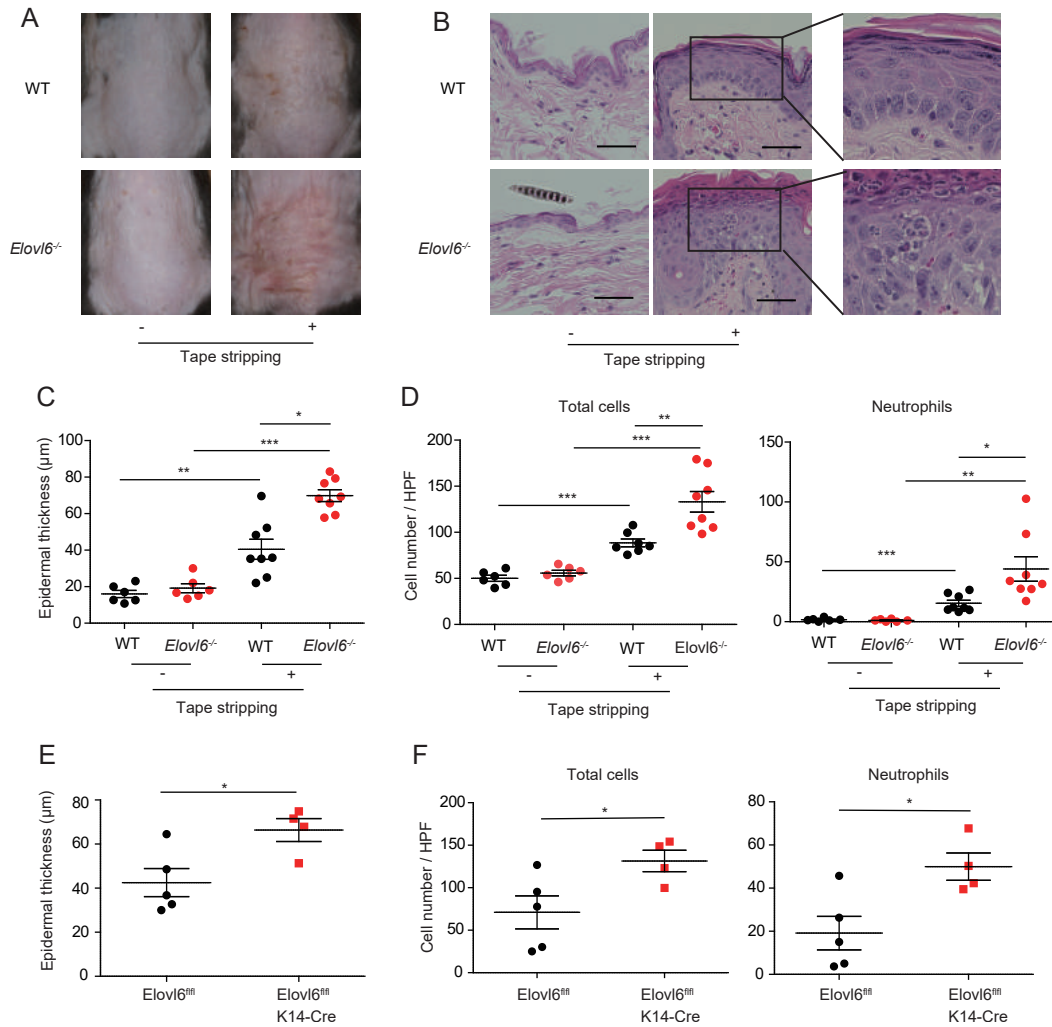
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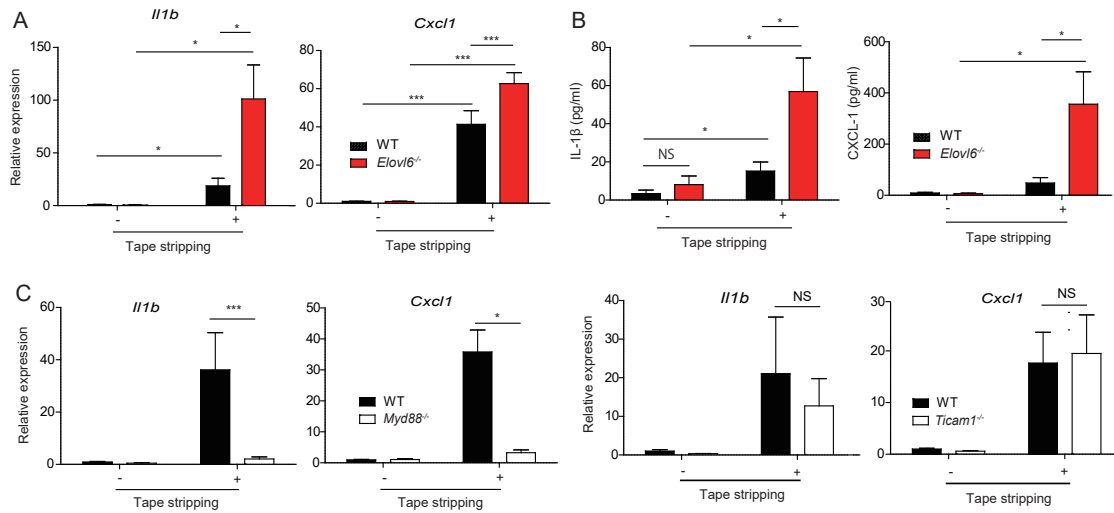
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- 612
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614 **Figures and legends**



615 **Fig. 1. *Elov6*^{-/-} mice show exacerbation of dermatitis.**
 616 (A to D) Representative gross findings (A), histology (hematoxylin and eosin staining)
 617 (B), epidermal thickness (C), and numbers of total infiltrating cells and neutrophils in
 618 the dorsal skin (D) of wild-type (n =6 or 8) and *Elov6*^{-/-} (n =6 or 8) mice before and on
 619 day 9 after the start of tape stripping. (E, F) Epidermal thickness (E) and numbers of
 620 total infiltrating cells and neutrophils in the skin (F) of *Elov6*^{fl/fl} (n = 5) and
 621 *Elov6*^{fl/fl} K14-Cre (n = 4) on day 9 after the start of tape stripping. Black bars indicate
 622 scale (50 µm) (B). Error bars indicate 1 SD; *, *P*<0.05; **, *P* < 0.01; ***, *P* < 0.001.
 623 Data are representative of three (A-D) and two (E, F) independent experiments.



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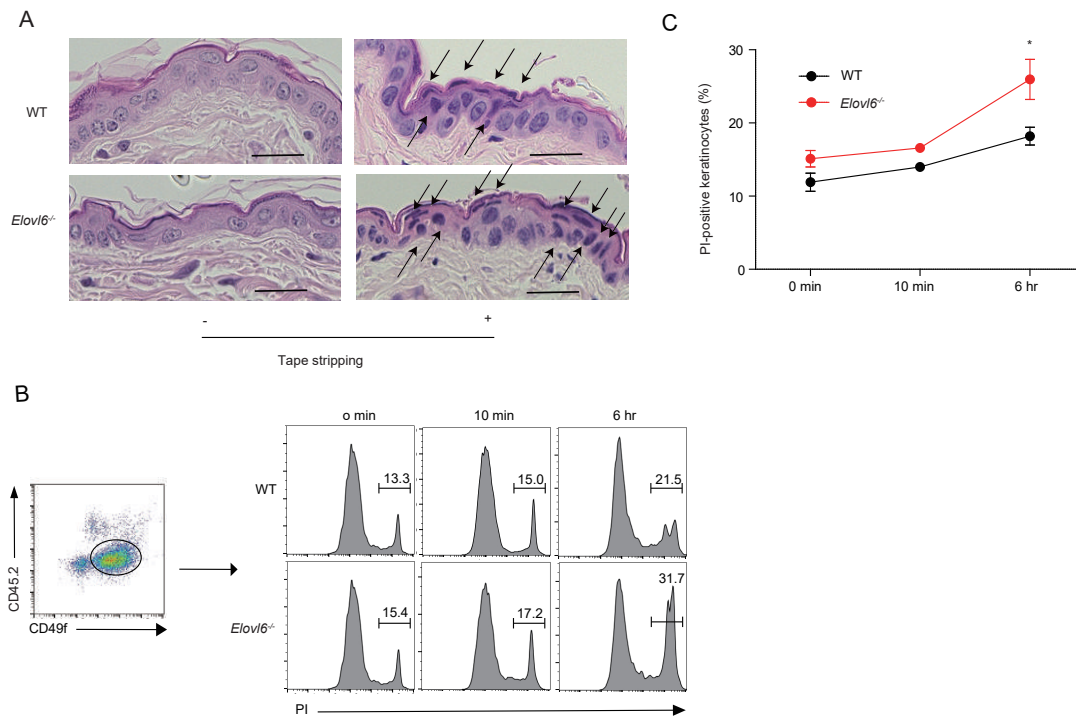
625 **Fig. 2. Elov6 suppresses mechanical damage-induced IL-1β and CXCL-1**
626 **production**

627 (A) Quantitative RT-PCR analysis of epidermis of wild-type and *Elov6*^{-/-} mice isolated
628 6 h after tape stripping (n = 10 in each group). (B) Epidermis was isolated before, and
629 12 h after, tape stripping from wild-type and *Elov6*^{-/-} mice and cultured for 24 h. The
630 concentrations of IL-1β and CXCL-1 in the supernatants were measured by using
631 cytometric bead array (n = 11 per group). (C) Quantitative RT-PCR analysis of *Il1b* and
632 *Cxcl1* in the epidermis of wild-type, *Myd88*^{-/-}, and *Ticam1*^{-/-} mice before, and 6 h after,
633 tape stripping (n = 7 to 11 per group). Error bars indicate 1 SD; *, P < 0.05; ***, P <
634 0.001. Data are representative of three independent experiments.

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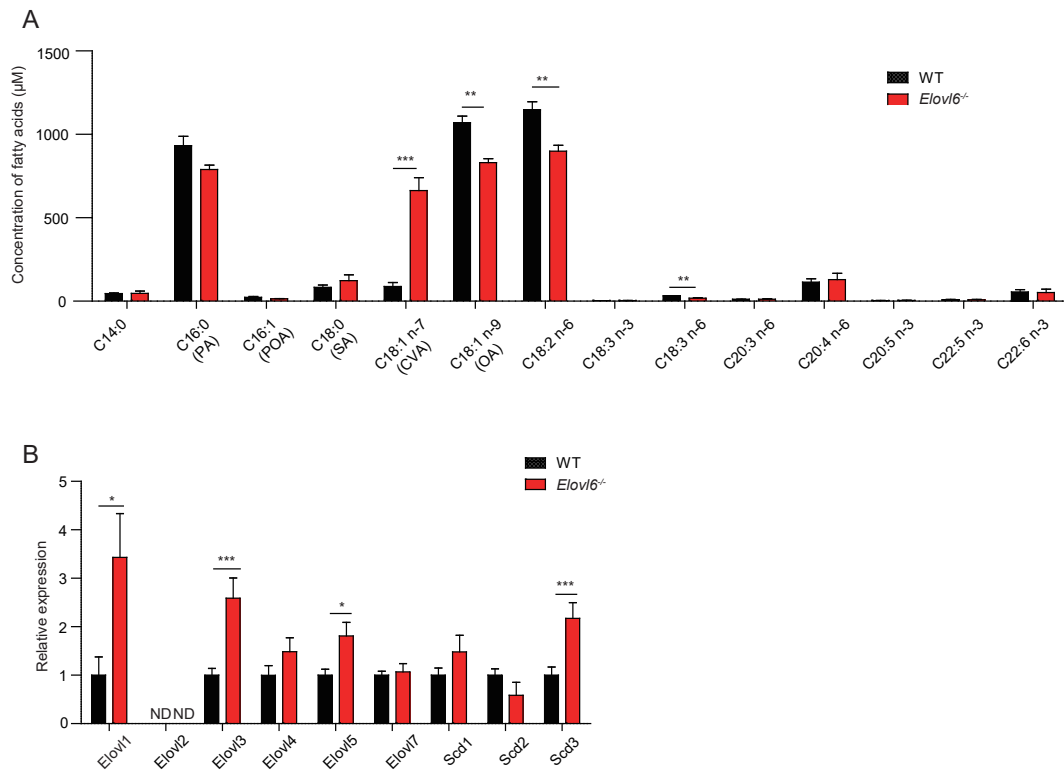
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640 **Fig. 3. Elov6 suppressed mechanical damage-induced keratinocyte death.**

641 (A) Representative histopathology of wild-type and *Elov6*^{-/-} mice 4 h after tape
642 stripping. Black arrows indicate degenerated keartinocytes with irregularly shaped
643 nuclei. Scale bar, 50 μm. (B, C) Flow cytometry of epidermal cells isolated from the
644 skin of wild-type and *Elov6*^{-/-} mice at the indicated time points after tape stripping.
645 Cells were stained with anti-CD45.2, anti-CD49f and propidium iodide (PI) and the
646 proportion of PI⁺ cells in CD45.2-CD49f⁺ cells were shown (n = 6 to 10 per group).
647 Error bars indicate 1 SD; *, P < 0.05. Data are representative of two independent
648 experiments.

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652 **Fig. 4. *Elov6* suppressed cis-vaccenic acid production in keratinocytes.**

653 (A) Fatty acid composition of epidermis in wild-type and *Elov6*^{-/-} mice (n = 4 per

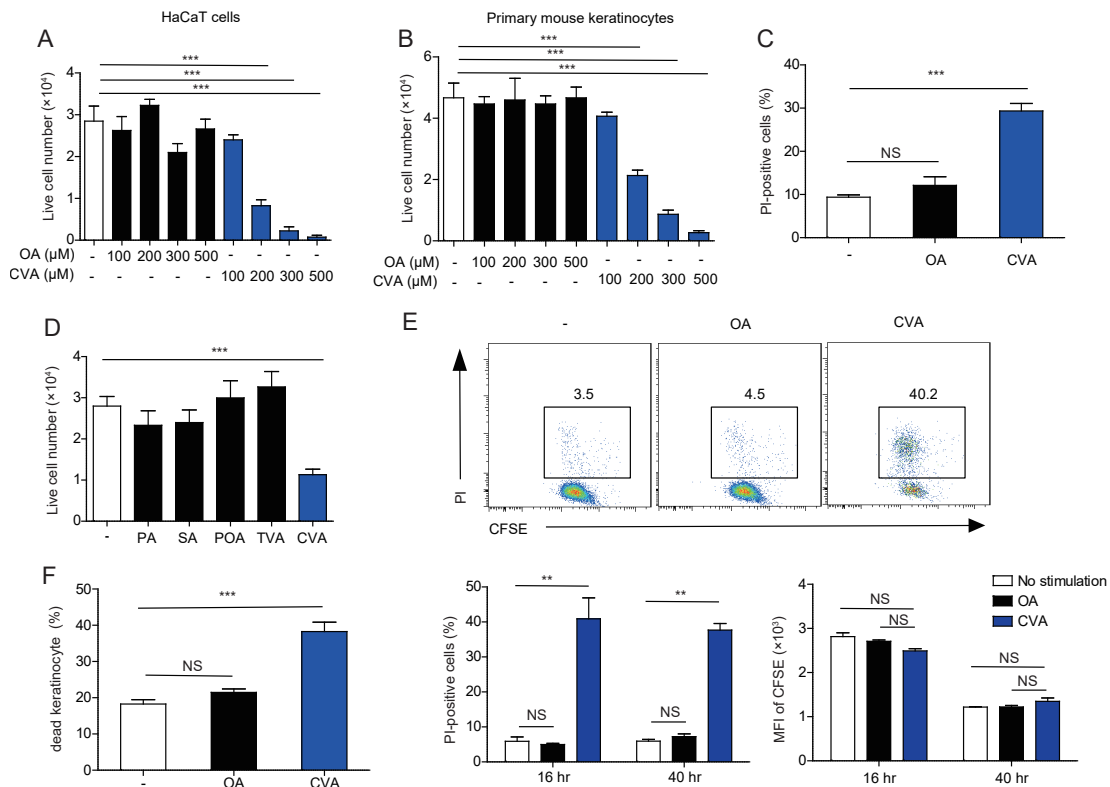
654 group). (B) Quantitative RT-PCR analysis of the epidermis for the expression of

655 long-chain fatty acid elongases and stearoyl-CoA desaturases in wild-type and *Elov6*^{-/-}

656 mice (n=10). Error bars indicate SD. ND, not done. *, *P*<0.05; **, *P*<0.01; ****P*<0.001.

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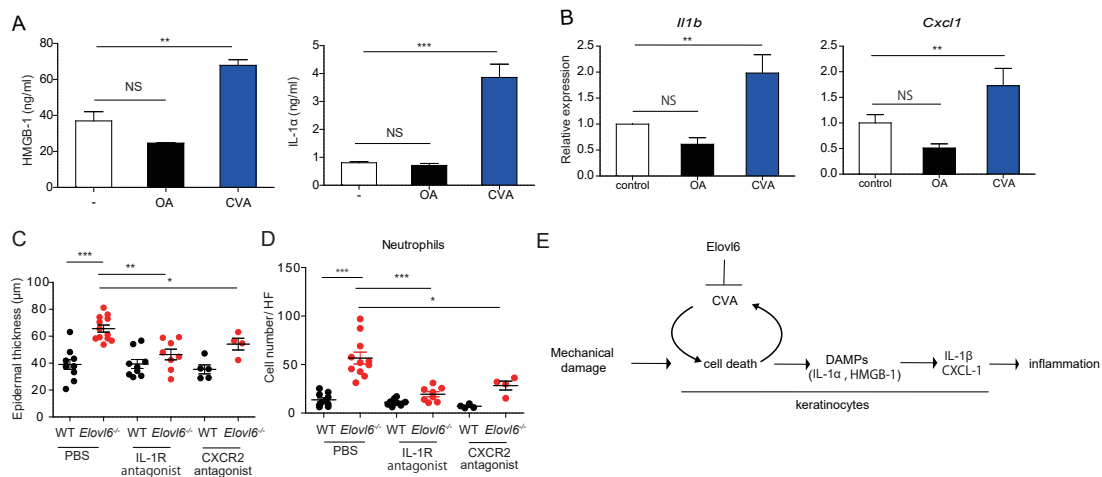
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660 **Fig. 5. Cis-vaccenic acid (CVA) induces keratinocyte death in the skin.**

661 (A, B, D) Number of live cells in cultures of HaCaT cells (A) and primary keratinocytes
662 (B, D) 16 h after stimulation with the indicated concentration of fatty acids (A, B) or
663 with 300 μM of the indicated fatty acid (D) (n = 3 per group). (C) Primary keratinocytes
664 were stimulated for 6 h with 300 μM of the indicated fatty acid and analyzed for
665 propidium iodide (PI)-positive (i.e. dead) cells by flow cytometry (n = 4 or 5 per group).
666 (E) HaCaT cells were labeled with 5-(and 6)-carboxyfluorescein diacetate succinimidyl
667 ester (CFSE) and stimulated or not with 300 μM of OA or CVA for 16 h or 40 h. Cells
668 were then stained with propidium iodide (PI) and analyzed by flow cytometry. (F) Flow
669 cytometry of epidermal cells isolated from the skin of wild-type mice that received
670 topical application of ethanol (control) or 45mM of OA or CVA to the dorsal skin for 6 h
671 (n = 6 per group). Error bars indicate SD. **, *P*<0.01; ***, *P*<0.001.

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674

675 **Fig. 6. CVA increased IL-1β and CXCL-1 production.**

676 (A) Enzyme-linked immunosorbent assay of HMGB-1 (n = 4 per group) and cytokine
 677 bead array of IL-1α (n = 3 per group) in the supernatant of cultured primary
 678 keratinocytes 10 h after initiation of stimulation with 300 μM OA or CVA. (B)
 679 Quantitative RT-PCR analysis of *Il1b* and *Cxcl1* in the epidermis of wild-type mice 6 h
 680 after topical application of ethanol (control) (n = 10) or 15mM of OA (n = 13) or CVA
 681 (n = 14) (B). (C, D) Wild-type and *Elovl6*^{-/-} mice were treated with PBS (n = 10 and 12,
 682 respectively), an IL-1 receptor antagonist (n = 9 and 8, respectively), or a CXCR-2
 683 antagonist (n = 5 and 4, respectively) daily for 9 days, from the beginning on the day of
 684 tape stripping. Epidermal thickness (C) and the number of infiltrating neutrophils (D)
 685 were analyzed on day 9. (E) A proposed signal pathway from mechanical damage onto
 686 the skin to skin inflammation. Error bars indicate SD; *, *P* < 0.05; **, *P* < 0.01, ***, *P*
 687 < 0.001; NS, not significant. Data are representative of at least two independent
 688 experiments.

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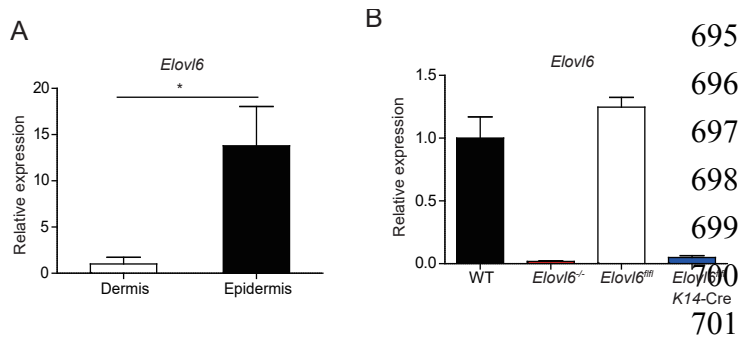
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693 **Supplementary Figures**

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702 **Fig. S1. Expression of *Elov16*.**

703 Quantitative RT-PCR analysis of *Elov16* from dermis and epidermis (n = 3 per group)
704 (A) and from the epidermis of wild-type (WT), *Elov16*^{-/-}, *Elov16*^{fl/fl}, *Elov16*^{fl/fl} K14-Cre
705 mice (n=3 to 5 in each group) (B). Error bars indicate SD. **P*<0.05. Data are
706 representative of more than two independent experiments.

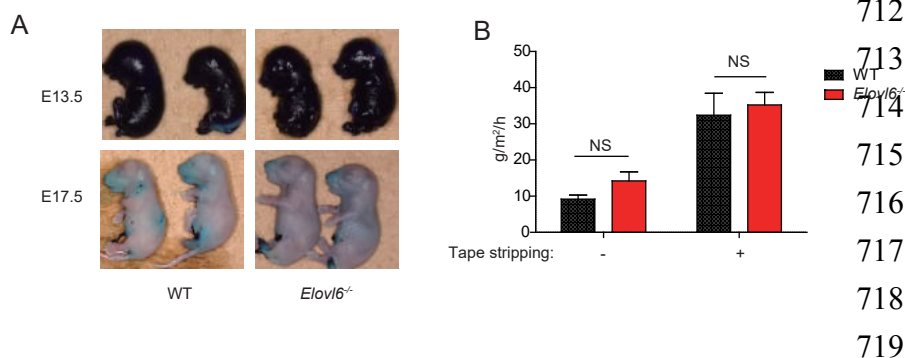
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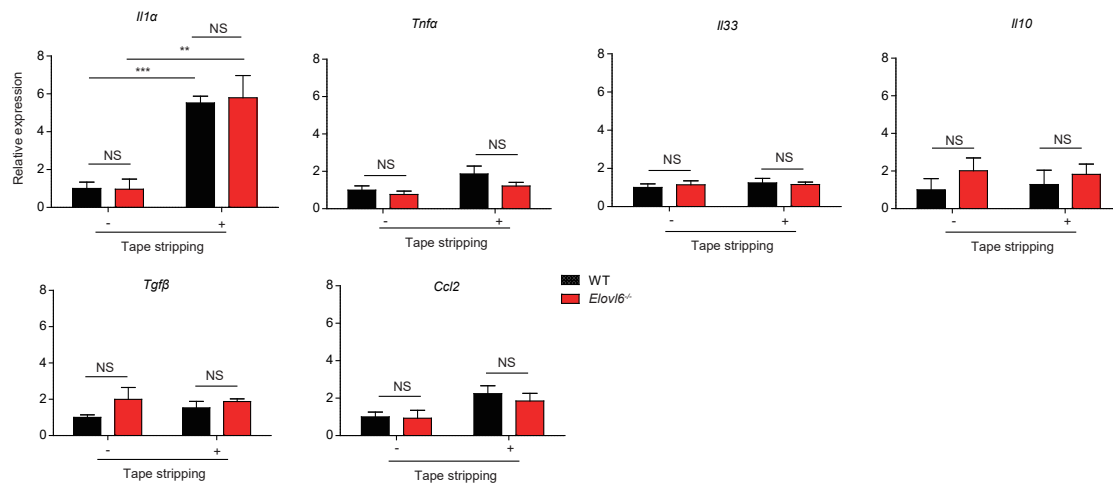


720 **Fig. S2. Comparable skin permeability barrier function between wild-type (WT)**
721 **and *Elov16*^{-/-} mice.**

722 (A) The fetuses at E13.5 and E17.5 from WT and *Elov16*^{-/-} mice were stained with 0.1%
723 toluidine blue for 24 h and photographed. (B) The transepidermal water loss of 6-8
724 weeks old WT and *Elov16*^{-/-} mice was measured before and after tape stripping (n = 14).
725 Error bars indicate SD. NS, not significant. Data are representative of three independent
726 experiments.

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731 **Fig. S3. Expression of cytokines**

732 Quantitative RT-PCR analysis of the epidermis isolated before and 12 h after tape

733 stripping from WT and *Elov6*^{-/-} mice and cultured for 24 h (n = 11 in each group). Error

734 bars indicate SD. NS, not significant. **P<0.01, ***P<0.001. Data are representative of

735 two independent experiments.

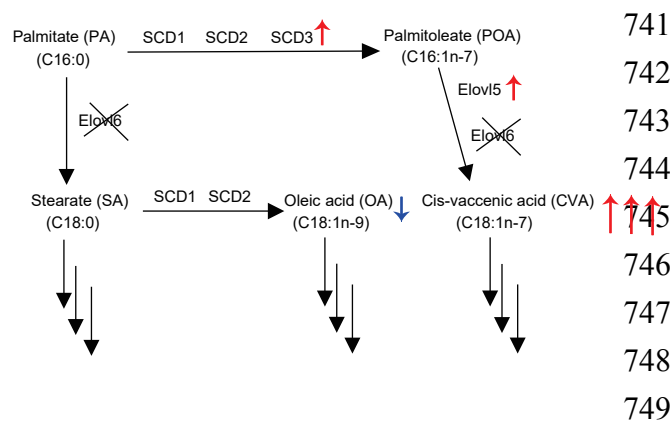
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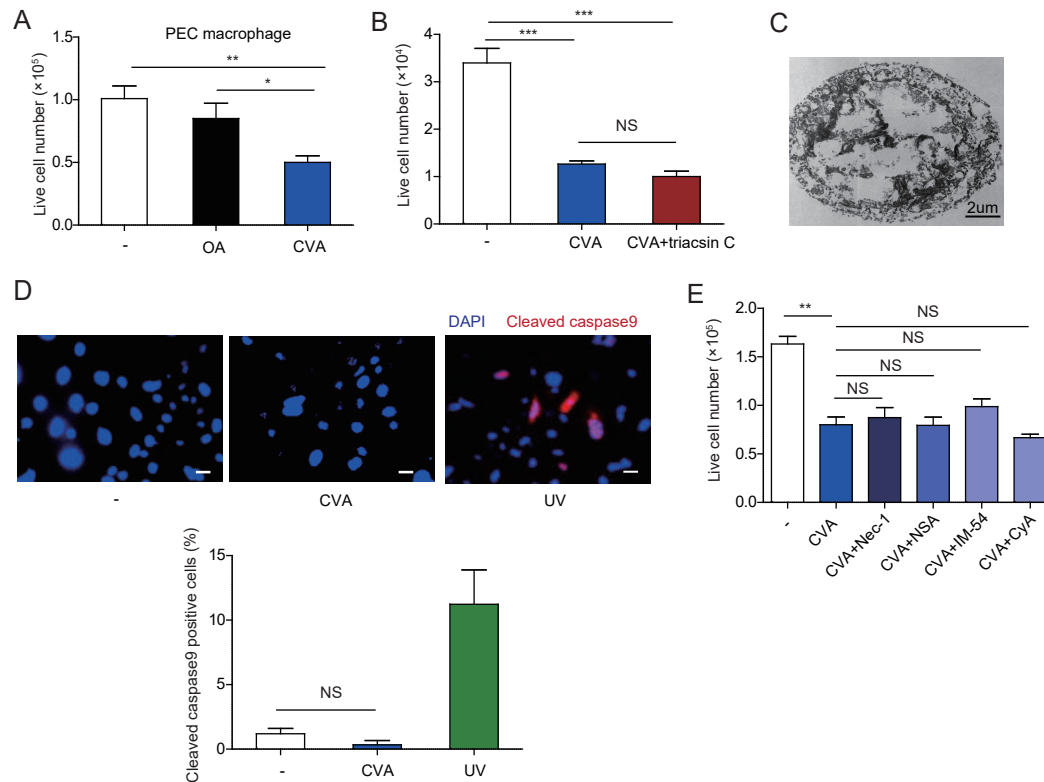
750 **Fig. S4. Schematic representation of the proposed pathway (black arrows)**

751 **controlling cis-vaccenic acid (CVA) generation in *Elov6*^{-/-} keratinocyte.**

752 Red and blue arrows indicate the increase in SCD3, Elov5, and CVA and the decrease

753 in OA, respectively.

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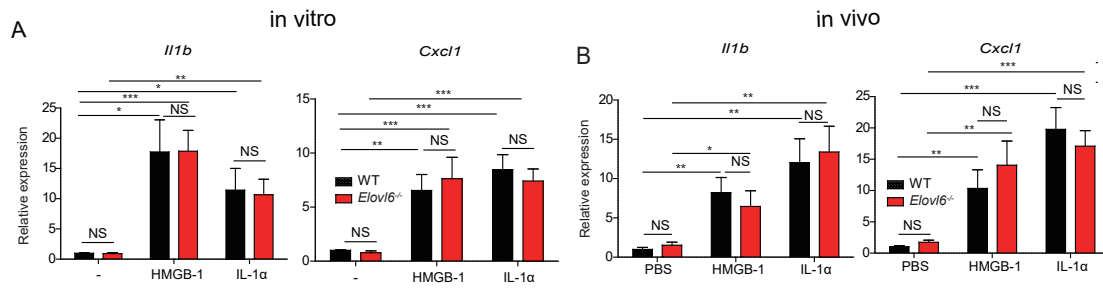
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757 **Fig. S5. CVA induced non-programmed cell death.**

758 (A) Live cell number of primary peritoneal macrophages 16 h after stimulation with
 759 oleic acid (OA) or CVA (300 μ M) (n = 3 in each group). (B, E) Primary mouse
 760 keratinocytes were cultured for 6 h in the presence or absence of 10 μ M of triacsin C
 761 (B), or 1 mM necrostatin (Nec-1), 1 mM necrosulfonamide (NSA), 2 mM IM-54, or 1
 762 mM cyclosporine (CyA) (E), followed by stimulation by adding 300 μ M CVA; live cells
 763 were counted 16 h afterward (n = 3). (C) A representative dead primary keratinocyte
 764 induced by stimulation with 300 μ M CVA for 10 h under a transmission electron
 765 microscope. (D) Immunofluorescence microscopic study of primary keratinocyte 10 h
 766 after stimulation with CVA or 6 h after ultraviolet irradiation. Cells were stained with
 767 anti-cleaved caspase 9, followed by Alexa Fluor 594-conjugated secondary antibody
 768 and DAPI. White bars indicate a scale (20 μ m). Percentage of cleaved caspase
 769 9-positive cells was calculated (n = 3). Error bars indicate SD. NS, not significant. *,
 770 $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Data are representative of more than two
 771 independent experiments.

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775 **Fig. S6. DAMPs increased IL-1 β and CXCL-1 production.**

776 Quantitative RT-PCR analysis of *Il1 β* and *Cxcl1* in keratinocytes of wild-type and
777 *Elov6*^{-/-} mice after stimulation or not with HMGB-1 or IL-1 α in vitro (n=10 per group)
778 (A) and in the epidermis isolated 4 h after injection intradermally with PBS, HMGB-1,
779 or IL-1 α (n = 8 per each group) (B).

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