

28 **ABSTRACT**

29 The NLRP3 inflammasome coordinates inflammation in response to different pathogen-
30 and damage-associated molecular patterns, being implicated in different infectious,
31 chronic inflammatory, metabolic and degenerative diseases. In chronic tendinopathies
32 lesions, different non-resolving mechanisms produce a degenerative condition that
33 impairs tissue healing, complicating their clinical management. Percutaneous needle
34 electrolysis consist in the application of a galvanic current and is emerging as a novel
35 treatment for tendinopathies. Here we found that galvanic current activates the NLRP3
36 inflammasome and the induction of an inflammatory response promoting a collagen-
37 mediated regeneration of the tendon. This study establish the molecular mechanism of
38 percutaneous electrolysis for the treatment of chronic lesions and the beneficial effects
39 of an induced inflammasome-related response.

40 INTRODUCTION

41 Tissue damage and infection triggers an inflammatory response that is coordinated by
42 the activation of the immune system. Inflammation promotes a response to recover
43 homeostasis by removing invading pathogens and repairing tissues (Medzhitov, 2008).
44 However, chronic inflammation can induce the continuous production of tissue
45 regenerative factors and an excessive accumulation of components of the extracellular
46 matrix leading to tissue fibrosis (Alegre et al., 2017; Borthwick et al., 2013; Gaul et al.,
47 2020; Wynn, 2008). Therefore, an equilibrated inflammatory response is required to
48 recover homeostasis and successfully achieve tissue healing (Borthwick et al., 2013;
49 Eming et al., 2017; Liston and Masters, 2017; Medzhitov, 2008). In response to tissue
50 damage, the nucleotide-binding oligomerization domain with leucine rich repeat and
51 pyrin domain containing 3 (NLRP3) inflammasome is activated and coordinates an
52 inflammatory response (Broz and Dixit, 2016; Schroder et al., 2011). NLRP3
53 inflammasome is a multiprotein complex mainly formed in myeloid cells after encounter
54 damage- or pathogen-associated molecular patterns, including elevated concentrations
55 of extracellular ATP, changes in extracellular osmolarity or detection of insoluble
56 particles and crystals, as uric acid crystals or amyloid deposition (Amores-Iniesta et al.,
57 2017; Compan et al., 2012; Heneka et al., 2013; Mayor et al., 2006). NLRP3 oligomers
58 recruit the accessory apoptosis-speck like protein with a caspase recruitment and
59 activation domain (ASC) that favor the activation of the inflammatory caspase-1
60 (Boucher et al., 2018; Li et al., 2018; Schmidt et al., 2016). Caspase-1 proteolytically
61 process immature pro-inflammatory cytokines of the interleukin (IL)-1 family to produce
62 the bioactive form of IL-1 β and IL-18 (Broz and Dixit, 2016; Schroder et al., 2011).
63 Caspase-1 also process gasdermin D protein (GSDMD), and its amino-terminal fragment
64 (GSDMD^{NT}) oligomerize in the plasma membrane forming pores allowing the release of
65 IL-1 β and IL-18 cytokines, as well as other intracellular content, including inflammasome
66 oligomers (Baroja-Mazo et al., 2014; Broz et al., 2020).

67 NLRP3 activation occurs in different chronic inflammatory, metabolic and degenerative
68 diseases such as gout, type 2 diabetes or Alzheimer (Daniels et al., 2016; Heneka et al.,
69 2013; Masters et al., 2010; Mayor et al., 2006), therefore selective small molecules that
70 block NLRP3 are emerging as novel anti-inflammatory therapies (Cocco et al., 2017; Coll
71 et al., 2015; Tapia-Abellán et al., 2019). However, in some pathological circumstances,
72 a boost, rather than an inhibition of NLRP3 would be beneficial to reduce clinical
73 complications, such as in immunosuppressed septic patients that accumulate high
74 mortality rates due to secondary infections associated to a profound deactivation of the
75 NLRP3 inflammasome (Martínez-García et al., 2019). In chronic non-resolving lesions,
76 as tendinopathies developed after prolonged extreme exercise, there are several
77 mechanisms establishing a degenerative condition of the tissue that impairs healing and
78 complicate clinical management (Cook and Purdam, 2009; Regan et al., 1992;
79 Soslowky et al., n.d.). Anti-inflammatory therapies have shown inefficient in randomized
80 trials for the treating of this type of lesions (Bisset et al., 2006; Coombes et al., 2013)
81 and novel treatments are emerging aiming at the regeneration of the tissue (Bubnov,
82 2013; Chellini et al., 2019), including the minimally invasive percutaneous needle
83 electrolysis (De-la-Cruz-Torres et al., 2020; Margalef et al., 2020; Valera-Garrido et al.,
84 2013, 2014, 2020). Percutaneous needle electrolysis consist in the application of a
85 galvanic current through an acupuncture needle, combining mechanical and electrical
86 stimulation in the tissues, resulting in a local controlled microtrauma that derives in an
87 inflammatory response and the repairment of the affected tissue (Valera-Garrido and
88 Minaya-Muñoz, 2019). However, the detailed molecular mechanism behind
89 percutaneous needle electrolysis inducing the induction of an inflammatory response has
90 not been yet described. In this study, we found that galvanic current applicated during
91 percutaneous needle electrolysis was able to activate the NLRP3 inflammasome and
92 induce the release of IL-1 β from macrophages. Mice deficient on NLRP3 failed to
93 increase IL-1 β in tendons after percutaneous needle electrolysis and resulted in a
94 reduction of TGF- β and type I collagen deposition, indicating that the NLRP3

- 95 inflammasome plays an important role in the regenerative response of the tendon
96 associated to percutaneous needle electrolysis.

97 **RESULTS**

98 **Galvanic current enhances macrophage pro-inflammatory M1 phenotype**

99 We initially designed and produced a device to apply galvanic current to adherent
100 cultured cells in 6 well cell culture plates (**Fig. S1**), this device allowed us to explore the
101 effect of galvanic currents in bone marrow derived mouse macrophages. Application of
102 2 impacts of 12 mA of galvanic current for 6 seconds each over LPS stimulated
103 macrophages, induced an increase of the expression of *Cox2* and *Ii6* genes (**Fig. 1A**).
104 However, it did not affect LPS-induced *Ii1b* or *Tnfa* pro-inflammatory gene expression
105 (**Fig. 1A**). Interestingly meanwhile *Tnfa* expression was upregulated with galvanic
106 current alone (**Fig. 1A**), galvanic currents were not inducing the expression of *Cox2*, *Ii6*
107 or *Ii1b* genes on non-LPS treated macrophages, or over IL-4 treated macrophages (**Fig.**
108 **1A**). When macrophages were polarized to M2 by IL-4, galvanic currents decreased the
109 expression of the M2 markers *Arg1*, *Fizz1* and *Mrc1* (**Fig. 1B**). These data suggest that
110 galvanic current could enhance the pro-inflammatory signature of M1 macrophages
111 whilst decrease M2 polarization. We next studied the concentration of released pro-
112 inflammatory cytokines from macrophages, and found that galvanic current was not able
113 to increase the concentration of IL-6 or TNF- α release after LPS stimulation (**Fig. 1C**),
114 but significantly augmented the release of IL-1 β in an intensity dependent manner (**Fig.**
115 **1C**). This data indicates that the increase of *Ii6* and *Tnfa* gene expression detected at
116 mRNA level would not be transcribing to higher amounts of released IL-6 and TNF- α
117 over LPS treatment, but galvanic current could be potentially activating an
118 inflammasome to induce the release of IL-1 β .

119

120 **Galvanic current activates the NLRP3 inflammasome**

121 Since IL-1 β release is increased by the activation of caspase-1 after the canonical or
122 non-canonical inflammasome formation (Broz and Dixit, 2016), we next studied the
123 release of IL-1 β induced by galvanic current in macrophages deficient on caspase-1 and

124 -11 to avoid both the canonical and non-canonical inflammasome signaling. We found
125 that *Casp1/11*^{-/-} macrophages fail to release IL-1 β induced by galvanic current (**Fig. 2A**).
126 We then found that galvanic current application on *Pycard*^{-/-} macrophages also failed to
127 induce the release of IL-1 β , denoting that the inflammasome adaptor protein ASC would
128 be also required for the inflammasome activation (**Fig. 2A**). Since current application
129 could be considered a sterile danger signal, we next assessed the implication of NLRP3,
130 an inflammasome sensor important to elicit an immune response in sterile dangerous
131 situations (Broz and Dixit, 2016; Liston and Masters, 2017). *Nlrp3*^{-/-} and the use of the
132 specific NLRP3 inhibitor MCC950 (Coll et al., 2015; Tapia-Abellán et al., 2019) impaired
133 the release of IL-1 β induced by galvanic current (**Fig. 2A,B**), demonstrating that the
134 NLRP3 inflammasome is activated during galvanic current application. As controls,
135 similar results were obtained in parallel with the specific NLRP3 activator nigericin (**Fig.**
136 **2B and S2A**). Mechanistically, the use of an extracellular buffer with 40 mM of KCl
137 decreased IL-1 β release induced by nigericin and galvanic current application, but not
138 the release of IL-1 β induced by *Clostridium difficile* toxin B, that activate the Pypin
139 inflammasome which is a K⁺-efflux independent inflammasome (**Fig. 2C**). However,
140 meanwhile we found a robust intracellular K⁺ decrease in macrophages treated with the
141 K⁺ ionophore nigericin, we fail to detect a decrease of intracellular K⁺ when galvanic
142 current was applied (**Fig. S2B**). This data suggests that either a small and/or transient
143 decrease of intracellular K⁺ could be induced by galvanic current or alternatively a dilution
144 of intracellular K⁺ concentration should occur when galvanic current is applied, and
145 this could also explain the smaller concentration of IL-1 β release induced by galvanic
146 current compared to nigericin application (**Fig. 2C**). After galvanic current application we
147 were able to detect the generation of the active p20 caspase-1 fragment, and processed
148 IL-1 β and GSDMD^{NT} (**Fig. 2D**). MCC950 was able to abrogate caspase-1 activation and
149 the processed forms of IL-1 β and GSDMD^{NT} (**Fig. 2D**), suggesting a functional caspase-
150 1 activation and downstream signaling due to canonical NLRP3 activation and discarding

151 the non-canonical NLRP3 activation that would result in GSDMD processing in the
152 presence of MCC950.

153

154 **Galvanic current does not induce inflammasome-mediated pyroptosis**

155 Since GSDMD was processed and the N-terminus detected upon galvanic current
156 application, we next assessed pyroptosis by means of Yo-Pro-1 uptake to cells and LDH
157 leakage from the cell. Two impacts of galvanic currents of different intensities (3, 6, 12
158 mA) for a period of 6 seconds (conditions that induce IL-1 β release) were only inducing
159 a significant, but slightly increase of cell death (**Fig. 3A**). This increase in cell death was
160 not associated with the activation of the inflammasome, since it was also present in
161 macrophages deficient on NLRP3, ASC or caspase-1/11 (**Fig. 3B**), suggesting that was
162 independently of pyroptosis. Increasing the number or the time of 12 mA impacts
163 applied, resulted in a time-dependent increase of cell death (**Fig. 3A**), correlating with
164 higher concentrations of IL-1 β release (**Fig. 3C**). However, meanwhile IL-1 β release was
165 blocked by MCC950 (**Fig. 3C**), LDH release was not dependent on NLRP3 activation
166 (**Fig. 3D**). This further corroborate that the NLRP3 activation is dependent on the
167 intensity and time of galvanic current application. Similarly, two impacts of 12 mA for a
168 period of 6 seconds were unable to induce plasma membrane permeabilization
169 measured by Yo-Pro-1 uptake during a period of 3 h (**Fig. 3E**). Yo-Pro uptake increased
170 over 3 h in an intensity dependent manner (3, 6, 12 mA) when 8 impacts were applied
171 during 6 seconds (**Fig. 3E**). This increase of plasma membrane permeabilization was
172 not reverted after NLRP3 blocking with MCC950 or when ASC-deficient macrophages
173 were used (**Fig. 3F**). All these results demonstrate that doses of galvanic current of 3 or
174 6 mA for impacts of 6 seconds do not compromise cell viability but are able to induce an
175 inflammatory response dependent on NLRP3 activation, in contrast with current
176 intensities of 12 mA that if prolonged in time could cause significant cell death
177 independently of the inflammasome.

178

179 **Galvanic current applied in tendon increases inflammation *in vivo***

180 In order to study the effect of galvanic current *in vivo*, we found that application of 3
181 impacts of 3 mA of galvanic current during 3 seconds in the calcaneal tendon of mice
182 resulted in an increase of the number of polymorphonuclear cells after 3 days when
183 compared with tendons treated with needling alone (a puncture without current
184 application, **Fig. 4A,B**). This increase returned to basal after 7 days and stayed low up
185 to 21 days after galvanic current application (**Fig. 4B**). Similarly, the number of F4/80⁺
186 macrophages increased after 3 days of galvanic current application when compared to
187 needling alone and returned to basal levels after 7 days (**Fig. 4C,D**). Other immune cell
188 types detected in the tendon, as mastocytes, were not significantly increased by galvanic
189 current application when compared to needling alone (**Fig. S3A**). Other histological
190 features of the tendon (number of tenocytes, shape and area of tenocyte nuclei or neo-
191 vascularization) were also not affected by the application of galvanic currents compared
192 to needling alone (**Fig. S3B-E**).

193 We next assessed the expression of different pro-inflammatory cytokines in the
194 calcaneal tendon after 3 days of 3 impacts of 3 mA of galvanic current application during
195 3 seconds. Expression of *Il6*, *Il1a* and *Il1b*, as well as the IL-1 receptor antagonist (*Il1rn*)
196 and the chemokine *Cxcl10* were all increasing after percutaneous electrolysis when
197 compared to needling alone (**Fig. 5A**). Different NLRP3 inflammasome genes also
198 exhibit an increase in expression (*Nlrp3*, *Pycard*, *Casp1*) when galvanic current was
199 applied, but this increase was not significant when compared to needling (**Fig. 5B**).
200 *Gsdmd* expression was not upregulated in the tendons after galvanic current application
201 (**Fig. 5B**). These data suggest that galvanic current induces an inflammatory response
202 driven by the infiltration of polymorphonuclear cells and macrophages, together an
203 increase of the expression of several cytokines and chemokines.

204

205 **The NLRP3 inflammasome controls the *in vivo* inflammatory response induced by**
206 **galvanic current**

207 In order to evaluate if the NLRP3 inflammasome mediates the inflammatory response in
208 tendons after percutaneous electrolysis, we applied galvanic currents in the calcaneal
209 tendon of *Nlrp3*^{-/-} mice. Application of 3 impacts of 3 mA of galvanic current for 3 seconds
210 in the calcaneal tendon of *Nlrp3*^{-/-} mice resulted in a significant reduction of *Il1b*, *Il1rn*
211 and *Cxcl10* expression after 3 days when compared to wild-type mice (**Fig. 6A**). Specific
212 inflammasome associated genes, as *Pycard*, *Casp1* or *Gsdmd* (except for *Nlrp3*) where
213 not affecting their expression in the calcaneal tendon of *Nlrp3*^{-/-} mice after 3 days of
214 galvanic current application when compared to wild type mice (**Fig. 6B**). Surprisingly,
215 galvanic current produced a tendency to increase the expression of *Il6* in the tendons of
216 *Nlrp3*^{-/-} after 3 days (**Fig. 6C**) and in parallel, the number of polymorphonuclear cells was
217 also increased (**Fig. 6D**). However, the number of macrophages was not affected in the
218 *Nlrp3*^{-/-} calcaneal tendon when galvanic current was applied (**Fig. 6D**). We also
219 confirmed a decrease of *Il1b* and *Cxcl10* expression in the tendons of *Pycard*^{+/-} mice after
220 3 days of galvanic current application (**Fig. S4**), suggesting that the NLRP3
221 inflammasome is important to modulate part of the inflammatory response after galvanic
222 current application.

223

224 **The NLRP3 inflammasome induces a tissue regenerative response to galvanic** 225 **current application**

226 Galvanic current application has been widely used to resolve chronic tendinopathies
227 (Abat et al., 2016; Rodríguez-Huguet et al., 2020; Valera-Garrido et al., 2014), and here
228 we present the case of a 6 weeks resolution of lateral epicondylitis after four sessions of
229 percutaneous electrolysis with an intensity of 3 mA of galvanic current application for 3
230 sec, 3 times (3:3:3) (**Fig. 7A**), according to the protocol previously described by Valera-
231 Garrido and Minaya-Muñoz (Valera-Garrido and Minaya-Muñoz, 2016). During this
232 tissue regeneration, the production of new extracellular matrix is a key process (Shook
233 et al., 2018; Wynn, 2008). In order to investigate if the inflammatory response mediated
234 by the NLRP3 inflammasome after galvanic current application is important for tissue

235 regeneration, we measured *Tgfb1* expression as a key factor inducing collagen
236 production. We found that *in vivo* the expression of *Tgfb1* after 3 days of galvanic current
237 application in the calcaneal tendon of mice was dependent on NLRP3 (**Fig. 7B**). In line,
238 after 7 days of percutaneous electrolysis the levels of type III collagen were decreased,
239 with a parallel increase of type I collagen when compared to needling alone (**Fig. 7C**,
240 **S5E**). However, percutaneous electrolysis did not affect collagen fiber properties
241 measured (width, length, strength or angle) when compared to needling alone (**Fig. S5A-**
242 **D**). The increase of type I collagen after 7 days of galvanic current application was
243 reduced in *Nlrp3*^{-/-} mice (**Fig. 7D**), suggesting that the NLRP3 inflammasome could
244 control the response of galvanic current inducing type I collagen. Overall, we found that
245 galvanic current application is able to activate the NLRP3 inflammasome and induce the
246 release of IL-1 β , initiating an inflammatory response that could lead to the regeneration
247 of the tendon (**Fig. S6**).

248 **DISCUSSION**

249 In this study we demonstrate how galvanic current application induces in macrophages
250 a pro-inflammatory signature, mainly characterized by the activation of the NLRP3
251 inflammasome and the release of mature IL-1 β . Inflammation is an important initial
252 response that promotes tissue repair and wound healing, however excessive
253 inflammation could lead to chronic inflammation and fibrosis (Alegre et al., 2017; Eming
254 et al., 2017; Gaul et al., 2020). The NLRP3 inflammasome is a key pathway to control
255 inflammation in the absence of pathogenic microorganisms (in sterile conditions) and its
256 stimulation favors the activation of caspase-1 and the processing of GSDMD, that
257 execute a pro-inflammatory type of cell death called pyroptosis (Broz et al., 2020; Broz
258 and Dixit, 2016; Liston and Masters, 2017). Pyroptosis compromises the integrity of the
259 cellular plasma membrane and results in the uncontrolled release of intracellular content,
260 including the release of the potent pro-inflammatory cytokines IL-1 β and IL-18 (Broz et
261 al., 2020). Pyroptosis also leads to the release of inflammasome oligomers that spread
262 pro-inflammatory signaling and drives fibrosis (Baroja-Mazo et al., 2014; Franklin et al.,
263 2014; Gaul et al., 2020). Here we found that galvanic current application, a technique
264 that has been widely used to resolve chronic lesions in clinic (Valera-Garrido et al.,
265 2014), was able to activate the NLRP3 inflammasome and induce IL-1 β release, but with
266 very little associated pyroptotic cell death. This could be due to two potentially different
267 mechanisms: (i) the finding of an alternative processing of GSDMD after galvanic current
268 application that was independent on NLRP3 and could inactivate its N-terminal lytic
269 domain, as has been previously found for caspase-3 processing GSDMD (Taabazuing
270 et al., 2017); and/or (ii) the small amounts of GSDMD^{NT} found that could result in a small
271 number of pores at the plasma membrane and facilitates their repair by the endosomal
272 sorting complexes required for transport machinery leading to an hyperactive state of the
273 macrophage (Evavold et al., 2018; Rühl et al., 2018). During this state of the
274 macrophage, IL-1 β is released in the absence of cell death (Evavold et al., 2018).

275 However, an increase of the intensity and the time of galvanic current application leads
276 to an increase in cell death, that was independent on the inflammasome and could be
277 related to the technique *per se*. Therefore, clinical application of current intensities above
278 6 mA would probably lead to necrosis of the tissue and not to an efficient reparative
279 process. Galvanic currents of 3 and 6 mA application for 2 impacts of 6 seconds are both
280 able to induce NLRP3 inflammasome activation *in vitro* and also lead to phenotypic
281 changes in the tendon *in vivo*. This is in line with the fact that 3 mA of galvanic current
282 are able to induce a clinically relevant regeneration of lesions (García Vidal et al., 2019;
283 Margalef et al., 2019; Medina i Mirapeix et al., 2019; Valera-Garrido et al., 2014). High
284 intensity doses for long periods of time or repeated impacts could induce massive tissue
285 necrosis and therefore are not recommended in the clinical practice.

286 The activation of the NLRP3 inflammasome induced by galvanic currents was found
287 dependent on K⁺ efflux, as extracellular high concentrations of K⁺ was able to block IL-
288 1β release, but surprisingly galvanic current application was not resulting in a detectable
289 intracellular K⁺ decrease. This oppose the effect of the well-studied K⁺ ionophore
290 nigericin that was able to induce a dramatic decrease of intracellular K⁺ in accordance to
291 previous publications (Muñoz-Planillo et al., 2013; Petrilli et al., 2007; Próchnicki et al.,
292 2016). It might be that galvanic currents would induce a slight decrease of intracellular
293 K⁺ not detectable by the technique used in this study, but enough to result in NLRP3
294 activation. In fact, the amount of IL-1β released from galvanic current activated
295 macrophages was lower than when macrophages were activated with nigericin, denoting
296 a correlation to the decrease in intracellular K⁺. The low NLRP3 activation induced by
297 galvanic current application could result in a moderate inflammatory response *in vivo*
298 beneficial for tissue regeneration. In fact, NLRP3 was important to induce an
299 inflammatory response *in vivo* with elevation of different cytokines including *Il1b* or
300 *Cxcl10*, but contrary affecting *Il6* production and the deficiency of NLRP3 leads to an
301 increase of polymorphonuclear cells. Also, exacerbated NLRP3 activation could led to
302 fibrosis (Alegre et al., 2017; Gaul et al., 2020), denoting that NLRP3 could control

303 collagen deposition. The mild activation of NLRP3 found after galvanic current
304 application was associated to increase production of *Tgfb1* and an increase of collagen
305 type I vs type III in tendons. This could explain the beneficial regenerative response of
306 the application of galvanic current in tendon lesions (Abat et al., 2016; Rodríguez-Huguet
307 et al., 2020; Valera-Garrido et al., 2014, 2013).

308 Therefore, this study reports how galvanic current is a feasible technique applied *in*
309 *vivo* to activate the NLRP3 inflammasome and induce a local inflammatory response to
310 enhance a collagen-mediated regeneration process in the tendon, establishing the
311 molecular mechanism of percutaneous electrolysis for the treatment of chronic lesions.

312 MATERIAL AND METHODS

313 **Animals and percutaneous needle puncture procedure.** All experimental protocols
314 for animal handling were refined and approved by the local animal research ethical
315 committee (references 241/2016 and 541/2019) and Animal Health Service of the
316 General Directorate of Fishing and Farming of the Council of Murcia (*Servicio de Sanidad*
317 *Animal, Dirección General de Ganadería y Pesca, Consejería de Agricultura y Agua de*
318 *la Región de Murcia*, reference A13160702). C57/BL6 mice (wild-type) were obtained
319 from the Jackson Laboratories. NLRP3-deficient mice (*Nlrp3^{-/-}*) and Caspase-1/11-
320 deficient mice (*Casp-1/11^{-/-}*) in C57/BL6 background were a generous gift of I. Coullin.
321 For all experiments, mice between 8-10 weeks of age were used. Mice were bred in
322 specific pathogen-free conditions with 12:12 h light-dark cycle and used in accordance
323 with the *Hospital Clínico Universitario Virgen de la Arrixaca* animal experimentation
324 guidelines, and the Spanish national (RD 1201/2005 and Law 32/2007) and EU
325 (86/609/EEC and 2010/63/EU) legislation. Percutaneous needle puncture was
326 performed with 16G and 13 mm needles (Agupunt) in the calcaneal tendon in isoflurane
327 (Zoetis) anesthetized mice; galvanic current was applied using Physio Invasiva®
328 equipment (Prim) delivering three impacts of 3 mA for 3 seconds and compared to a
329 puncture without current application. Paws without puncture were also used as controls.
330 3, 7, 14 and 21 days after puncture, animals were euthanized and paws were collected
331 for histopathology or gene expression. Only calcaneal tendon was dissected for gene
332 expression and the zone between gastrocnemius and calcaneus, including tendon,
333 adipose tissue, tibia and peroneus, was dissected for histopathology.

334

335 **Patient.** A male patient of 36 year old with lateral epicondylalgia in the right elbow for 6
336 months of evolution, with pain and functional impairment. Resistant to conventional
337 treatments (physiotherapy, oral non-steroidal anti-inflammatory and local corticoid
338 infiltrations). Ultrasound analysis show extensor joint tendon degeneration correlating
339 with positive orthopedic tests. The patient was subjected to four sessions of

340 percutaneous electrolysis with an intensity of 3 mA of galvanic current application for 3
341 sec, 3 times (3:3:3), according to the protocol by Valera-Garrido and Minaya-Muñoz
342 (Valera-Garrido and Minaya-Muñoz, 2016).

343

344 **Cell culture and treatments.** Bone marrow-derived macrophages (BMDMs) were
345 obtained from wild-type, *Casp1/11^{-/-}*, *Nlrp3^{-/-}* and *Pycard^{-/-}* mice. Cells were differentiating
346 for 7 days in DMEM (Lonza) supplemented with 25% of L929 medium, 15% fetal bovine
347 serum (FCS, Life Technologies), 100 U/ml penicillin/streptomycin (Lonza), and 1% L-
348 glutamine (Lonza). After differentiation, cells were primed for 2 h with 1 µg/ml *E. coli*
349 lipopolysaccharide (LPS) serotype O55:B5 at (Sigma-Aldrich) or for 4 h with 20 ng/ml
350 recombinant mouse IL-4 (BD Pharmigen). Cells were then washed twice with isotonic
351 buffer composed of 147 mM NaCl, 10 mM HEPES, 13 mM glucose, 2 mM CaCl₂, 1 mM
352 MgCl₂, and 2 mM KCl, pH 7.4, and then treated in OptiMEM (Lonza) with different
353 intensities and time of galvanic current (as indicated in the text and figure legends) using
354 an ad hoc adaptor for 6 well plates (**Fig. S1**), and then cultured for 6 h. Alternatively and
355 as a positive control, after LPS-priming macrophages were treated for 6 h in OptiMEM
356 with 1.5 µM nigericin (Sigma-Aldrich) or 1 µg/ml *Clostridium difficile* toxin B (Enzo Life
357 Sciences) to activate NLRP3 and Pypin inflammasomes respectively. In some
358 experiments, cells were treated with 10 µM of the NLRP3 inflammasome inhibitor
359 MCC950 (CP-456773, Sigma-Aldrich) after LPS priming and during inflammasome
360 activation.

361

362 **LDH release, Yo-Pro uptake assay and K⁺ measurements.** The presence of lactate
363 dehydrogenase (LDH) in cell culture supernatants was measured using the Cytotoxicity
364 Detection kit (Roche), following manufacturer's instructions. It was expressed as the
365 percentage of the total amount of LDH present in the cells. For Yo-Pro uptake,
366 macrophages were preincubated for 5 min at 37 °C with 2.5 µM of Yo-Pro-1 iodide (Life
367 Technologies) after galvanic current application or 1% triton X100 (Sigma-Aldrich)

368 application. Yo-Pro-1 fluorescence was measured after the treatments every 5 minutes
369 for the first 30 min and then every 30 min for the following 3 h with an excitation
370 wavelength of 478 ± 20 nm and emission of 519 ± 20 nm in a Synergy neo2 multi-mode
371 plate reader (BioTek). Intracellular K^+ was quantified from macrophages lysates as
372 already reported (Compan et al., 2012) and measured by indirect potentiometry on a
373 Cobas 6000 with ISE module (Roche).

374

375 **Western blot and ELISA.** After cell stimulation, cells extracts were prepared in cold lysis
376 buffer and incubated at 4°C for 30 min and then centrifuged at 12856 xg for 10 min at
377 4°C . Cells supernatants were centrifuged at 12856 xg for 30 seconds at 4°C and
378 concentrated by centrifugation at 11000 xg for 30 min at 4°C through a column with a 10
379 kDa cut-off (Merk-Millipore). Cell lysates and concentrated supernatants were mixed with
380 loading buffer (Sigma), boiled at 95°C for 5 min, resolved in 15% polyacrylamide gels
381 and transferred to nitrocellulose membranes (BioRad). Different primary antibodies were
382 used for the detection of interest proteins: anti-IL-1 β rabbit polyclonal (1:1000, H-153,
383 SC-7884, Santa Cruz), anti-caspase-1 (p20) mouse monoclonal (1:1000, casper-1, AG-
384 20B-0042, Adipogen), anti-gasdermin D rabbit monoclonal (1:2000, EPR19828,
385 ab209845, Abcam) and anti- β -Actin mouse monoclonal (1:10000, Santa Cruz).
386 Appropriate secondary antibody conjugated with HRP was used at 1:5000 dilution
387 (Sigma) and developed with ECL plus (Amherstan Biosciences) in a ChemiDoc HDR
388 (BioRad). Uncropped Western blots are shown in **Figure 2-source data 1 and 2**. The
389 concentration of IL-1 β , TNF- α and IL-6 in cell supernatants was determined by ELISA
390 following the manufacturer's instructions (R&D Systems). Results were read in a
391 Synergy Mx plate reader (BioTek).

392

393 **Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis.**
394 Total RNA extraction was performed using macrophages or mice tendons dissected as
395 described above. Macrophage total RNA extraction was performed using the RNeasy

396 Mini Kit (Qiagen) following manufacturer's instructions. Total RNA extraction from mice
397 tendons was performed using Qiazol lysis reagent (Qiagen) and samples were
398 homogenized using an Omni THQ homogenizer. After homogenization, samples were
399 incubated 5 min at room temperature and centrifuged at 12000 xg for 15 min at 4°C.
400 After centrifugation, upper phase was collected and one volume of 70% ethanol was
401 added. Samples were loaded in RNeasey Mini Kit columns and total RNA isolation was
402 performed following manufacturer's instructions. In both cases a step with a treatment
403 with 10 U/ μ l DNase I (Qiagen) was added during 30 min. Reverse transcription was
404 performed using iScript cDNA Synthesis kit (BioRad). The mix SYBR Green Premix
405 ExTaq (Takara) was used for quantitative PCR in an iCyclerMyiQ thermocycler (BioRad).
406 Specific primers were purchased from Sigma (KiCqStart SYBR Green Primers) for the
407 detection of the different genes. Relative expression of genes was normalized to the
408 housekeeping gene *Actb* using the $2^{-\Delta Ct}$ method and for the expression in tendon then
409 normalized to mean Ct value of non-treated samples using the $2^{-\Delta\Delta Ct}$ method (value
410 shown in figures). When expression in non-treated samples was below threshold and
411 was non detected (ND), $2^{-\Delta Ct}$ values are shown in the figures. To compare gene
412 expression between wild-type and knock-out mice, the fold change of $2^{-\Delta\Delta Ct}$ values of the
413 knock-out mice was calculated respect the average of the $2^{-\Delta\Delta Ct}$ values of the wild-type
414 mice.

415

416 **Histopathology.** Mice paws were fixed using 4% *p*-formaldehyde (Sigma-Aldrich) for at
417 least 24 h, processed, paraffin-embedded, and sectioned in 4 μ m slides. Hematoxylin
418 and eosin stained slices were initially evaluated in a 0 to 3 qualitative scale, being 0
419 control (healthy tendon) conditions, 1 mild, 2 medium, and 3 severe, for inflammatory
420 infiltrate and tendon cellularity grade (the median value for each of the paws was used
421 as the final value represented in the figures), the number of polymorphonuclear cells was
422 quantified by counting 3 different fields for each sample, attending to nuclear morphology
423 of the cells in adipose tissue next to calcaneal tendon, the number and area of tenocytes

424 nuclei was evaluated using the FIJI macro based on a manual threshold to select nuclei
425 and evaluating the different parameters measured with the “Analyze particles” tool. Sirius
426 red staining was performed in the slides using the Picro Sirius red stain kit (Abcam)
427 following the manufacturer’s instructions and polarized light pictures (**Fig. S5E**) were
428 used to quantify the type of collagen by converting pictures to SHG color and then using
429 the CT-Fire algorithm to calculate width, length, straightness and angle of collagen fibers
430 in these pictures (Liu et al., 2017). Immunohistochemistry with anti-F4/80 rat monoclonal
431 antibody (MCA497GA, BioRad) was used for the quantification of macrophages by
432 counting 3 different fields for each sample, attending to stained cells in adipose tissue
433 next to calcaneal tendon. All slides were examined with a Zeiss Axio Scope AX10
434 microscope with 20x and 40x objectives (Carl Zeiss) and pictures were taken with an
435 AxioCam 506 Color (Carl Zeiss).

436

437 **Statistics.** Statistical analyses were performed using GraphPad Prism 7 (Graph-Pad
438 Software, Inc). A Shapiro-Wilk normality test was initially performed to all groups to
439 decide the analysis type to be used. For two-group comparisons, nonparametric Mann-
440 Whitney *U* test (without making the assumption that values are normally distributed) or
441 the parametric unpaired *t*-test (for normal distributed data) were used to determine the
442 statistical significance. For more than two group comparisons, one-way ANOVA test (for
443 normal distributed data) or nonparametric Krustal-Wallis test (without making the
444 assumption that values are normally distributed) were used to determine the statistical
445 significance. Data are shown as mean values and error bars represent standard error
446 from the number of independent assays indicated in the figure legend, which are also
447 overlaid in the histograms as dot-plotting. *p* value is indicated as **p* <0.05; ***p* <0.01;
448 ****p* <0.001; *****p* <0.0001; *p* >0.05 not significant (*ns*).

449 **Acknowledgments** We thanks M.C. Baños (IMIB-Arrixaca, Murcia, Spain) for technical
450 assistance with molecular and cellular biology, Antonio García Martínez (CESMAR
451 Electromedicina) for electrode development for *in vitro* application of galvanic current,
452 Darío Peñín Franch for helping in the development of the plugin to measure different
453 types of collagen and F. Noguera and M. Martínez (IMIB-Arrixaca, Murcia, Spain) for
454 running Hitachi ion detection system and the members of the Pelegrin's laboratory for
455 comments and suggestions thought the development of this project. We also want to
456 acknowledge the support of the SPF-animal house from IMIB-Arrixaca.

457

458 **Funding:** A.P-F. was supported by MVClinic and Prim. This work was supported by
459 grants to P.P. from *FEDER/Ministerio de Ciencia, Innovación y Universidades – Agencia*
460 *Estatad de Investigación* (grant SAF2017-88276-R and PID2020-116709RB-I00),
461 *Fundación Séneca* (grants 20859/PI/18 and 21081/PDC/19), and the European
462 Research Council (ERC-2013-CoG grant 614578 and ERC-2019-PoC grant 899636).

463

464 **Author contributions**

465 A.P-F., performed all the experimental work; A.P-F., J.A.G-V., C.M.M., P.E-R. performed
466 *in vivo* animal manipulation and histology; A.P-F. and P.P. analyzed the data, interpreted
467 results, conceived the experiments, prepared the figures and paper writing; F.V-G. and
468 F.Mi-M. provided clinical data and conceptual supervision of the study. F.Me-M. and P.P.
469 conceived the project, provided funding and overall supervision of this study.

470

471 **Declaration of interests**

472 F.Mi.-M. and F.V.-G. are employees of MVClinic Institute. A.P-F. contract was supported
473 by MVClinic Institute and Prim. P.P. declares that he is an inventor in a patent filled on
474 March 2020 by the Fundación para la Formación e Investigación Sanitaria de la Región
475 de Murcia (PCT/EP2020/056729) for a method to identify NLRP3-immunocompromised

477 sepsis patients. P.P. is consultant of Glenmark Pharmaceutical. The remaining authors
478 declare no competing interests.

479 **REFERENCES**

- 480 Abat F, Sánchez-Sánchez JL, Martín-Nogueras AM, Calvo-Arenillas JI, Yajeya J,
481 Méndez-Sánchez R, Monllau JC, Gelber PE. 2016. Randomized controlled trial
482 comparing the effectiveness of the ultrasound-guided galvanic electrolysis
483 technique (USGET) versus conventional electro-physiotherapeutic treatment on
484 patellar tendinopathy. *J Exp Orthop* **3**:34. doi:10.1186/s40634-016-0070-4
- 485 Alegre F, Pelegrin P, Feldstein AE. 2017. Inflammasomes in Liver Fibrosis. *Semin*
486 *Liver Dis* **37**. doi:10.1055/s-0037-1601350
- 487 Amores-Iniesta J, Barberà-Cremades M, Martínez CMCM, Pons JAJA, Revilla-Nuin B,
488 Martínez-Alarcón L, Di Virgilio F, Parrilla P, Baroja-Mazo A, Pelegrín P. 2017.
489 Extracellular ATP Activates the NLRP3 Inflammasome and Is an Early Danger
490 Signal of Skin Allograft Rejection. *Cell Rep* **21**:3414–3426.
491 doi:10.1016/j.celrep.2017.11.079
- 492 Baroja-Mazo A, Martín-Sánchez F, Gomez AI, Martínez CM, Amores-Iniesta J,
493 Compan V, Barberà-Cremades M, Yagüe J, Ruiz-Ortiz E, Antón J, Buján S,
494 Couillin I, Brough D, Arostegui JI, Pelegrín P, Martín-Sánchez F, Gomez AI,
495 Martínez CM, Amores-Iniesta J, Compan V, Barberà-Cremades M, Yagüe J, Ruiz-
496 Ortiz E, Antón J, Buján S, Couillin I, Brough D, Arostegui JI, Pelegrin P. 2014. The
497 NLRP3 inflammasome is released as a particulate danger signal that amplifies the
498 inflammatory response. *Nat Immunol* **15**:738–748. doi:10.1038/ni.2919
- 499 Bisset L, Beller E, Jull G, Brooks P, Darnell R, Vicenzino B. 2006. Mobilisation with
500 movement and exercise, corticosteroid injection, or wait and see for tennis elbow:
501 randomised trial. *BMJ* **333**:939. doi:10.1136/bmj.38961.584653.AE
- 502 Borthwick LA, Wynn TA, Fisher AJ. 2013. Cytokine mediated tissue fibrosis. *Biochim*
503 *Biophys Acta - Mol Basis Dis* **1832**:1049–1060. doi:10.1016/j.bbadis.2012.09.014
- 504 Boucher D, Monteleone M, Coll RC, Chen KW, Ross CM, Teo JL, Gomez GA, Holley
505 CL, Bierschenk D, Stacey KJ, Yap AS, Bezbradica JS, Schroder K. 2018.
506 Caspase-1 self-cleavage is an intrinsic mechanism to terminate inflammasome

- 507 activity. *J Exp Med* **215**:827–840. doi:10.1084/jem.20172222
- 508 Broz P, Dixit VM. 2016. Inflammasomes: mechanism of assembly, regulation and
509 signalling. *Nat Rev Immunol* **16**:407–420. doi:10.1038/nri.2016.58
- 510 Broz P, Pelegrín P, Shao F. 2020. The gasdermins, a protein family executing cell
511 death and inflammation. *Nat Rev Immunol* **20**:143–157. doi:10.1038/s41577-019-
512 0228-2
- 513 Bubnov R. 2013. Ultrasound guided injections of Platelets Rich Plasma for muscle
514 injury in professional athletes. Comparative study. *Med Ultrason* **15**:101–105.
515 doi:10.11152/mu.2013.2066.152.rb1vy2
- 516 Chellini F, Tani A, Zecchi-Orlandini S, Sassoli C. 2019. Influence of Platelet-Rich and
517 Platelet-Poor Plasma on endogenous mechanisms of skeletal muscle
518 repair/regeneration. *Int J Mol Sci* **20**:683. doi:10.3390/ijms20030683
- 519 Cocco M, Pellegrini C, Martínez-Banaclocha H, Giorgis M, Marini E, Costale A, Miglio
520 G, Fornai M, Antonioli L, López-Castejón G, Tapia-Abellán A, Angosto D, Hafner-
521 Bratkovič I, Regazzoni L, Blandizzi C, Pelegrín P, Bertinaria M. 2017.
522 Development of an Acrylate Derivative Targeting the NLRP3 Inflammasome for
523 the Treatment of Inflammatory Bowel Disease. *J Med Chem* **60**:3656–3671.
524 doi:10.1021/acs.jmedchem.6b01624
- 525 Coll RC, Robertson AAB, Chae JJ, Higgins SC, Muñoz-Planillo R, Inserra MC, Vetter I,
526 Dungan LS, Monks BG, Stutz A, Croker DE, Butler MS, Haneklaus M, Sutton CE,
527 Núñez G, Latz E, Kastner DL, Mills KHG, Masters SL, Schroder K, Cooper MA,
528 O'Neill LAJ. 2015. A small-molecule inhibitor of the NLRP3 inflammasome for the
529 treatment of inflammatory diseases. *Nat Med* **21**:248–255. doi:10.1038/nm.3806
- 530 Compan V, Baroja-Mazo A, López-Castejón G, Gomez AI, Martínez CM, Angosto D,
531 Montero MT, Herranz AS, Bazán E, Reimers D, Mulero V, Pelegrín P. 2012. Cell
532 Volume Regulation Modulates NLRP3 Inflammasome Activation. *Immunity*
533 **37**:487–500. doi:10.1016/j.immuni.2012.06.013
- 534 Cook JL, Purdam CR. 2009. Is tendon pathology a continuum? A pathology model to

- 535 explain the clinical presentation of load-induced tendinopathy. *Br J Sports Med*
536 **43**:409–416. doi:10.1136/bjism.2008.051193
- 537 Coombes BK, Bisset L, Brooks P, Khan A, Vicenzino B. 2013. Effect of corticosteroid
538 injection, physiotherapy, or both on clinical outcomes in patients with unilateral
539 lateral epicondylalgia: a randomized controlled trial. *JAMA* **309**:461–9.
540 doi:10.1001/jama.2013.129
- 541 Daniels MJD, Rivers-Auty J, Schilling T, Spencer NG, Watremez W, Fasolino V, Booth
542 SJ, White CS, Baldwin AG, Freeman S, Wong R, Latta C, Yu S, Jackson J,
543 Fischer N, Koziel V, Pillot T, Bagnall J, Allan SM, Paszek P, Galea J, Harte MK,
544 Eder C, Lawrence CB, Brough D. 2016. Fenamate NSAIDs inhibit the NLRP3
545 inflammasome and protect against Alzheimer’s disease in rodent models. *Nat*
546 *Commun* **7**. doi:10.1038/ncomms12504
- 547 De-la-Cruz-Torres B, Barrera-García-Martín I, Valera-Garrido F, Minaya-Muñoz F,
548 Romero-Morales C. 2020. Ultrasound-Guided Percutaneous Needle Electrolysis
549 in Dancers with Chronic Soleus Injury: A Randomized Clinical Trial. *Evidence-*
550 *Based Complement Altern Med* **2020**:1–8. doi:10.1155/2020/4156258
- 551 Eming SA, Wynn TA, Martin P. 2017. Inflammation and metabolism in tissue repair and
552 regeneration. *Science (80-)* **356**:1026–1030. doi:10.1126/science.aam7928
- 553 Evavold CL, Ruan J, Tan Y, Xia S, Wu H, Kagan JC. 2018. The pore-forming protein
554 gasdermin D regulates interleukin-1 secretion from living macrophages. *Immunity*
555 **48**:35-44.e6. doi:10.1016/j.immuni.2017.11.013
- 556 Franklin BS, Bossaller L, De Nardo D, Ratter JM, Stutz A, Engels G, Brenker C,
557 Nordhoff M, Mirandola SR, Al-Amoudi A, Mangan MS, Zimmer S, Monks BG,
558 Fricke M, Schmidt RE, Espevik T, Jones B, Jarnicki AG, Hansbro PM, Busto P,
559 Marshak-Rothstein A, Hornemann S, Aguzzi A, Kastenmüller W, Latz E. 2014.
560 The adaptor ASC has extracellular and “prionoid” activities that propagate
561 inflammation. *Nat Immunol* **15**:727–737. doi:10.1038/ni.2913
- 562 García Vidal J, Pelegrín P, Escolar Reina P, Medina i Mirapeix F. 2019. Inflammatory

563 response of two invasive techniques in the mouse with collagenase induced
564 tendinopathy. *Rev Fisioter Invasiva / J Invasive Tech Phys Ther* **02**:080–080.
565 doi:10.1055/s-0039-3401862

566 Gaul S, Leszczynska A, Alegre F, Kaufmann B, Johnson CD, Adams LA, Wree A,
567 Damm G, Seehofer D, Calvente CJ, Povero D, Kisseleva T, Eguchi A, McGeough
568 MD, Hoffman HM, Pelegrin P, Laufs U, Feldstein AE. 2020. Hepatocyte pyroptosis
569 and release of inflammasome particles induce stellate cell activation and liver
570 fibrosis. *J Hepatol* **S0168-8278**:30522–30525. doi:10.1016/j.jhep.2020.07.041

571 Heneka MT, Kummer MP, Stutz A, Delekate A, Schwartz S, Vieira-Saecker A, Griep A,
572 Axt D, Remus A, Tzeng T, Gelpi E, Halle A, Korte M, Latz E, Golenbock DT. 2013.
573 NLRP3 is activated in Alzheimer’s disease and contributes to pathology in
574 APP/PS1 mice. *Nature* **493**:674–678. doi:10.1038/nature11729

575 Li Y, Fu T-M, Lu A, Witt K, Ruan J, Shen C, Wu H. 2018. Cryo-EM structures of ASC
576 and NLRC4 CARD filaments reveal a unified mechanism of nucleation and
577 activation of caspase-1. *Proc Natl Acad Sci U S A* **115**:10845–10852.
578 doi:10.1073/pnas.1810524115

579 Liston A, Masters SL. 2017. Homeostasis-altering molecular processes as
580 mechanisms of inflammasome activation. *Nat Rev Immunol* **17**:208–214.
581 doi:10.1038/nri.2016.151

582 Liu Y, Keikhosravi A, Mehta GS, Drifka CR, Eliceiri KW. 2017. Methods for Quantifying
583 Fibrillar Collagen Alignment. pp. 429–451. doi:10.1007/978-1-4939-7113-8_28

584 Margalef R, Bosque M, Monclús P, Flores P, Minaya-Muñoz F, Valera-Garrido F,
585 Santafé MM. 2020. Percutaneous Application of Galvanic Current in Rodents
586 Reverses Signs of Myofascial Trigger Points. *Evidence-Based Complement Altern*
587 *Med* **2020**:1–9. doi:10.1155/2020/4173218

588 Margalef R, Minaya Muñoz F, Valera Garrido F, Santafe M. 2019. Vasodilation
589 secondary to exposure to galvanic currents. *Rev Fisioter Invasiva / J Invasive*
590 *Tech Phys Ther* **02**:107–107. doi:10.1055/s-0039-3401880

- 591 Martínez-García JJ, Martínez-Banaclocha H, Angosto-Bazarra D, de Torre-Minguela C,
592 Baroja-Mazo A, Alarcón-Vila C, Martínez-Alarcón L, Amores-Iniesta J, Martín-
593 Sánchez F, Ercole GAGA, Martínez CMCM, González-Lisorge A, Fernández-
594 Pacheco J, Martínez-Gil P, Adriouch S, Koch-Nolte F, Luján J, Acosta-Villegas F,
595 Parrilla P, García-Palenciano C, Pelegrin P. 2019. P2X7 receptor induces
596 mitochondrial failure in monocytes and compromises NLRP3 inflammasome
597 activation during sepsis. *Nat Commun* **10**:2711. doi:10.1038/s41467-019-10626-x
- 598 Masters SL, Dunne A, Subramanian SL, Hull RL, Tannahill GM, Sharp FA, Becker C,
599 Franchi L, Yoshihara E, Chen Z, Mullooly N, Mielke LA, Harris J, Coll RC, Mills
600 KHG, Mok KH, Newsholme P, Nunez G, Yodoi J, Kahn SE, Lavelle EC,
601 O’Neill LAJ. 2010. Activation of the NLRP3 inflammasome by islet amyloid
602 polypeptide provides a mechanism for enhanced IL-1 β in type 2 diabetes. *Nat*
603 *Immunol* **11**:897–904. doi:10.1038/ni.1935
- 604 Mayor A, Tardivel A, Martinon F, Pe V, Pétrilli V, Mayor A, Tardivel A, Tschopp J. 2006.
605 Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature*
606 **440**:237–241. doi:10.1038/nature04516
- 607 Medina i Mirapeix F, García Vidal J, Escolar Reina P, Martínez Cáceres C. 2019.
608 Histopathological analysis of the inflammatory response of two invasive
609 techniques in the calcaneal tendon of a mouse. *Rev Fisioter Invasiva / J Invasive*
610 *Tech Phys Ther* **02**:091–091. doi:10.1055/s-0039-3401870
- 611 Medzhitov R. 2008. Origin and physiological roles of inflammation. *Nature* **454**:428–35.
612 doi:10.1038/nature07201
- 613 Muñoz-Planillo R, Kuffa P, Martínez-Colón G, Smith BL, Rajendiran TM, Nunez G,
614 Núñez G, Munoz-Planillo R, Kuffa P, Martinez-Colon G, Smith BL, Rajendiran TM,
615 Nunez G. 2013. K⁺ Efflux Is the Common Trigger of NLRP3 Inflammasome
616 Activation by Bacterial Toxins and Particulate Matter. *Immunity* **38**:1142–1153.
617 doi:10.1016/j.immuni.2013.05.016S1074-7613(13)00243-4 [pii]
- 618 Petrilli V, Papin S, Dostert C, Mayor A, Martinon F, Tschopp J. 2007. Activation of the

- 619 NALP3 inflammasome is triggered by low intracellular potassium concentration.
620 *Cell Death Differ* **14**:1583–1589. doi:10.1038/sj.cdd.4402195
- 621 Próchnicki T, Mangan MS, Latz E. 2016. Recent insights into the molecular
622 mechanisms of the NLRP3 inflammasome activation. *F1000Research* **5**:1415–
623 1469. doi:10.12688/f1000research.8614.1
- 624 Regan W, Wold LE, Coonrad R, Morrey BF. 1992. Microscopic histopathology of
625 chronic refractory lateral epicondylitis. *Am J Sports Med* **20**:746–749.
626 doi:10.1177/036354659202000618
- 627 Rodríguez-Huguet M, Góngora-Rodríguez J, Rodríguez-Huguet P, Ibañez-Vera AJ,
628 Rodríguez-Almagro D, Martín-Valero R, Díaz-Fernández Á, Lomas-Vega R. 2020.
629 Effectiveness of Percutaneous Electrolysis in Supraspinatus Tendinopathy: A
630 Single-Blinded Randomized Controlled Trial. *J Clin Med* **9**:1837.
631 doi:10.3390/jcm9061837
- 632 Rühl S, Shkarina K, Demarco B, Heilig R, Santos JC, Broz P. 2018. ESCRT-dependent
633 membrane repair negatively regulates pyroptosis downstream of GSDMD
634 activation. *Science* **362**:956–960. doi:10.1126/science.aar7607
- 635 Schmidt FI, Lu A, Chen JW, Ruan J, Tang C, Wu H, Ploegh HL. 2016. A single domain
636 antibody fragment that recognizes the adaptor ASC defines the role of ASC
637 domains in inflammasome assembly. *J Exp Med* **213**:771–90.
638 doi:10.1084/jem.20151790
- 639 Schroder K, Tschopp J, Couillin I, Pétrilli V, Martinon F, Schroder K, Tschopp J,
640 Couillin I, Petrilli V, Martinon F. 2011. The Inflammasomes. *Cell* **140**:821–832.
641 doi:10.1016/j.cell.2010.01.040
- 642 Shook BA, Wasko RR, Rivera-Gonzalez GC, Salazar-Gatzimas E, López-Giráldez F,
643 Dash BC, Muñoz-Rojas AR, Aultman KD, Zwick RK, Lei V, Arbiser JL, Miller-
644 Jensen K, Clark DA, Hsia HC, Horsley V. 2018. Myofibroblast proliferation and
645 heterogeneity are supported by macrophages during skin repair. *Science (80-)*
646 **362**:eaar2971. doi:10.1126/science.aar2971

- 647 Soslowsky LJ, Thomopoulos S, Tun S, Flanagan CL, Keefer CC, Mastaw J, Carpenter
648 JE. n.d. Overuse activity injures the supraspinatus tendon in an animal model: a
649 histologic and biomechanical study. *J Shoulder Elb Surg* **9**:79–84.
- 650 Taabazuing CY, Okondo MC, Bachovchin DA. 2017. Pyroptosis and Apoptosis
651 Pathways Engage in Bidirectional Crosstalk in Monocytes and Macrophages. *Cell*
652 *Chem Biol* **24**:507-514.e4. doi:10.1016/j.chembiol.2017.03.009
- 653 Tapia-Abellán A, Angosto-Bazarra D, Martínez-Banaclocha H, de Torre-Minguela C,
654 Cerón-Carrasco JPJP, Pérez-Sánchez H, Arostegui JIJ, Pelegrin P. 2019.
655 MCC950 closes the active conformation of NLRP3 to an inactive state. *Nat Chem*
656 *Biol* **15**:560–564. doi:10.1038/s41589-019-0278-6
- 657 Valera-Garrido F, Jiménez-Rubio S, Minaya-Muñoz F, Estévez-Rodríguez JL,
658 Navandar A. 2020. Ultrasound-Guided Percutaneous Needle Electrolysis and
659 Rehab and Reconditioning Program for Rectus Femoris Muscle Injuries: A Cohort
660 Study with Professional Soccer Players and a 20-Week Follow-Up. *Appl Sci*
661 **10**:7912. doi:10.3390/app10217912
- 662 Valera-Garrido F, Minaya-Muñoz F. 2019. Fundamentos y principios de la electrolysis
663 percutánea musculoesquelética In: Valera-Garrido F, Minaya-Muñoz F, editors.
664 Fisioterapia Invasiva. Barcelona: Elsevier. pp. 390–391.
- 665 Valera-Garrido F, Minaya-Muñoz F. 2016. Aplicaciones clínicas de la electrolysis
666 percutánea In: Valera-Garrido F, Minaya-Muñoz F, editors. Fisioterapia Invasiva.
667 Barcelona: Elsevier. p. 425.
- 668 Valera-Garrido F, Minaya-Muñoz F, Medina-Mirapeix F. 2014. Ultrasound-Guided
669 Percutaneous Needle Electrolysis in Chronic Lateral Epicondylitis: Short-Term
670 and Long-Term Results. *Acupunct Med* **32**:446–454. doi:10.1136/acupmed-2014-
671 010619
- 672 Valera-Garrido F, Minaya-Muñoz F, Sánchez-Ibáñez JM, García-Palencia P,
673 Valderrama-Canales F, Medina-Mirapeix F, Polidori F. 2013. Comparison of the
674 acute inflammatory response and proliferation of dry needling and electrolysis

675 percutaneous intratissue (EPI) in healthy rat achilles tendons. *Br J Sports Med*
676 **47**:e2.52-e2. doi:10.1136/bjsports-2013-092459.56
677 Wynn T. 2008. Cellular and molecular mechanisms of fibrosis. *J Pathol* **214**:199–210.
678 doi:10.1002/path.2277
679

680 **FIGURE LEGENDS**

681

682 **Figure 1. Galvanic current increases the M1 phenotype of macrophages.**

683 (A) Quantitative PCR for M1 genes *Cox2*, *Il6*, *Il1b* and *Tnfa* expression from mouse bone
684 marrow derived macrophages (BMDMs) treated for 2 h with LPS (1 μ g/ml) or 4 h with IL-
685 4 (20 ng/ μ l) as indicated and then 2 impacts of 12 mA of galvanic current for 6 seconds
686 was applied. Cells were then further cultured for 6 h before analysis. Center values
687 represent the mean and error bars represent s.e.m.; $n= 5-10$ samples of 5 independent
688 experiments; for *Cox2* unpaired *t*-test except in the two first columns where Mann-Whitney
689 test were performed, for *Il6* Mann-Whitney test except in LPS vs LPS+galvanic current
690 comparison where unpaired *t*-test were performed, for *Il1b* Mann-Whitney test, for *Tnfa*
691 unpaired *t*-test except in LPS vs LPS+galvanic current comparison where Mann-Whitney
692 were performed, *** $p<0.0005$, ** $p<0.005$, * $p<0.05$, and *ns* $p>0.05$.

693 (B) Quantitative PCR for M2 genes *Arg1*, *Fizz1* and *Mrc1* expression from BMDMs
694 treated as in (A). Center values represent the mean and error bars represent s.e.m.; $n=$
695 3-10 samples of 5 independent experiments; unpaired *t*-test except LPS vs
696 LPS+galvanic current comparison where Mann-Whitney test were performed,
697 *** $p<0.0005$, * $p<0.05$, and *ns* $p>0.05$.

698 (C) IL-6, TNF- α and IL-1 β release from BMDMs treated as in (A) but different intensities
699 of galvanic current (3, 6, 12 mA) were applied. Center values represent the mean and
700 error bars represent s.e.m.; $n= 2$ for untreated cells and $n= 4-6$ for treatment groups from
701 4 independent experiments; one-way ANOVA were performed comparing treated groups
702 with control group, **** $p<0.0001$, * $p<0.05$, and *ns* $p>0.05$.

703

704

705

706 **Figure 2. IL-1 β release induced by galvanic current is dependent on the NLRP3**

707 **inflammasome.**

708 (A) IL-1 β release from wild type, *Casp1/11*^{-/-}, *Pycard*^{-/-} and *Nlrp3*^{-/-} mouse bone marrow
709 derived macrophages (BMDMs) treated for 2 h with LPS (1 μ g/ml) and then 2 impacts of
710 different intensities of galvanic current (3, 6, 12 mA) for 6 seconds was applied. Cells
711 were then further cultured for 6 h before cytokine measurement in supernatant. Center
712 values represent the mean and error bars represent s.e.m.; $n= 6-16$ samples of 10
713 independent experiments; LPS vs LPS+galvanic current in wild-type and wild-type vs
714 *Nlrp3*^{-/-} unpaired *t*-test, wild-type vs *Casp1/11*^{-/-} and wild-type vs *Pycard*^{-/-} Mann-Whitney
715 test, ** $p<0.005$.

716 (B) IL-1 β release from wild type BMDMs treated as in A but applying the NLRP3
717 specific inhibitor MCC950 (10 μ M) 30 min before the galvanic current application and
718 during the last 6 h of culture. As a control, cells were treated with nigericin (1,5 μ M)
719 instead galvanic current application. Center values represent the mean and error bars
720 represent s.e.m.; $n= 5-10$ samples of 5 independent experiments; nigericin vs
721 nigericin+MCC950 Mann-Whitney test, galvanic current vs galvanic current+MCC950
722 unpaired *t*-test, **** $p<0.0001$ and *** $p<0.0005$.

723 (C) IL-1 β release from wild type BMDMs treated as in A but applying a buffer with 40
724 mM of KCl (high K⁺ buffer) during the last 6 h of culture. As controls, cells were treated
725 with nigericin (1,5 μ M) or *Clostridium difficile* toxin B (1 μ g/ml) instead galvanic current
726 application. Center values represent the mean and error bars represent s.e.m.; $n= 3-12$
727 samples of 4 independent experiments; unpaired *t*-test, **** $p<0.0001$, ** $p<0.005$ and *ns*
728 $p>0.05$.

729 (D) Immunoblot of cell extract and supernatants for caspase-1, IL-1 β , GSDMD and β -
730 actin from wild type BMDMs treated as in B, but with 8 impacts . Representative of $n= 2$
731 independent experiments.

732

733 **Figure 3. Galvanic current does not induce inflammasome-mediated pyroptosis.**

734 (A) Extracellular amount of LDH from mouse bone marrow derived macrophages
735 (BMDMs) treated for 2 h with LPS (1 μ g/ml) and then 2 or 8 impacts of different intensities
736 of galvanic current (3, 6, 12 mA) for 6 or 12 seconds were applied as indicated. Cells
737 were then further cultured for 6 h before LDH determination in supernatant. Center
738 values represent the mean and error bars represent s.e.m.; $n= 3-4$ samples of 6
739 independent experiments; Kruskal-Wallis test to compare LPS with increasing intensities
740 of galvanic current, LPS vs LPS+ galvanic current (12mA-12s)x2 unpaired t -test, LPS vs
741 LPS+ galvanic current (12mA-6s)x8, **** $p<0.0001$, *** $p<0.0005$ and * $p<0.05$.

742 (B) Extracellular amount of LDH from wild type, *Nlrp3*^{-/-}, *Pycard*^{-/-} and *Casp1/11*^{-/-} mouse
743 BMDMs treated as in A. Center values represent the mean and error bars represent
744 s.e.m.; $n= 6-17$ samples of 12 independent experiments; unpaired t -test except for
745 *Casp1/11*^{-/-} comparison, *** $p<0.0005$ and * $p<0.05$.

746 (C) IL-1 β release from wild type BMDMs treated as in A, but applying the NLRP3
747 specific inhibitor MCC950 (10 μ M) during the last 6 h of culture. Center values represent
748 the mean and error bars represent s.e.m.; $n= 6-10$ samples of 5 independent
749 experiments; unpaired t -test, **** $p<0.0001$ and * $p<0.005$.

750 (D) Extracellular amount of LDH from wild type BMDMs treated as in C. Center values
751 represent the mean and error bars represent s.e.m.; $n= 5-10$ samples of 5 independent
752 experiments; unpaired t -test, $ns p>0.005$.

753 (E) Kinetic of Yo-Pro-1 uptake (upper panel) or slope of the uptake (lower panel) in wild
754 type BMDMs treated for 2 h with LPS (1 μ g/ml) and then with different intensities of
755 galvanic current (as indicated) or with the detergent triton X-100 (1 %) during 3.5 h.
756 Center values represent the mean and error bars represent s.e.m.; $n= 3-6$ of 3
757 independent experiments; Kruskal-Wallis test, *** $p<0.0005$, ** $p<0.005$ and $ns p>0.05$.

758 (F) Kinetic of Yo-Pro-1 uptake (upper panel) or slope of the uptake (lower panel) in wild
759 type or *Pycard*^{-/-} BMDMs treated as in E, but when indicated the NLRP3 specific inhibitor

760 MCC950 (10 μ M) was added after galvanic current application. Center values represent
761 the mean and error bars represent s.e.m.; $n= 3-6$ samples of 3 independent experiments;
762 unpaired t -test, ** $p<0.005$, * $p<0.05$ and $ns p>0.05$.

763

764 **Figure 4. Galvanic current induces polymorphonuclear and macrophage infiltrate**
765 **in the calcaneal tendon of mice.**

766 (A) Representative hematoxylin and eosin images of wild type mice calcaneal tendon
767 after 3 days application of 3 punctures with needle (needling, green) or 3 impacts of 3
768 mA for 3 sec (blue). Scale bar: 50 μ m. Magnification show the presence of
769 polymorphonuclear cells (arrowheads).

770 (B) Quantification of polymorphonuclear cells per field of view of calcaneal tendon
771 sections treated and stained as described in A. Center values represent the mean and
772 error bars represent s.e.m.; $n= 7-8$ independent animals; unpaired t -test, * $p<0.005$.

773 (C) Representative immunostaining images for the macrophage marker F4/80 from the
774 calcaneal tendon of wild type mice treated as described in A. Scale bar: 50 μ m.
775 Magnification show the presence of F4/80 positive cells (arrowheads).

776 (D) Quantification of F4/80 positive cells per field of view of calcaneal tendon sections
777 treated and stained as described in C. Center values represent the mean and error bars
778 represent s.e.m.; $n= 8$ independent animals; Mann-Whitney test, * $p<0.005$.

779

780 **Figure 5. Galvanic current induces proinflammatory cytokine expression in the**
781 **calcaneal tendon of mice.**

782 (A,B) Quantitative PCR for the indicated genes normalized to *Actb* in the calcaneal
783 tendon of wild type mice after 3 days application of 3 punctures with needle (needling,
784 green) or 3 impacts of 3 mA for 3 sec (blue), and compared to the expression of genes
785 in non-treated tendons. Center values represent the mean and error bars represent
786 s.e.m.; $n= 4-12$ independent animals; for *Il6*, *Nlrp3*, *Pycard* and *Gsdmd* unpaired t -test,

787 for *Il1b* untreated vs galvanic current Mann-Whitney and puncture vs galvanic current
788 unpaired *t*-test, for *Cxcl10*, *Il1rn* and *Casp1/11* Mann-Whitney test, for *Il1a* one sample
789 Wilcoxon test (ND: non detected), ****p*<0.0005, ***p*<0.005, **p*<0.05 and *ns p*>0.05.

790

791 **Figure 6. Inflammatory response in the calcaneal tendon of *Nlrp3*^{-/-} mice after**
792 **galvanic current application.**

793 **(A-C)** Quantitative PCR for the indicated genes in the calcaneal tendons of *Nlrp3*^{-/-} mice
794 (calculated as 2^{-ΔΔCt}) normalized to the expression in wild type (calculated as 2^{-ΔΔCt}) after
795 3 days application of 3 impacts of 3 mA for 3 sec. Center values represent the mean and
796 error bars represent s.e.m.; *n*= 3-12 independent animals; for *Il1b*, *Nlrp3*, *Pycard*,
797 *Casp1/11* and *Gsdmd* unpaired *t*-test, for *Il1rn* and *Cxcl10* Mann-whitney test, **p*<0.05
798 and *ns p*>0.05.

799 **(D)** Quantification of polymorphonuclear (top) and F4/80 positive cells (bottom) per field
800 of view from wild type and *Nlrp3*^{-/-} mice calcaneal tendon treated as in A. Center values
801 represent the mean and error bars represent s.e.m.; *n*= 6-8 independent animals;
802 unpaired *t*-test, **p*<0.05 and *ns p*>0.05. representative hematoxylin and eosin images
803 (top) and F4/80 immunostaining (bottom) of calcaneal tendon quantified. Scale bar: 50
804 μm. Magnification show the presence of polymorphonuclear (top) or F4/80 cells (bottom)
805 denoted by arrowheads.

806

807 **Figure 7. Galvanic current increase of type I collagen via NLRP3 inflammasome.**

808 **(A)** Ultrasound scanning of the right elbow of a patient of with lateral epicondylalgia for
809 6 months of evolution, with pain an functional impairment. Baseline represent the image
810 before galvanic current application. Discharge, represent the image after 4 sessions of
811 percutaneous electrolysis with an intensity of 3 mA of galvanic current application for 3
812 sec, 3 times (3:3:3). 6 w, represent the image after 6 weeks of the last session of
813 percutaneous electrolysis.

814 **(B)** Quantitative PCR for *Tgfb1* in the calcaneal tendons of *Nlrp3^{-/-}* mice (calculated as
815 $2^{-\Delta\Delta Ct}$) normalized to the expression in wild type (calculated as $2^{-\Delta\Delta Ct}$) after 3 days
816 application of 3 impacts of 3 mA for 3 sec. Center values represent the mean and error
817 bars represent s.e.m.; $n= 6-12$ independent animals; Mann-Whitney test, $***p<0.0005$.
818 **(C,D)** Quantification of the collagen type I and III in calcaneal tendon sections stained
819 with picosirius red from wild type (C,D) and *Nlrp3^{-/-}* (D) mice after 3, 7 or 14 days (C) or
820 3 days (D) application of punctures with needle (needling, green) or 3 impacts of 3 mA
821 for 3 sec (blue), or in treated tendons (white). Center values represent the mean and
822 error bars represent s.e.m.; $n= 3-12$ independent animals; for 3 days and panel D
823 unpaired *t*-test, for 7 days non-treated vs needling Mann-Whitney test and non-treated
824 vs percutaneous electrolysis unpaired *t*-test, for 14 days untreated vs needling unpaired
825 *t*-test and untreated vs percutaneous electrolysis Mann-Whitney test, $**p<0.005$, $*p<0.05$
826 and *ns* $p>0.05$.













