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## **Cav1.4 calcium channels control cytokine production by human peripheral Th17 cells and psoriatic skin-infiltrating T cells**

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1 **Ca<sub>v</sub>1.4 calcium channels control cytokine production by human peripheral Th17-cells**  
2 **and psoriatic skin-infiltrating T cells**

3  
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29

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31 3918 words (Introduction through Discussion only).

32 **Abstract**

33 **Background:** Type-17 inflammation characterizes psoriasis, a chronic skin disease. As several  
34 inflammatory cytokines contribute to psoriasis pathogenesis, inhibiting the simultaneous  
35 production of these cytokines in Th17-cells may be beneficial in psoriasis. We found that  
36 Ca<sub>v</sub>1.4, encoded by *CACNA1F*, was the only Ca<sub>v</sub>1 calcium channel expressed in Th17-cells.

37 **Objective:** We investigated the role of Ca<sub>v</sub>1.4 expression in early Th17-activation events and  
38 effector functions, as well as its association with Th17 signature genes in lesional psoriatic (LP)  
39 skins. **Methods:** Transcriptional gene signatures associated with *CACNA1F* expression were  
40 examined in LP skins by RT-PCR and *in situ* hybridization. Ca<sub>v</sub>1 inhibitor and/or shRNA  
41 lentivectors were used to assess the contribution of Ca<sub>v</sub>1.4 on Th17 activation and effector  
42 functions in a 3D skin reconstruction model. **Results:** *CACNA1F* expression correlated with  
43 inflammatory cytokine expression that characterizes LP skins and was preferentially associated  
44 with *RORC* expression in CD4<sup>+</sup> and CD4<sup>-</sup> cells from LP biopsies. Nicardipine, a Ca<sub>v</sub>1 channel  
45 antagonist, markedly reduced inflammatory cytokine production by Th17-cells from blood or  
46 LP skin. This was associated with decreased TCR-induced early calcium events at cell  
47 membrane and proximal signaling events. The knockdown of Ca<sub>v</sub>1.4 in Th17-cells impaired  
48 cytokine production. Finally, Ca<sub>v</sub>1 inhibition reduced the expression of the keratinocyte genes  
49 characteristic of Th17-mediated psoriasis inflammation in human skin equivalents.  
50 **Conclusion:** Ca<sub>v</sub>1.4 channels promote Th17-cell functions both at the periphery and in  
51 inflammatory psoriatic skin. **Clinical implication:** These data open a new field of therapy based  
52 on the development of drugs that inhibit Ca<sub>v</sub>1.4 calcium channels in the treatment of psoriasis.

53 **Capsule Summary**

54  $Ca_v1.4$  calcium channels selective for Th17 among effector T-cells are detected in skin psoriasis  
55 lesions and their inhibition reduces inflammatory cytokine production, opening a new field in  
56 psoriasis treatment.

57

58 **Key words**

59 Psoriasis, calcium channels,  $Ca_v1.4$ , inflammatory cytokine, Th17 cells, TCR activation

**60 Abbreviations**

61 AD: atopic dermatitis

62 Ca<sup>2+</sup>: calcium

63 CsA: ciclosporin A

64 DC: dendritic cells

65 ECE: Elementary calcium events

66 GMFI: geometric mean fluorescence intensity

67 IHF: immunohistofluorescence

68 ISH: in situ hybridization

69 HD: healthy donor

70 LP: lesional psoriasis skin

71 NLP: non-lesional psoriasis skin

72 PBMC: peripheral blood mononuclear cell

73 PP: psoriasis patient

74 SE: skin equivalent

## 75 **Introduction**

76 Psoriasis is a chronic inflammatory skin disease frequently associated with comorbidities,  
77 including arthritis, cardiometabolic disease, and a high emotional burden. It affects 2-3% of the  
78 world's population. The skin lesions present as erythematous scaly patches characterized by  
79 keratinocyte hyperproliferation and infiltration of the epidermis and dermis by inflammatory  
80 cells<sup>1, 2</sup>. Psoriasis results from the abnormal dialog between innate and adaptive immune cells,  
81 with an important role for T-helper 17 (Th17) cells<sup>3</sup>. Skin injury causes cell death and the  
82 release of self DNA-anti-microbial products such as LL37, which can activate plasmacytoid  
83 dendritic cells (DC) in the skin, resulting in interferon  $\alpha$  (IFN $\alpha$ ) production and DC activation.  
84 Activated DCs migrate into the draining lymph node cells, where they polarize a population of  
85 self-reactive CD4<sup>+</sup> T lymphocytes producing interleukin (IL) 17, IL22, IFN $\gamma$  and tumor necrosis  
86 factor  $\alpha$  (TNF $\alpha$ ) through the secretion of IL23 mainly and IL12<sup>4</sup>. These effector T-cells are  
87 recruited into the skin and produce inflammatory cytokines that induce keratinocyte  
88 proliferation and promote a positive feedback loop between the epithelial and immune cells,  
89 resulting in sustained inflammation<sup>3, 5</sup>.

90 In accordance with this pathophysiological scheme, targeting the TNF $\alpha$ /IL23/IL17  
91 cytokine axis is effective in the treatment of psoriasis<sup>6-8</sup>. Although neutralizing anti-cytokine  
92 antibodies are protective in psoriasis, they present some disadvantages in terms of cost,  
93 administration route, and potential adverse events<sup>9, 10</sup>. As Th17-derived cytokines are key  
94 effectors of psoriasis pathogenesis<sup>11</sup>, developing new therapies to reduce their productions  
95 could be beneficial in psoriasis.

96 In this context, we focused on the singularities of signaling pathways that control  
97 cytokine production in T-helper cells. We previously reported the discriminative expression of  
98 Ca<sub>v</sub>1 calcium (Ca<sup>2+</sup>) channel family members in different T-cell populations<sup>12-14</sup>. Ca<sub>v</sub>1 channels  
99 are a multi-protein complex composed of a pore-forming  $\alpha$ 1 subunit encoded by four genes

100 (*CACNAIS*, *CACNAIC*, *CACNAID* and *CACNAIF* encoding for the  $Ca_v1.1$  to  $Ca_v1.4$  channels,  
101 respectively), and auxiliary subunits  $\beta$  and  $\alpha2-\delta$  (each encoded by four genes)<sup>15</sup>. In T  
102 lymphocytes,  $Ca_v1$  expression is plastic depending on their activation and differentiation  
103 status<sup>13, 14, 16</sup>. The  $Ca_v1.2$  and  $Ca_v1.3$  channels are selectively expressed in mouse and human  
104 Th2-cells<sup>12-14</sup> and  $Ca_v1.4$  is the only  $Ca_v1$  calcium channel to be expressed in Th17 cells from  
105 the peripheral blood of healthy donors (HD)<sup>14</sup>. Nicardipine, a  $Ca_v1$  channel antagonist,  
106 decreased TCR-induced IL17A production by human Th17 (CCR6<sup>+</sup>) cells<sup>14</sup>.

107 In the present study, we highlighted the presence of  $Ca_v1.4$  calcium channels in ROR $\gamma$ t<sup>+</sup>  
108 CD4<sup>+</sup> T cells from lesional psoriatic (LP) skin biopsies.  $Ca_v1$  inhibition dampened  
109 inflammatory cytokine production by Th17, regardless of their origin (blood or LP).  $Ca_v1$   
110 channels acted early during Th17 activation contributing to the elementary  $Ca^{2+}$  events (ECE)  
111 preceding the global  $Ca^{2+}$  response. Finally, the  $Ca_v1$  blocker, nicardipine was beneficial in a  
112 3D model of psoriasis-like reconstructed skin induced by the infiltration of activated Th17-  
113 cells.



## 114 **Methods**

115

### 116 **Healthy donors and patients**

117 This study complied with the ethical principles of the Declaration of Helsinki and with  
118 applicable French regulations. All human participants provided written informed consent.  
119 PBMCs from blood of anonymous, healthy donors (HD, 18 to 65 year-old male) were from the  
120 Toulouse blood transfusion center (Etablissement Français du Sang). Study (n°2016-A00863-  
121 48) from psoriasis patients (PP) and atopic dermatitis (AD) patients were accepted by the  
122 Committee for the Protection of Persons in the South-West and Overseas France III (Bordeaux)  
123 and The National Agency for the Safety of Medicines and Health Products (ANSM). For more  
124 details see the Online Repository.

125

### 126 **Th17 cell sorting and expansion**

127 From PBMC, CD4<sup>+</sup>CXCR3<sup>-</sup> T cells were enriched using EasySep™ Human Th17 Cell  
128 Enrichment Kit (STEMCELL Technologies). Then, CD4<sup>+</sup>CD45RA<sup>-</sup>CCR6<sup>+</sup>CXCR3<sup>-</sup> (Th17)  
129 cells were cell sorted on a FACS Aria cell sorter (BD Biosciences). Cells (0.2x10<sup>6</sup>) were  
130 expanded during 14 days with ImmunoCult™ Human CD3/CD28/CD2 T Cell Activator  
131 (STEMCELL) in Immunocult-XF (STEMCELL) medium containing IL2 (5 ng/mL), anti-IFN $\gamma$   
132 antibody (5  $\mu$ g/ml, B27, BD Biosciences, to limit Th1 bias) and etanercept (10 $\mu$ g/ml, to favor  
133 cell proliferation).

134

### 135 **Total internal reflection fluorescence (TIRF) microscopy**

136 Cells were loaded with 5  $\mu$ M Fluo-5-AM in culture medium containing 5% heat-inactivated  
137 FBS and 100  $\mu$ M EGTA-AM (buffering Ca<sup>2+</sup> at the channel mouth) for 30 minutes at 37°C.  
138 After washing and plating cells in medium, we recorded elementary calcium events (ECE) at

139 the cell membrane every 2 ms before and after stimulation with ImmunoCult™ Human  
140 CD3/CD28 T Cell Activator in presence or not of 10 µg/ml of nicardipine. We analyzed  
141 spatiotemporal fluorescence changes expressed as F/F<sub>0</sub>, the number of opened channels and the  
142 ECE opening frequencies. Complete procedures are given in the Online Repository.

143

144 **Cytometry, calcium measurements, ELISA, western-blotting, qPCR,**  
145 **immunohistofluorescence (IHF) and in situ hybridization (ISH) on skin sections, Th17-**  
146 **cell transduction with shRNA, T-cell recovery and amplification from LP biopsies, the**  
147 **generation of human inflammatory psoriasis skin equivalents populated with Th17 cells,**  
148 **murine imiquimod-induced psoriasis like model and statistical analyses were detailed in**  
149 **the Online Repository.**

150 **Results**

151

152 ***CACNAIF* expression, encoding the Ca<sub>v</sub>1.4 calcium channel, expression is higher in**  
153 **lesional psoriatic compared with non-lesional psoriatic or atopic dermatitis biopsies**

154 We first characterized gene expression profile in lesional (LP) versus non-lesional (NLP)  
155 psoriatic biopsies and for comparison we included lesional atopic dermatitis (AD) biopsies. As  
156 expected, the LP biopsies were characterized by the upregulation of genes known to have a  
157 pathological role in psoriasis, including genes encoding the cytokines related to the IL17/IL22  
158 axis, (*IL17A*, *IL26*, *IL23A*, *IFNG*, *CSF2*, *IL22*, *IL6*), chemokines (*CCL20* and *CXCL8*) involved  
159 in both Th1/ Th17 lymphocytes and neutrophil recruitment, and skin barrier function (elafin  
160 (*PI3*),  $\beta$ -defensin2 (*DEFB4B*), late cornified envelop (*LCE3A*), psoriasin (*S100A7*), *MPR6*  
161 (*S100A8*), mKi67, keratin16 (*KRT16*) and involucrin (*IVL*)). These genes were significantly  
162 more expressed in LP than in NLP skins (**Figure 1A R1 and see Table E1 in the Online**  
163 **repository**) and in AD biopsies for most of them (**Figure 1A R1 and see Table E2 in the**  
164 **Online repository**). The AD biopsies were characterized by the upregulation of genes involved  
165 in type 2-cell function/recruitment (*IL13*, *CCL26*, carbonic anhydrase 2 (*CA2*) and neural EGFL  
166 like 2 (*NELL2*)), (**Figure 1A R1 and see Table E2 in the Online repository**). Hierarchical  
167 clustering based on gene expression levels showed two clusters with respect to inflammation  
168 status: one containing the LP and AD biopsies and the other the NLP biopsies and two AD  
169 samples with low levels of anti-microbial peptide expression.

170 We observed significant *CACNAIF* overexpression in the LP versus NLP or AD skin  
171 (four and five-fold, respectively), (**Figure 1B R1**). The cytokines (*IL17A*, *IFNG*, and *IL26*)  
172 expression were significantly upregulated in LP compared with NLP and AD samples (**Figure**  
173 **E1A R1 in the Online repository**), and the expression of *CACNAIF* positively correlated with  
174 these cytokines (**Figure 1C R1**), but not in the different disease-states taken separately.

175

176 **In LP skin, Ca<sub>v</sub>1.4 calcium channel is preferentially detected in RORC<sup>+</sup> compared with**  
177 **RORC<sup>-</sup> cells**

178 Using *in situ* hybridization (ISH), we next analyzed whether Ca<sub>v</sub>1.4 expressing RORγt<sup>+</sup> CD4<sup>+</sup>  
179 (Th17) lymphocytes can be detected in LP skins. CD4<sup>+</sup> T cells co-expressing *CACNA1F* and  
180 *RORC* (encoding for the transcription factor RORγt) mRNAs were detected in all LPs (**Figure**  
181 **1D R1**). Among CD4<sup>+</sup> cells, which represented 21% (20.7 ± 9.1) of nucleated cells, 29% (28.5  
182 ± 10.1) were RORC<sup>+</sup> (**Figure 1D and 1E R1**). *CACNA1F* was found in 47% (47.2 ± 18.9) of  
183 RORC<sup>+</sup> CD4<sup>+</sup> cells versus 28% (27.8 ± 17.3) of RORC<sup>-</sup> CD4<sup>+</sup> cells (**Figure 1E R1**). Thus, there  
184 was a strong enrichment in *CACNA1F*<sup>+</sup> cells among RORC<sup>+</sup> CD4<sup>+</sup> cells compared with RORC<sup>-</sup>  
185 CD4<sup>+</sup> (**Figure E1B R1 in the Online repository**). Among the CD4<sup>-</sup> cells, 19% (19.2 ± 10)  
186 were RORC<sup>+</sup>, with 40% (39.8 ± 15.6) being *CACNA1F*<sup>+</sup> versus 21% (21.2 ± 10) of *CACNA1F*<sup>+</sup>  
187 in RORC<sup>-</sup> (**Figure 1E R1**). As in the CD4<sup>+</sup> cells, there was an enrichment in *CACNA1F*<sup>+</sup> cells  
188 among RORC<sup>+</sup> CD4<sup>-</sup> cells compared with RORC<sup>-</sup> CD4<sup>-</sup> (**Figure E1C R1 in the Online**  
189 **repository**). There was a positive correlation between the percentage of CD4<sup>+</sup> RORC<sup>+</sup>  
190 *CACNA1F*<sup>+</sup> and CD4<sup>-</sup> RORC<sup>+</sup> *CACNA1F*<sup>+</sup> (**Figure E1D R1 in the Online repository**  
191 ), suggesting a robust association between *CACNA1F* and *RORC* gene expression within the  
192 same cells.

193 At the protein level, we confirmed the detection of cells co-labeled with anti-Ca<sub>v</sub>1.4,  
194 anti-CD4 and anti-RORγt antibodies in the dermis (**Figure 1F R1**). Notably, higher numbers  
195 of Ca<sub>v</sub>1.4<sup>+</sup> RORγt<sup>+</sup> CD4<sup>+</sup> cells were associated with T cell clustering in LP skins (**Figure 1F**  
196 **R1 and New Figure 1G R1**). Ca<sub>v</sub>1.4 staining was not detected in the epidermis.

197

198 **TCR-induced cytokine production by T cells infiltrating psoriatic lesions depends on Ca<sub>v</sub>1**  
199 **channels**

200 To investigate the role of  $Ca_v1.4$  in LP skins, infiltrating T-cells were recovered and amplified  
201 by stimulation with anti-CD3/CD28/CD2 in the presence of IL2 and IL23, as previously  
202 described<sup>17</sup>, (**Figure 2A**). Fourteen days later, all cells were  $CD3^+$ , dividing nearly equally into  
203  $CD4^+$  and  $CD8^+$  T cells (**Figure 2B**). We defined Th1, Th1/Th17, and Th17 cells as  
204  $CD4^+CD45RA^-$  cells that were  $CXCR3^+CCR6^-$ ,  $CXCR3^+CCR6^+$  and  $CXCR3^-CCR6^+$   
205 respectively according to previous works<sup>18</sup>. Expanded skin T-cells were composed of Th17  
206 (30%), Th1/Th17 (30%) and Th1 (20%) cells (**Figure 2C**). Upon anti-CD3/CD28 stimulation,  
207 T cells produced IL17A, IL22,  $IFN\gamma$  and  $TNF\alpha$  (**Figure 2D**). Nicardipine, a  $Ca_v1$  channel  
208 antagonist (used at 10  $\mu\text{g/ml}$ , a concentration that inhibits  $Ca_v1$  function in human Th2 cells<sup>14</sup>)  
209 reduced the production of IL17A (36%), IL22 (35%),  $IFN\gamma$  (27%) and  $TNF\alpha$  (53%) upon TCR  
210 stimulation (**Figure 2D** and **Figure E2A in the Online repository**). Cytokine gene  
211 transcription depended on  $Ca_v1$  channels, as nicardipine inhibited the TCR-induced  
212 transcription of *IL17A* (52%), *IL22* (57%) and at a lower level *IFNG* (37%) and *TNFA* (27%)  
213 (**Figure 2E** and see **Figure E2B in the Online repository**). In addition, *IL17F* and *CSF2*  
214 transcript expressions induced by TCR-stimulation were reduced by nicardipine (by 54% and  
215 42% respectively, **Figure 2E** and **Figure E2B in the Online repository**). Altogether, these  
216 data suggest that TCR-induced cytokine expression and production were partially controlled by  
217  $Ca_v1$  calcium channels in LP-infiltrating T-cells.

218

### 219 **TCR-induced cytokine transcription and production by Th17 cells expanded from healthy** 220 **and psoriatic patient PBMCs are also partially controlled by $Ca_v1$ channel activation**

221 Based on chemokine receptor expression, we showed that the only population to be  
222 significantly increased in PP compared with HD was the Th17 cells (see **Figure E3 in the**  
223 **Online repository**). To investigate the role of  $Ca_v1.4$  in Th17 cell functions of PP and HD, we  
224 set up a protocol of amplification to obtain sufficient Th17 cells from the peripheral blood. We

225 sorted the Th17 cells ( $CD4^+CD45RA^-CCR6^+CXCR3^-$ ) and expanded them by anti-  
226 CD3/CD28/CD2 stimulation for 14-21 days in the presence of IL2, anti-IFN $\gamma$  and anti-TNF $\alpha$   
227 antibodies. The expanded Th17 cells still displayed their ability to produce IL17A, IL22, and  
228 IFN $\gamma$  (although at a lower level for the two latter cytokines) when compared with *ex vivo*  
229 production (see **Figure E4A in the Online repository**). The expression of *RORC* and  
230 *CACNA1F* remained detectable in expanded Th17-cells (see **Figure E4B in the Online**  
231 **repository**).

232 The Th17 cells from PP and HD produced IL17A, IL22, IFN $\gamma$ , and TNF $\alpha$  after anti-  
233 CD3/CD28 stimulation (**Figure 3**). Nicardipine decreased not only the percentage of cytokine-  
234 producing cells (**Figure 3**) but also the amount of cytokines per cells (GMFI) and in culture  
235 supernatants, and their transcription (see **Figure E5 and E6 in the Online repository**). The  
236 inhibitory effect of nicardipine on cytokines was similar in HD and PP, with respect to cytokine  
237 secretion, production, or transcription (see **Figure E5 and E6 in the Online repository**).  
238 Together, these results show that  $Ca_v1$   $Ca^{2+}$  channels contribute to optimal cytokine production  
239 by TCR-activated Th17 lymphocytes from HD and PP.

240

### 241 **$Ca_v1$ channels are involved in TCR-driven initial calcium events and transduction**

### 242 **signaling in Th17 cells**

243 To demonstrate that  $Ca_v1$  channels play a role in the global  $Ca^{2+}$  response of Th17 cells, we  
244 analyzed changes in the intracellular  $Ca^{2+}$  concentration ( $[Ca^{2+}]_i$ ) induced by TCR stimulation  
245 of expanded Th17 cells from the peripheral blood of HD. As shown in **Figure 4A-B**, nicardipine  
246 strongly reduced the TCR-induced increase in  $[Ca^{2+}]_i$ . We also analyzed the potential role of  
247  $Ca_v1$  channels in the regulation of endoplasmic reticulum (ER)  $Ca^{2+}$  stores and store-operated  
248  $Ca^{2+}$  entry (SOCE) by examining the effect of nicardipine on the  $Ca^{2+}$  signal elicited by  
249 thapsigargin in the presence or absence of calcium in the extracellular medium. Nicardipine

250 neither affected intracellular  $\text{Ca}^{2+}$  store release into the cytoplasm, nor SOCE resulting from  
251 the ER calcium depletion induced by thapsigargin (see **Figure E7 in the Online repository**).  
252 In order to deepen the role of  $\text{Ca}_v1$  channels in the influx of  $\text{Ca}^{2+}$  following TCR engagement,  
253 we developed total internal reflection fluorescence microscopy (TIRFM) imaging to record  
254 rapid  $\text{Ca}^{2+}$  changes close to the plasma membrane. This allows the imaging of peaks  
255 corresponding to spatiotemporal single elementary calcium events (ECE) likely due to the  
256 activity of one channel or a cluster of  $\text{Ca}^{2+}$  channels<sup>19</sup>. Under resting conditions, the expanded  
257 Th17-cells from the peripheral blood of HD displayed few ECEs and their number significantly  
258 increased by 1.82 fold after 2-10 seconds of stimulation with anti-CD3-biotin/streptavidin  
259 complexes (**Figure 4C-E**). In addition, the ECE opening frequency significantly increased by  
260 TCR stimulation (**Figure 4F**), suggesting that both an increase in the number of open channels  
261 and higher opening frequencies are responsible for initial TCR-driven localized  $\text{Ca}^{2+}$  fluxes.  
262 Nicardipine decreased the number of spontaneous ECEs in resting cells (**Figure 4C-E**) and  
263 precluded the increase in ECE numbers and opening frequencies upon TCR stimulation (**Figure**  
264 **4C-F**). Thus,  $\text{Ca}_v1$  channels support early  $\text{Ca}^{2+}$  influx at the plasma membrane in both resting  
265 and TCR-activated Th17 cells, even before the global  $[\text{Ca}^{2+}]_i$  rise.

266 We then studied the consequences of this early decrease in calcium entry after TCR  
267 engagement on proximal transduction signaling. The TCR-induced phosphorylated-tyrosine  
268 protein pattern (**Figure 4G**) and phosphorylation of the MAPKinase, ERK2, were decreased by  
269 nicardipine (**Figure 4H**). Nicardipine also inhibited the nuclear translocation of the  $\text{Ca}^{2+}$ -  
270 dependent transcription factor, NFATc2 (**Figure 4I**). Altogether, these data suggest that  $\text{Ca}_v1$   
271 channels control an early calcium entry after TCR engagement, which is essential for the full  
272 TCR-driven activation of Th17 cells.

273

274 **Knockdown of  $\text{Ca}_v1.4$  decreases IL17A and IL22 production by Th17 cells**

275 As nicardipine antagonizes all Ca<sub>v</sub>1 channels, we tested the specific role of Ca<sub>v</sub>1.4 in Th17  
276 functions by knocking down its expression using GFP reporter shRNAs. Th17-cells from the  
277 peripheral blood of HD transduced with gene-specific shRNAs resulted in a strong reduction  
278 of *CACNA1F* expression (50%, **Figure 4J**) associated with a reduction of Ca<sub>v</sub>1.4 channel at the  
279 protein level (87%, **Figure 4K**) in GFP<sup>+</sup> sorted cells compared with cells transduced with  
280 control shRNA. We did not detect compensatory *CACNA1C* and *CACNA1D* expression in the  
281 Th17 cells transduced with shRNAs against *CACNA1F* (**Figure 4J**). The decreased Ca<sub>v</sub>1.4  
282 expression was associated with the reduced production of IL17A and IL22 after TCR  
283 stimulation by 55 and 59%, respectively (**Figure 4L**). These data provide evidence for the  
284 selective role of Ca<sub>v</sub>1.4 in IL17A and IL22 production by Th17 cells.

285

### 286 **Ca<sub>v</sub>1 channels are involved in the development of psoriatic inflammation in a human 3D** 287 **skin reconstruction model and in murine imiquimod-induced psoriasis like model**

288 To study the role of Ca<sub>v</sub>1 channels in the pathogenesis of Th17 cells in psoriasis, we established  
289 a three-dimensional (3D) skin reconstruction model, in which the expression by keratinocytes  
290 of psoriasis inflammatory markers is induced by populating the skin with activated Th17-  
291 cells<sup>20</sup>. To evaluate the role of Ca<sub>v</sub>1 channels in the development of psoriasis inflammation,  
292 nicardipine was added to Th17 cells before stimulation and on each day of the culture (**Figure**  
293 **5A**). Cyclosporin A (CsA), used as a control, was only added the last two days of the culture,  
294 as previously described<sup>20</sup>. Activation of Th17 cells was confirmed by CD69 expression and was  
295 not modified by nicardipine (data not shown). Nicardipine did not prevent Th17 cells from  
296 migrating into skin equivalents (SE), as shown by the number of infiltrating CD3<sup>+</sup> T-cells after  
297 four days of culture (**Figure 5B-C**). According to<sup>20</sup>, addition of Th17-cells did not modify the  
298 epidermis morphology, with no effect of nicardipine or CsA (see **Figure E8 in the Online**  
299 **repository**). Nicardipine tended to reduce IL17A production and significantly decreased IL22



300 production (**Figure 5D**). The infiltration of Th17 cells in SE induced a significant increase in  
301 the expression of psoriasis-associated genes, such as *KRT16*, *PI3*, and *DEFB4* (encoding for  
302 cytokeratin 16, elafin, and  $\beta$ -defensin 2, respectively), after four days of culture (**Figure 5E**).  
303 Nicardipine decreased the expression of these genes to the same level as CsA (**Figure 5E**).  
304 These data suggest that inhibition of  $Ca_v1$  channels can relieve the psoriatic inflammation in a  
305 3D skin reconstruction model, probably by acting on Th17-cells functions, in accordance with  
306 locally decreased IL17A and IL22 production.

307 Finally, we investigated the role of  $Ca_v1$  calcium channels in local lesions in murine  
308 imiquimod-induced psoriasis like model. We applied Imiquimod to the shaved back and on one  
309 ear of BALB/c mice that received or not intraperitoneal injections of nicardipine. As shown in  
310 the **New Figure E9A R1** below, imiquimod-treated mice presented dermal inflammation and  
311 epidermal acanthosis compared with untreated mice, in treated-ear and -back skin (see **New**  
312 **Figure E9A and 1B R1 in the Online repository**) and a significant increase of IMQ-treated-  
313 ear and -back skin thickness (see **New Figure E9C R1 in the Online repository**). The  
314 inhibition of  $Ca_v1$  channels by nicardipine greatly reduced both dermal inflammation,  
315 epidermal acanthosis and decreased the ear and back-skin thickness in this experimental model.

316 **Discussion**

317 This study aimed to investigate the singularities of the  $\text{Ca}^{2+}$  toolkit of human Th17  $\text{CD4}^+$  T cell  
318 activation and its possible involvement in psoriasis, a Th17 skewed chronic skin disease. We  
319 focused on  $\text{Ca}_v1.4$ , since this channel, which is present in naive  $\text{CD4}^+$  T cells, disappears upon  
320 activation and differentiation of effector human T cells, except in Th17 cells<sup>14</sup>. Our results  
321 showed a preferential association of *CACNA1F* with *RORC* expression in  $\text{CD4}^+$  and  $\text{CD4}^-$  cells,  
322 and the detection of  $\text{CD4}^+$   $\text{ROR}\gamma\text{t}^+$   $\text{Ca}_v1.4^+$  triple-positive cells in the LP skin dermis. The  
323 inhibition of  $\text{Ca}_v1$  channels decreased Th17 cytokine transcription and production. The  $\text{Ca}_v1$   
324 channels acted early during Th17-cell activation, allowing optimal Th17 cell activation, as  
325 indicated by the effect of nifedipine on global early tyrosine phosphorylation, MAP Kinase  
326 activation and NFAT nuclear translocation. The  $\text{Ca}_v1.4$  knockdown decreased IL17A and IL22  
327 production, supporting the role of this channel in the Th17-cells. Moreover, the  $\text{Ca}_v1$  channel  
328 blocker decreased psoriatic inflammation in a model of skin equivalent model populated with  
329 Th17 cells.

330 The expression of *CACNA1F* was higher in LP than in NLP or AD skin and correlated  
331 with the expression of the inflammatory cytokines *IL17A*, *IFNG*, and *IL26* only when the  
332 different groups (lesional and non-lesional psoriasis and atopic dermatitis) were analyzed  
333 together. Moreover, *CACNA1F* expression was enriched among *RORC*<sup>+</sup> over *RORC*<sup>-</sup> cells in  
334 both  $\text{CD4}^+$  and  $\text{CD4}^-$  dermis-infiltrating cells from PP. We did not check the expression of  
335 *CACNA1F* in the regulatory T cells, but these cells have been shown to turn into *RORC*  
336 expressing cells which produce IL17 in psoriatic skin<sup>21</sup>. The  $\text{Ca}_v1.4$  channel was also present  
337 in *RORC*<sup>+</sup>  $\text{CD4}^-$  cells (which might include CD8 T cells, neutrophils, and ILC3), supporting the  
338 role of  $\text{Ca}_v1.4$  in the IL17-associated inflammatory axis<sup>22</sup>.  $\text{Ca}_v1.4$  expression was not observed  
339 in the epidermis, suggesting that the keratinocytes do not express this  $\text{Ca}^{2+}$  channel. In the  
340 dermis,  $\text{Ca}_v1.4$  was essentially found in inflammatory zones containing clusters of  $\text{CD4}^+$  T cells.

341 We expanded T cells from the biopsies and showed that they transcribed the pro-inflammatory  
342 cytokines *IL17A*, *IL17F*, *IL22*, *IFNG*, *CSF2* and *TNFA* after TCR stimulation. Nicardipine, a  
343  $\text{Ca}_v1$  channel antagonist, reduced both TCR-induced cytokine transcription and production by  
344 the  $\text{CD4}^+$  T cells infiltrating the PP skin. Although the  $\text{CD4}^+$  T cells were the main producers  
345 of *IL17A* and *IL22* (the  $\text{CD8}^+$  T cells produced very few *IL17A* and *IL22*; data not shown),  
346  $\text{IFN}\gamma$  and  $\text{TNF}\alpha$  were also produced by infiltrating  $\text{CD8}^+$  T cells, and nicardipine inhibited their  
347 production (not shown), in accordance with  $\text{Ca}_v1.4$  expression by the  $\text{CD8}^+$  T cells<sup>14</sup>. It is  
348 interesting to note the potential inhibitory action of nicardipine on all cytokines and in the  
349 majority of PPs, since these cytokines act synergistically and amplify the inflammatory loop in  
350 psoriatic skin inflammation<sup>23, 24</sup>.

351 The  $\text{CCR6}^+\text{CXCR3}^- \text{CD4}^+$  T cells that expressed *RORC*, the master switch transcription  
352 factor of Th17 cells, were the only subset to increase in the blood of PP compared to HD, in  
353 accordance with Blauvelt et al.<sup>22</sup>. The Th17 cells expressed *CACNA1F* and maintained this  
354 expression as well as their pattern of TCR-induced cytokine expression after expansion,  
355 indicating that they represent a valuable tool for studying Th17 functions. TCR stimulation of  
356 Th17 cells induced similar amounts of cytokines (transcription and production) from both PP  
357 and HD, which were inhibited to a similar extent by nicardipine. These data confirm the  
358 quantitative bias toward more Th17 cells in psoriasis and show that nicardipine reduced  
359 cytokine production by the Th17 cells obtained from the blood or even from inflamed tissues.  
360 Among the four  $\text{Ca}_v1$  channels,  $\text{Ca}_v1.4$  was the only  $\text{Ca}_v1$  channel expressed in the Th17 cells.  
361 Furthermore, the transduction of these cells with specific shRNA reduced inflammatory  
362 cytokine production. Altogether, these results support the role of  $\text{Ca}_v1.4$  in Th17-cell functions.

363 Nicardipine decreased the transcription and production of all inflammatory cytokines  
364 tested, indicating that  $\text{Ca}_v1$  channels regulate common pathways upstream of cytokine gene  
365 transcription. The crucial role of  $\text{Ca}^{2+}$  in Th17 functions and their pathological potential<sup>25</sup>

366 explains why ciclosporin A, which inhibits  $\text{Ca}^{2+}$ -dependent phosphatase calcineurin, is  
367 effective in the treatment of PP<sup>26</sup>. Our investigations described an early role of  $\text{Ca}_v1$  channels  
368 during TCR activation as nicardipine fully abrogated early ECE induced by TCR stimulation  
369 and even decreased the few  $\text{Ca}^{2+}$  fluxes observed under resting conditions, leading to the  
370 conclusion that  $\text{Ca}_v1.4$  is involved in both  $\text{Ca}^{2+}$  homeostasis and the TCR response. Contrary  
371 to what is shown in murine naive T lymphocytes<sup>16</sup>, the  $\text{Ca}_v1.4$  channels do not regulate the  
372 intracellular  $\text{Ca}^{2+}$  stores nor the functionality of STIM/ORAI (SOCE) since the response to  
373 thapsigargin was not affected by nicardipine in human Th17 cells. However, nicardipine  
374 reduced the TCR-driven global  $\text{Ca}^{2+}$  response suggesting a link between the ECE and the  
375 cellular  $\text{Ca}^{2+}$  response and that the initial defect cannot be overcome to induce a full response  
376 in the absence of functional  $\text{Ca}_v1$  channels. It is conceivable that the ECEs locally modify the  
377 cell environment in the vicinity of the immune synapse allowing an optimal assembly of the  
378 TCR transduction machinery. This is in accordance with the role of local ECE preceding full  
379 TCR activation, required to initiate cell transduction, possibly through an effect on cytoskeleton  
380 rearrangement<sup>27, 28</sup>. In murine  $\text{CD8}^+$  T cells,  $\text{Ca}_v1.4$  can associate with  $\text{p56}^{\text{lck}}$  and  $\text{vav1}$ , which  
381 both play a key role in TCR-dependent signal transduction<sup>29</sup>. Nicardipine reduced the early  
382 profile of tyrosine phosphorylation, ERK2 activation and NFATc2 nuclear translocation,  
383 supporting the role of  $\text{Ca}_v1$  channels in the initiation of global signal transduction. Our data  
384 suggest that  $\text{Ca}_v1.4$ , which is known to activate at a relatively negative cell membrane potential,  
385 closer to the resting cell membrane potential than the other  $\text{Ca}_v1$  channels, could deliver  $\text{Ca}^{2+}$   
386 in a very fast and localized manner facilitating the transduction machinery assembly. Several  
387 groups have shown that ORAI channels play a major role in the calcium response induced by  
388 TCR engagement in human pathogenic Th17 cells<sup>25, 30</sup>, raising the question of the respective  
389 places of  $\text{Ca}_v1$  and OrAI  $\text{Ca}^{2+}$  channels in Th17 cell  $\text{Ca}^{2+}$  signaling. TIRF experiments performed  
390 here favor the earliest involvement of  $\text{Ca}_v1$  channels in the initiation of T-cell activation (very

391 few seconds) followed by STIM/ORAI channel activation. Accordingly, the Ca<sub>v</sub>1 channels  
392 were described to act earlier than the ORAI channels following cell activation and to convey  
393 ten more calcium ions than the ORAI channels<sup>31</sup>.

394 In order to establish a potential beneficial use of Ca<sub>v</sub>1.4 inhibitors in the treatment of  
395 PP, we tested the effects of nifedipine on a human psoriatic inflammation 3D skin model  
396 populated with Th17 cells. Nifedipine did not prevent the migration of Th17 cells into the  
397 dermis, but it tended to diminish the expression of specific psoriasis keratinocyte markers with  
398 the same effects as CsA. As the cytokines produced by T cells appeared to be the main factor  
399 responsible for the psoriatic inflammatory phenotype<sup>20</sup> and that nifedipine diminished IL17A  
400 and IL22 cytokines in the skin-equivalent infiltrated with Th17 cells, we propose that Ca<sub>v</sub>1  
401 channel blockade reduced psoriatic gene expression by inhibiting the cytokine production by T  
402 cells. Moreover, we showed Ca<sub>v</sub>1 calcium channel blocker was beneficial in the treatment of  
403 psoriasis-like inflammation in mice since nifedipine inhibits the dermal inflammation,  
404 epidermal acanthosis and the ear and back-skin thickening in murine imiquimod-induced  
405 psoriasis like model.

406 The effect of nifedipine on the cytokine production by Th17 cells was partial, but  
407 affected all tested cytokines that participate in a positive feedback inflammatory loop<sup>26</sup>.  
408 *CACNA1F* was also associated with *RORC* expression in the CD4<sup>+</sup> cells that also participate in  
409 psoriasis pathogenesis<sup>32</sup>. Therefore, targeting this channel might be beneficial to treat psoriasis.  
410 Finally, psoriasis is associated with cardiovascular comorbidities such as blood hypertension<sup>33</sup>;  
411 therefore, Ca<sub>v</sub>1 channel blockers could be also beneficial in this regard.

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501

**502 Author contributions**

503 MMA performed all the experiments. IN, CL, SB and CR performed ISH and HIF of psoriatic  
504 skin biopsies and confocal analysis. MMo and LP performed TIRF experiments. MMo  
505 developed software for TIRFM analysis and analyzed the number of open channels and their  
506 opening frequency. SL helped to analyze the nuclear translocation of NFAT. CP and MT  
507 designed and implemented the clinical study, recruited patients with psoriasis and atopic  
508 dermatitis. EG and CD developed analysis of qPCR data and performed heatmap. JCG, HD and  
509 DR contributed to the design of experiments and criticized the results. MB and MDT managed  
510 and supervised the clinical project. FL did the experimental design, performed and analysed the  
511 results of qPCR and criticized the results. LP and MS did the experimental design, analysed the  
512 results, and wrote the paper. MS supervised the work. All authors critically reviewed the  
513 manuscript.

514 **Figure legends**

515

516 **New Figure 1 R1. Ca<sub>v</sub>1.4 calcium channel expression in Th17-cells infiltrating psoriasis**  
517 **skins**

518 (A) Hierarchical gene expression heat map in lesional (LP), non-lesional (NLP) psoriasis and  
519 atopic dermatitis (AD) skins. (B) Violin plots showing *CACNA1F* expression. One symbol by  
520 patient; the median and the interquartile ranges are indicated. (C) Correlation between  
521 *CACNA1F* and cytokine expression in LP, NLP and AD skins. (D) Representative images with  
522 *CD4* (red), *RORC* (green) and *CACNA1F* (white) encoding mRNAs by ISH of LP skins. (E)  
523 Quantifications of cell numbers per field as described in the Online Repository. (+/-) groups  
524 together cells that are positive or negative for the marker. Means  $\pm$  sem; one symbol by patient.  
525 (F) Representative images with *CD4* (red), *ROR $\gamma$ t* (green) and Ca<sub>v</sub>1.4 (white) proteins by IHF  
526 of LP skins. Bar = 50  $\mu$ m for general view of skin section and 10  $\mu$ m for inserts. (New G) IHF  
527 scores of LP skins. 0 = any CD4<sup>+</sup> ROR $\gamma$ t<sup>+</sup> Ca<sub>v</sub>1.4<sup>+</sup> cells in any zone; 1 = one CD4<sup>+</sup> ROR $\gamma$ t<sup>+</sup>  
528 Ca<sub>v</sub>1.4<sup>+</sup> cells in one zone; 2 = several zones containing one CD4<sup>+</sup> ROR $\gamma$ t<sup>+</sup> Ca<sub>v</sub>1.4<sup>+</sup> cells ; 3 =  
529 several zones containing at least three CD4<sup>+</sup> ROR $\gamma$ t<sup>+</sup> Ca<sub>v</sub>1.4<sup>+</sup> cells. Means  $\pm$  sem; one symbol  
530 by patient.

531

532

533 **Figure 2. Ca<sub>v</sub>1-dependent inflammatory cytokine production by T-cell-infiltrating**  
534 **lesional psoriasis skin**

535 (A) Experimental procedure. (B) Lymphocyte frequencies after T-cell expansion. (C) Th17,  
536 Th1/Th17 and Th1-cell frequencies in expanded T-cells. (D-E) Cytokine production (ELISA,  
537 D) and expression (qPCR, E) in expanded T-cells after anti-CD3/CD28 stimulation with or  
538 without nicardipine. One symbol by patient. P values were determined by one-way ANOVA

539 followed by Turkey's multiple comparison test. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\* $p < 0.005$ ;  
540 \*\*\*\* $p < 0.0001$ .

541

542 **Figure 3.  $Ca_v1$ -dependent inflammatory cytokine production by peripheral blood Th17**  
543 **cells**

544 Th17 cells expanded from healthy donor (HD) and psoriasis patient (PP) blood were stimulated  
545 with anti-CD3/CD28/CD2 antibodies plus or minus (nicar) for 4h before intracellular cytokine  
546 staining. Comparisons with one-way ANOVA followed by Turkey's multiple comparison tests.  
547 \*  $p < 0.05$ ; \*\*\* $p < 0.005$ ; \*\*\*\* $p < 0.0001$ .

548

549 **New Figure 4.  $Ca_v1$  channels contribute to TCR-induced  $[Ca^{2+}]_i$  rise and transduction**  
550 **signaling in Th17 cells and knocking-down *CACNA1F* reduces Th17 cytokine production**

551 **(A-B)** HD Th17 cells were loaded with Fura2-AM and fluorescence was recorded before (F0)  
552 and after (F) TCR stimulation  $\pm$  nicardipine (nicar). **(A)** Mean  $\pm$  SEM from 50-100 cells from  
553 3 HD. **(B)** the area under the curve. **(C-F)** 3-D images of ECE from Th17 cells loaded with  
554 Fluo-5AM **(C)**, number of ECE (one symbol by cell; pool from 2 HD) **(D)**, relative ECE number  
555 **(E)** and opening frequency (one symbol by ECE; 5-7 cells from 2 donors) **(F)**. **(New G-H)**  
556 Th17 cells were stimulated with ImmunoCult™ Human CD3/CD28/CD2 T Cell Activator for  
557 2 min (Stim) or not (NS) in presence or absence of nicardipine (nicar). Equal amounts of lysate  
558 proteins were blotted and probed with antibody against phosphorylated tyrosine (P-Tyr) **(New**  
559 **G)**, P-ERK2 **(New H)** or  $\beta$ -actin (Actin, loading control). Graphs show the quantification of  
560 staining intensities relative to actin intensity and to unstimulated samples of 3-4 donors. **(New**  
561 **I)** Th17 cells were stimulated with ImmunoCult™ Human CD3/CD28 T Cell Activator for 4  
562 hours (Stim) or not (NS) in presence or absence of nicardipine (nicar). Nuclear translocation of  
563 NFATc2 is analyzed by confocal. Bar = 5  $\mu$ m **(J-L)** HD Th17 cells were transduced with control

564 (shCtr) or *CACNA1F* GFP reporter shRNAs. *CACNA1* expression (**J**), Cav1.4 protein level  
565 (**New K**, Bar = 5  $\mu$ m) and TCR-induced cytokine production (**L**) in GFP<sup>+</sup>-sorted cells; 2-3  
566 biological replicates from 3 experiments. P values were determined by Student's t-test (**B**, **D**-  
567 **F**, **New I** and **New K**) and by paired Wilcoxon test (**L**). \* p<0.05; \*\* p<0.01; \*\*\*p<0.005;  
568 \*\*\*\*p<0.0001.

569

570 **Figure 5. Antagonizing Cav1 channels diminishes cytokine and psoriasis-like gene**  
571 **expression in skin equivalent infiltrated by Th17 cells**

572 (**A**) Experimental procedure. (**B-C**) Enumeration of CD3<sup>+</sup> T cells 4 days after introducing Th17  
573 cells under the skin equivalent (SE). The mean is indicated and the dotted line shows the  
574 background without T-cells. (**D**) IL17A and IL22 production 24h after Th17 cells introduction  
575 in SE Each point corresponds to one SE. (**E**) *KRT16*, *PI3* and *DEFB4B* expression after 4 days  
576 of culture. P values were determined by paired Wilcoxon test (**D**) and Student's t-test (**E**). \*  
577 p<0.05; \*\*p<0.01 Bar = 50 $\mu$ m.