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1 Curcumin modulates endothelial permeability and monocyte
2 transendothelial migration by affecting endothelial cell dynamics

3

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23 **ABSTRACT**

24 Curcumin is a phenolic compound that exhibits beneficial properties for cardiometabolic health. We
25 previously showed that curcumin reduced the infiltration of immune cells into the vascular wall and
26 prevented atherosclerosis development in mice. This study aimed to investigate the effect of curcumin
27 on monocyte adhesion and transendothelial migration (TEM) and to decipher the underlying
28 mechanisms of these actions.

29 Human umbilical vein endothelial cells (HUVECs) were exposed to curcumin (0.5-1 μ M) for 3 hours
30 prior to their activation by Tumor Necrosis Factor alpha (TNF- α). Endothelial permeability, monocyte
31 adhesion and transendothelial migration assays were conducted under static condition and shear stress
32 that mimics blood flow. We further investigated the impact of curcumin on signaling pathways and on
33 the expression of genes using macroarrays.

34 Pre-exposure of endothelial cells to curcumin reduced monocyte adhesion and their transendothelial
35 migration in both static and shear stress conditions. Curcumin also prevented changes in both
36 endothelial permeability and the area of HUVECs when induced by TNF- α . We showed that curcumin
37 modulated the expression of 15 genes involved in the control of cytoskeleton and endothelial junction
38 dynamic. Finally, we showed that curcumin inhibited NF- κ B signaling likely through an antagonist
39 interplay with several kinases as suggested by molecular docking analysis.

40 Our findings demonstrate the ability of curcumin to reduce monocyte TEM through a multimodal
41 regulation of the endothelial cell dynamics with a potential benefit
42 on the vascular endothelial function barrier.

43

44

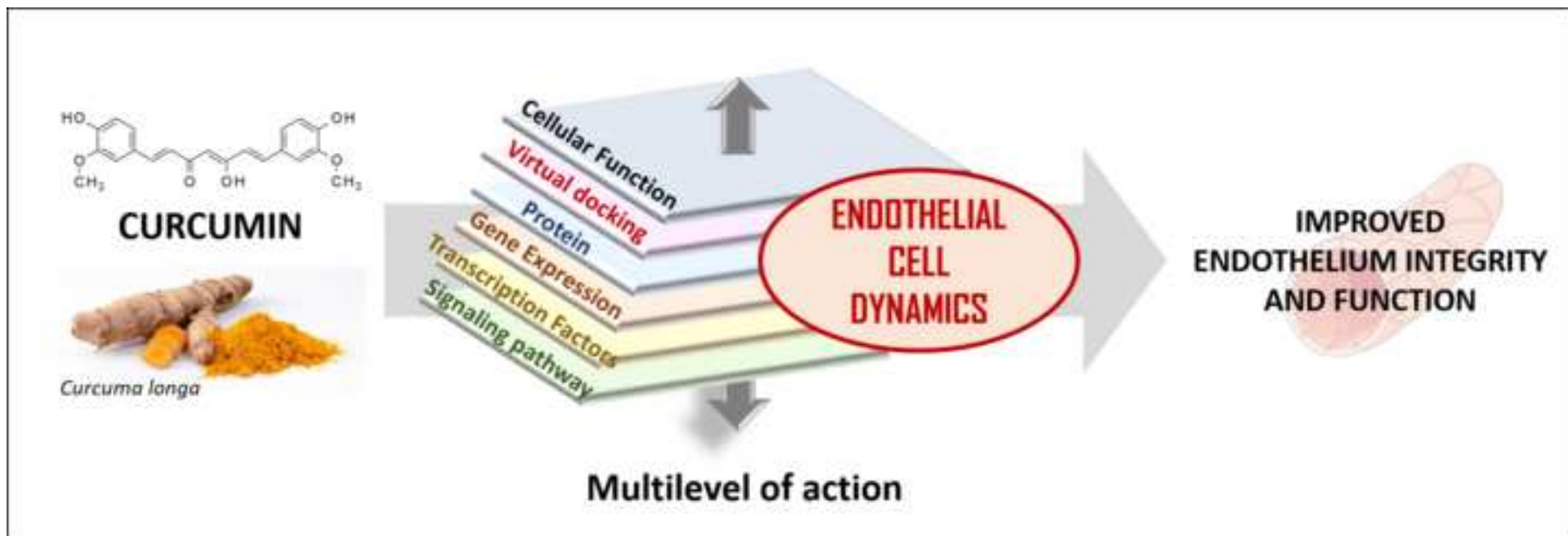
45 **Keywords ;**

46 Curcumin

47 Endothelial cell

48 Endothelial permeability

49 Monocyte adhesion



51 INTRODUCTION

52 Impairment of the endothelial cell function is a major factor in the development of vascular
53 damage. Endothelial cell dysfunction is characterized by the abnormal expression of genes including
54 those coding for adhesion molecules, which leads to the firm adhesion of leukocytes to the
55 endothelium and their subsequent infiltration into the vascular wall, which is one of the initial events
56 in atherosclerosis onset [1].

57 Curcumin is a polyphenolic compound of turmeric derived from the rhizome of *Curcuma longa*.
58 This compound is commonly used as a dietary spice and food coloring agent. Curcumin exhibits
59 potent antioxidant and anti-inflammatory properties that could be beneficial for vascular health [2].
60 Some clinical data showed that daily curcumin intake, from 450 mg/day to 8 g/day for 1 to 4 months,
61 can reduce some cardiovascular risk factors such as systolic blood pressure [3], serum triglycerides
62 and low density lipoprotein (LDL) [4], and can improve endothelial function [5]. Moreover, curcumin
63 has been shown to reduce the development of atheromatous lesions in different animal models of
64 atherosclerosis [6-11]. Coban et al. revealed a decrease in macrophages in the atherosclerotic lesions
65 of ApoE^{-/-} mice that were supplemented with dietary curcumin [6]. In the same study, the authors
66 identified that most of the differentially expressed genes in response to curcumin supplementation
67 were entailed in leukocyte recruitment and transmigration. Um et al. showed a lower expression of
68 endothelial adhesion molecules (VCAM-1, ICAM-1) in the aortas of rabbits fed with a high
69 cholesterol diet supplemented with curcumin [9].

70 *In vitro* studies have demonstrated that curcumin reduces monocyte adhesion to human
71 endothelial cells activated by the pro-inflammatory tumor necrosis factor alpha (TNF- α) by
72 modulating the expression of adhesion molecules [6, 12-15]. Down-regulation of such molecules has
73 been related to an inhibitory effect of curcumin on several cell-signaling molecules, of which NF- κ B
74 is one of the major transcription factors activated by TNF- α [16, 17].

75 Following their firm adhesion to the endothelium, monocytes can migrate through the
76 endothelium. This process, also known as diapedesis, transendothelial migration (TEM) or
77 extravasation, is a dynamic process entailing endothelial cytoskeleton remodeling and disruption of

78 endothelial cell junctions that cause an increase in vascular permeability [18, 19]. These cellular
79 modifications can be induced *in vitro* by the stimulation of endothelial cells with TNF- α [20, 21]. So
80 far, little is known about the effect of curcumin on the regulation of cytoskeleton and junctional
81 dynamics in the endothelium. It has been reported that oral supplementation of rats with curcumin may
82 reduce coronary artery permeability to a fluorescent macromolecule [22]. To our knowledge, no
83 studies have investigated the impact of curcumin on leukocyte transmigration through the
84 endothelium.

85 Based on the above considerations, this study aimed to investigate the effect of curcumin on
86 monocyte transendothelial migration and on the expression of genes involved in this process. Special
87 attention was paid to reproduce the physiological environment of vascular endothelial cells as closely
88 as possible. For that, the cells were exposed to low concentrations of curcumin for a 3-hour period, in
89 respect to pharmacokinetics data of curcumin [23]. Moreover, effects of curcumin were also
90 investigated in endothelial cells exposed to physiological shear stress that mimicked blood flow.

91

92 **2. MATERIALS AND METHODS**

93

94 **Chemicals**

95 Curcumin was purchased from Extrasynthese (France). Reagents for cell culture (RPMI 1640,
96 penicillin/streptomycin, Trypsin/EDTA, Foetal Bovine Serum) were purchased from PAN biotech
97 (Germany). Gelatin, paraformaldehyde (PFA), FITC-dextran, dimethylsulfoxide (DMSO), anti-
98 protease cocktail, anti-phosphatase cocktail, Nonidet P40 and DAPI were obtained from Sigma-
99 Aldrich (France). Recombinant-human-TNF- α and MCP-1 were obtained from R&D systems and
100 Miltenyi Biotec SAS (Paris, France) respectively.

101

102 **Cell Culture**

103 Human umbilical vein endothelial cells (HUVECs) (Lonza, Amboise, France) were cultured in a
104 phenol-red-free endothelial growth medium supplemented with 2% fetal bovine serum, 0.4% human
105 fibroblast growth factor, 0.1% human epidermal growth factor, 0.1% vascular endothelial growth

106 factor, 0.1% insulin-like growth factor, 0.1% ascorbic acid, 0.1% heparin, 0.1%
107 gentamicin/amphotericin-B and 0.04% hydrocortisone (all from Lonza, France). A monocyte THP1
108 cell line obtained from ACC (Manassas, VA) was cultured in RPMI 1640 supplemented with 10%
109 fetal bovine serum and 1% penicillin/streptomycin. Both cultures were maintained at 37°C in
110 humidified atmosphere with 5% CO₂.

111

112 **Cell adhesion assay**

113 HUVECs were seeded into 0.2% gelatin-coated 24-well plates at a density of 50,000 cells/well. At 60-
114 70% confluence, cells were made quiescent for 24 hours in medium with 0.2% FBS, and then were
115 exposed to 0.5 μM, 1 μM curcumin or a DMSO vehicle (0.02%) for 3 hours. Following pre-incubation
116 with curcumin, inflammatory stress was induced by 1 ng/ml of TNF-α for 4 hours. After TNF-α
117 stimulation, 5x10⁵ THP1 cells were added to each well, and cells were co-incubated for 15 minutes.
118 Non-adhering THP1 cells were rinsed away by washing with 1X DPBS. The remaining attached cells
119 were subsequently detached using trypsin/EDTA (0.05%/0.02%), fixed with 1% PFA and stored at
120 4°C prior to immunostaining and analysis by flow cytometry.

121

122 **Flow cytometry**

123 Cell immunostaining was performed according to the manufacturer instructions (Miltenyi Biotec SAS,
124 Paris, France). Briefly, fixed cells from the adhesion assay were pelleted by centrifugation at 300 g for
125 10 minutes and treated with FcR blocking reagent for 10 minutes. Subsequently, they were stained
126 with CD45 antibody conjugated to FITC for 30 minutes in the dark. IgG2a isotype antibodies
127 conjugated to FITC were used as a negative control. The number of CD45-positive cells (THP1) and
128 unstained cells (HUVECs) was measured by BD FACSCalibur (BD Bioscience, San Jose, CA), set at
129 20,000 events, and analyzed using CellQuest Pro software, version 5.1.1 (BD Bioscience, San Jose,
130 CA) for Macintosh. Relative monocyte adhesion to endothelial cells was expressed as the ratio of
131 monocytes to HUVECs.

132

133 **Monocyte transendothelial migration assay**

134 HUVECs were seeded at 15,000 cells/well onto Corning Transwell filters (5 μm pore, 6.5 mm
135 diameter) (Corning, USA) previously coated with 0.2% gelatin and grown to confluence. Cells were
136 made quiescent for 24 hours in medium with 0.2% FBS and were then exposed to 0.5 μM and 1 μM
137 curcumin or vehicle, respectively (DMSO, 0.02%), for 3 hours. Following this incubation, 100 ng/mL
138 of MCP-1 were added to the lower chamber, and 5×10^5 THP1 above HUVECs. Cells were incubated
139 for 3 hours at 37°C and 5% CO_2 . THP1 cells that had transmigrated to the lower chamber were
140 harvested and counted using a Beckman cell coulter (Beckman Coulter, USA). The results are
141 expressed as a percentage of transmigrated monocytes relative to the condition 'vehicle + $\text{TNF-}\alpha$ '.

142

143 **Endothelium permeability assay**

144 As for monocyte transendothelial migration assay, confluent cultured HUVECs loaded onto Coning
145 Transwell filters were made quiescent for 24 hours in medium with 0.2% FBS before being exposed to
146 0.5 μM or 1 μM curcumin or vehicle (0.02% DMSO) for 3 hours, followed by stimulation with 1
147 ng/ml of $\text{TNF-}\alpha$ for 4 hours. Permeability of the endothelium was evaluated by passage of FITC-
148 dextran at 40 kDa through endothelial monolayer. One hundred microliters of FITC-dextran at 40 kDa
149 (2 mg/mL) was added to the upper chamber and allowed to equilibrate for 20 minutes, after which
150 FITC fluorescence (excitation 488 nm; emission 520 nm) in the lower chamber was measured using a
151 Biotek synergy 2 plate reader (BioTek, France). Four independent experiments were performed.

152

153 **Endothelial cell area measurement**

154 HUVECs were seeded on 0.2% gelatin-coated glass coverslips (13 mm diameter). At 60-70% of
155 confluence, HUVECs were treated with $\text{TNF-}\alpha$ as previously described for the monocyte adhesion
156 assay. After 15 min of THP1-HUVECs co-incubation, cells were rinsed to remove non-adherent cells
157 and then fixed with 1% PFA for 15 min at 4°C. Fixed cells were permeabilized by a 1% Triton
158 X100/1X PBS/3% BSA solution, and the actin cytoskeleton was stained with rhodamine-phalloidin
159 (Invitrogen). Nuclei were counterstained with DAPI (100 nM). Fluorescence was detected and imaged
160 using a Zeiss AxioPlan E microscope (Karl Zeiss, Jena, Germany) and a ZeissAxioCam MRM (Carl
161 Zeiss Vision, Hallbergmoos, Germany). The mean endothelial cell area was measured in 10 fields of

162 view per condition from 2 independent experiments using “ImageJ” image analysis software
163 (<http://www.imagej.nih.gov/>).

164

165 **Chemoattractant capacity of TNF- α activated HUVECs**

166 Chemoattractant potency of HUVECs was evaluated using a transwell assay and conditioned media
167 harvested from HUVECs exposed 3 hours to 1 μ M curcumin or DMSO vehicle, with or without
168 stimulation by 1 ng/mL TNF- α for 4 hours. Conditioned media were added to the lower chamber and
169 5×10^5 THP1 was added above the filter. After incubation for 3 hours at 37°C with 5% CO₂,
170 transmigrated THP1 cells were harvested and counted using a Beckman cell counter (Beckman
171 Coulter, USA). The results were expressed as a percentage of transmigrated monocytes compared to
172 that measured in response to the supplementation of basal conditioned medium with 100 ng/mL MCP-
173 1.

174

175 **Monocyte adhesion and transendothelial migration assessment under shear stress**

176 HUVECs were seeded at 15,000 cells/cm² in gelatin-coated microchannels of the Vena8 Endothelial
177 Biochip (Cellix, Ireland). After adhesion (2 hours), biochips were connected to the Kima pump
178 (Cellix), and cells were grown under a 1 Hz-pulsatile aortic shear stress of 7 dyne/cm² for 72 h. Cells
179 were exposed to low concentrations of curcumin (0.5-1 μ M) or a vehicle (DMSO 0.02%) for 3 hours
180 prior to their stimulation with 1 ng/ml of TNF- α for 4 hours under shear stress. Following TNF- α
181 stimulation, a suspension of 2×10^6 THP1/mL was perfused through the microchannels at 0.5 dyne/cm²
182 for 10 min. Interactions between monocytes and HUVECs were visualized with videomicroscopy
183 using phase contrast optics (Diaphot-TMD microscope and DS-Fi 1 CCD camera, Nikon, USA). Each
184 experimental condition was recorded on a single field for the 10 min minutes. Additionally, images of
185 ten fields were captured for each condition and distributed along the channel. Firmly adherent
186 monocytes to HUVECs accumulated throughout the experiment, and those that had transmigrated
187 were quantified at the end of the experiment. Adherent and transmigrated monocytes were identified
188 based on their phase white and round appearance and on their phase dark and flattened shape,

189 respectively [24]. Quantifications were performed on pictures obtained from 2-3 independent
190 experiments using “ImageJ” image analysis software (www.imagej.nih.gov).

191

192 **Quantitative real-time PCR analysis**

193 Total RNAs were extracted using Trizol Reagent (Thermo Fisher Scientific, France) following
194 manufacturer conditions. RNA concentration was measured using a spectrophotometer NanoDrop ND-
195 1000 (LabTech, Ringmer, UK) and their quality checked by 1% agarose gel electrophoresis. 1 µg total
196 RNA extract was used as template for single-strand cDNA synthesis using High Capacity cDNA RT
197 kit (Applied Biosystems, USA) in a total volume of 20 µl containing 1 X RT buffer, 4 mM dNTP mix,
198 1 X random primers, 50 U reverse transcriptase and 20 U RNase inhibitor. The reverse transcription
199 reactions were ran under the following conditions: 25°C for 10 min, 37°C for 120 min and 85°C for 5
200 s. PCR was carried out in the final volume of 20 µl containing 10 µl Power SYBR Green PCR Master
201 Mix kit (Applied Biosystems, USA), 0.4 µl of each primer at 10 pmol/µl and 2 µl of the cDNA
202 solution. The primers used were: h-MCP-1-F: TCA TAG CAG CCA CCT TCA TTC C, h-MCP-1-R:
203 GGA CAC TTG CTG CTG GTG ATT C; GAPDH-F: CTG GGC TAC ACT GAG CAC C, GAPDH-
204 R: AAG TGG TCG TTG AGG GCA ATG. RT-PCR amplification was performed using an Applied
205 Biosystems Prism 7900HT (Applied Biosystems, France) system with the following thermal cycler
206 conditions: 2 min at 50°C and 10 min at 94.5°C, followed by 40 cycles of 30 s at 97°C and 30 s at
207 59.7°C. Raw data were analyzed using Sequence Detection System (SDS) Software v2.4 (Applied
208 Biosystems, Courtaboeuf, France) and compared by the $\Delta\Delta C_t$ method. Results are expressed relative
209 to the housekeeping gene transcript quantity and normalized to untreated cells.

210 Impact of curcumin on the expression of 93 other genes in HUVECs involved in endothelial cell
211 function was performed using TaqMan Low Density Array (TLDA) (Applied Biosystem). Genes were
212 chosen according to their role in atherosclerosis development from published articles (supplemental
213 table S1). Two hundreds ng (2 µl) cDNA of each sample was combined with, 98 µl of nuclease-free
214 water and 100 µl 2X TaqMan Universal PCR Master Mix (Applied Biosystems, USA) for the
215 quantitative real-time PCR (qPCR) measurements. This mixture was divided equally over two sample-
216 loading ports of the TLDA. The arrays were centrifuged once (1 min, 1300 rpm at room temperature)

217 to equally distribute the sample over the wells. Subsequently, the card was sealed to prevent an
218 exchange between wells. qPCR amplification was performed using an Applied Biosystems Prism
219 7900HT system (Applied Biosystem, USA) with the following thermal cycler conditions: 2 min at
220 50°C and 10 min at 94.5°C, followed by 40 cycles of 30 s at 97°C and 30 s at 59.7°C. Raw data were
221 analyzed using Sequence Detection System (SDS) Software v2.4 (Applied Biosystems). Analyses
222 were performed in triplicates. Bioinformatics analyzes with these genes were performed using the
223 miRWalk database (link) to predict the validated miRNAs modulated by curcumin.

224

225 **Western blot analyses**

226 HUVECs pre-exposed to curcumin or vehicle were harvested at the end of a 4-hour-TNF- α activation
227 period and washed twice with ice-cold PBS. Total proteins were extracted using lysis buffer
228 containing 50 mmol/L Tris pH 7.8, 150 mmol/L NaCl, 0.5% sodium deoxycholate, 1% NP40, anti-
229 protease and anti-phosphatase. Protein concentration was determined using BCA protein assay reagent
230 Kit (Interchem). Lysates were loaded onto a 10% SDS-polyacrylamide gel for electrophoresis and then
231 transferred onto immobilon-P membrane (GE Healthcare). The membrane was incubated in 5%
232 (wt/vol) dried milk protein in TBS containing 0.05% Tween-20 for 1 hour, and then further reacted
233 with primary antibodies: rabbit anti-iCAM (Santa Cruz, 1:1000), rabbit anti-vCAM-1 (1:1000, GTX),
234 rabbit anti-NF κ B total (1:1000, Cell Signaling), rabbit anti-NF κ B phosphor-ser536 (1:1000, Cell
235 Signaling), rabbit anti-I κ B α total (1:1000, Cell Signaling) and rabbit anti-I κ B α phosphor-ser32
236 (1:1000, Cell Signaling). After extensive washes, membrane was incubated with anti-rabbit IgG
237 antibody conjugated to HRP (1:5000, Santa Cruz, USA). Protein bands were visualized using ECL
238 detection kit (Millipore, USA) and then analyzed using Image J software (www.imagej.nih.gov).
239 Graphs represent protein level expressed as the mean +/- standard deviation of 4 independent
240 experiments.

241

242 **Molecular docking analysis**

243 Prior to virtual screening (docking), an academic license of *MarvinSketch* was used for drawing,
244 displaying of 2D structure and 3D optimization of curcumin and generation of the required input file
245 for docking (Tripos MOL2 file format), MarvinSketch 16.10.24.0, 2016, ChemAxon
246 (<https://www.chemaxon.com>). Docking also requires the 3D structure of the target and it was
247 necessary to cross-reference 2 on-line databases in order to identify the possible targets and their high-
248 resolution complete 3D structures – a resolution better than 2.0 Å being recommended for docking
249 [25]: The Universal Protein Resource – UniProt (<http://www.uniprot.org>) and RCSB Protein Data
250 Bank – PDB (<http://www.rcsb.org>). Since our search criteria did not had positive feed-back, were
251 constructed homologues models with the help of SWISS-MODEL[26]. Virtual screening of curcumin
252 against the selected targets was conducted with *PyRx* – Python Prescription 0.9.2 using AutoDock
253 Vina [27] as docking software. AutoDock Vina uses a scoring function inspired by X-score and tuned
254 with PDBbind dataset [28] to predict the noncovalent binding of macromolecules and automatically
255 calculates the grid maps and clusters the results. Data extraction and rendering of docking poses were
256 performed with Molegro Molecular Viewer 2.5 (MMV – Molegro, A CLC bio company, Aarhus N,
257 Denmark). The "backface culling" method was used for simulation of the molecular surface of
258 proteins. This method allowed us to determine whether a polygon of a graphical object is visible (e.g.,
259 primary structure, secondary structure, or inner part of binding side shown as a molecular surface);
260 when it was not visible, the polygon was "culled" from rendering process, which increases efficiency
261 by reducing the number of polygons the hardware has to draw [29]. All docking runs were conducted
262 in an extended search space, set to cover the entire target, with a volume bigger than 27.0 Å³ and the
263 exhaustiveness was manually increased to 80 in order to improve the accuracy of predictions.

264

265 **Statistical analyses**

266 Prism software, version 6.0.c (GraphPad, La Jolla, CA) for Macintosh, was used for the statistical
267 analysis of data. Data were analyzed using one-way analysis of variance (ANOVA) and Dunnett's
268 multiple comparison test. For the analysis of gene expression data, two-way ANOVA was performed,
269 followed by Dunnett's multiple comparison test with a single pooled variance. A value of $p < 0.05$ was
270 considered significant.

271

272

273

274 **3. Results**

275

276 **Curcumin decreases both monocyte adhesion to endothelial cells and their transendothelial**
277 **migration (TEM)**

278 As expected, activation of HUVECs by TNF- α stimulated the firm adhesion of monocytes to the
279 endothelial surface compared to those incubated with the non-stimulated HUVECs (3-fold increase;
280 $p < 0.001$) (fig. 1A). This adhesion was significantly reduced by 25% ($p < 0.001$) in HUVECs pre-
281 exposed to curcumin at 0.5 μM and 1 μM compared to vehicle-treated endothelial cells.

282 Regarding TEM, fig. 1B shows that the chemoattractant protein MCP-1 induced a 4.5-fold higher
283 monocyte migration in a transwell assay compared to the cells incubated without MCP-1 ($p < 0.001$).
284 The magnitude of this MCP-1-induced migration was significantly reduced when HUVECs were pre-
285 treated with 1 μM curcumin (-24%, $p < 0.05$).

286

287 **Curcumin preserves endothelium permeability and cell surface area during inflammation**

288 Cell permeability assays, performed using Transwell system, revealed that stimulation of HUVECs by
289 TNF- α resulted in a significant 4-fold increase of endothelial permeability to FITC-dextran (fig. 2A).
290 This increase in permeability was inhibited by 34% with a pre-incubation of endothelial cells with 1
291 μM curcumin ($p = 0.028$). In addition, the endothelial cell surface within the endothelium cell
292 monolayer was modified in response to TNF- α induced monocyte adhesion to HUVECs (fig. 2B). A
293 50% decrease of the mean cell area of HUVECs was observed after 1 hour of monocyte/TNF-
294 α -activated endothelial cells interaction ($p < 0.01$). In TNF- α activated HUVECs pre-exposed to 1 μM
295 curcumin, the surface of HUVECs decreased by only 25%. Thus, exposure of endothelial cells to 1
296 μM curcumin reduced by 50% the TNF- α -induced contraction of HUVECs ($p < 0.05$).

297

298 **Curcumin affects neither chemoattractive capacity of TNF- α -activated endothelial cells nor the**
299 **expression of MCP-1**

300 Fig. 3A shows that monocyte chemotaxis induced by the culture medium from TNF- α -activated

301 endothelial cells was similar to the chemotaxis induced by the chemokine MCP-1. In addition, we
302 observed that the treatment of endothelial cells with 1 μ M curcumin did not affect the endothelial
303 mediated monocyte chemotaxis. The expression of the gene coding for MCP-1 in endothelial cells was
304 quantified by RT-qPCR (fig. 3B). A 4-hour treatment of HUVECs with TNF- α highly increased the
305 expression of this gene ($p < 0.01$). Pre-exposure of endothelial cells to curcumin before their activation
306 by TNF- α had no significant effect on the TNF- α -induced expression of the MCP-1 gene.

307

308 **Curcumin decreases monocyte-endothelial interaction under shear stress**

309 Under physiological shear stress (~ 7 dyne/cm²), TNF- α significantly increased the tight adhesion of
310 monocytes to endothelial cells by 20-fold (Fig. 4A and suppl. Movie). The pretreatment of HUVECs
311 by 1 μ M curcumin significantly reduced monocyte adhesion by 36% ($p = 0.017$). A tendency for a
312 decrease was observed with 0.5 μ M of curcumin but did not reach significance.

313 Time-lapse microscopy assessing monocyte transendothelial migration under shear stress showed that
314 the pretreatment of HUVECs with 0.5 μ M or 1 μ M curcumin tended to decrease the number of
315 migrated monocytes in comparison to migration observed for the vehicle-treated cells under shear
316 stress (Fig. 4B and suppl. Movie).

317

318 **Curcumin modulates expression of genes in HUVECs**

319 Of 93 genes for which the expression of TEM was evaluated, seventeen were identified as not
320 expressed in our condition (fig. 5A and suppl. Table 1). Our nutrigenomic study revealed that the
321 exposure of endothelial cells to curcumin prior their activation by TNF- α significantly modulated the
322 expression of sixteen genes. Bioinformatic analyses showed that these differentially expressed genes
323 are involved in different cellular processes, such as antioxidant defense (SOD-1), metabolism
324 (IGFR1), cell signaling (CAV1, CASK, PDPK1), focal adhesion (PXN, VCL, ADAM10), intercellular
325 junction (TJP2) and cytoskeleton organization (CDC42 subunits, ARPC2, PAK4, ACTN1, VIM). For
326 three genes, tendencies in the modulation of their expression were observed; these genes encode two

327 small Rho GTPases involved in the regulation of the cytoskeleton (RHOC and RAC1) and for the
328 CALD1 gene which encodes caldesmon 1.

329 Additionally, bioinformatic analysis, performed using the miRWalk database, identified 459 putative
330 miRNAs potentially modulated by curcumin and involved in the regulation of the aforementioned
331 genes differentially expressed (data not shown).

332 Moreover, in TNF- α non-stimulated endothelial cells, a low basal expression of cell adhesion proteins
333 (VCAM-1 and ICAM-1) was observed (fig. [5B6 A-C](#)). A 4-hour treatment of endothelial cells with
334 TNF- α highly increased the expression of these two proteins. The expression of these proteins was not
335 affected by the exposure of HUVECs to curcumin prior to TNF- α -activation.

336

337 **Curcumin modulates phosphorylation of NF- κ B cell-signaling proteins**

338 We observed that a 3-hour exposure to 0.5 μ M or 1 μ M of curcumin in TNF- α non-stimulated
339 endothelial cells did not modify the basal phosphorylation level of NF- κ B p65 and I κ B α (fig. 6).

340 Following stimulation with TNF- α , levels of phosphorylated NF- κ B p65 (ser536) and phosphorylated
341 I κ B α (ser32) were increased, and the total amount of I κ B α was reduced compared to those observed

342 in the non-stimulated endothelial cells. As a result, the phosphorylated/total ratio of NF- κ B p65 and
343 I κ B α were higher in TNF- α -activated cells compared to the non-stimulated cells. Pre-exposure of

344 HUVECs to 0.5 μ M curcumin prior to their stimulation to TNF- α did not modify the
345 phosphorylated/total ratio of NF- κ B p65 and I κ B α . In contrast, the exposure to 1 μ M curcumin

346 significantly reduced the level of phosphorylation of NF- κ B p65 ([fig.6 A and D](#)) without affecting that
347 of I κ B α ([fig.6 A and E](#)).

348

349 **Curcumin reveals favorable binding properties to cell-signaling proteins**

350 The analysis of protein binding between curcumin and cell-signaling proteins of the NF- κ B signaling
351 pathway revealed that of the 13 proteins evaluated (Table 1 & suppl. Table 2), 2 protein structures

352 were not available, and 7 computational predictions had a low degree of confidence due to low
353 resolution of the 3D structure of the target (resolution > 2.0 Å is recommended for docking) [25]. *In*

354 *silico* predictions demonstrated that curcumin could interact directly with TAK1 and PDK1 to form a
355 covalent bond within the active site of these proteins with a binding affinity of -7.8 Kcal/mol and -6.5
356 Kcal/mol, respectively (Table 1). Curcumin can also interplay with the kinases AKT 1 and AKT 2
357 through a non-covalent interaction of -7.3 kCal/mol and -6.3 kCal/mol, respectively. These
358 interactions can be seen in fig. 7.

359

360

361

362 **4. Discussion**

363 In this study, we set out to identify the cellular effects and the molecular mechanisms responsible
364 for the ability of curcumin to prevent impairments in endothelial cell function. A special focus was on
365 the impact of curcumin on endothelial cell processes involved in the interactions between vascular
366 endothelium and circulating immune cells.

367 The bioavailability of pure curcumin is known to be very low in humans, with plasma
368 concentrations most often not detectable for administered dose below 4 grams and peaking at 1.77 μM
369 after the intake of 8 g of curcumin [30-33]. However, in recent years many strategies have been
370 developed to markedly increase the bioavailability of oral curcumin, including the use of natural
371 enhancers or of encapsulation systems to improve its delivery [34], with a positive impact on the bio-
372 efficacy of curcumin in clinical studies [35]. With such approaches, the concentrations of curcumin
373 tested in the present study (0.5 and 1 μM) could be easily reached in the plasma of human subjects
374 consuming a reasonable dose of curcumin. Our study showed a reduction of monocyte adhesion to
375 endothelial cells exposed to curcumin (0.5 μM and 1 μM) for 3 hours that corresponds to the resident
376 time of curcumin within blood circulation after curcumin intake [23]. This effect did not seem to result
377 from an altered monocyte chemotaxis as revealed by the lack of effect of curcumin on chemoattractive
378 potency of activated endothelial cells. This result is not consistent with previous studies reporting that
379 curcumin can alter the expression of the gene coding for chemoattractant chemokine (MCP-1) in
380 several cell lines [36]. However, these studies used curcumin at much higher concentrations than those
381 used in the present study.

382 To mimic the endothelial dysfunction observed in vascular diseases [21], endothelial cells were
383 activated by the inflammatory cytokine TNF- α . This activation induces the expression of cell adhesion
384 molecules such as ICAM and VCAM, which are involved in the firm adhesion of monocytes [20]. In
385 our *in vitro* conditions, curcumin at 1 μ M significantly reduced monocyte adhesion, but it did not
386 modify the expression of adhesion molecules induced by TNF- α . This observation is not in line with
387 results from other studies showing a reduction of monocyte adhesion to endothelial cells along with a
388 decrease in the expression of adhesion molecules. This discrepancy could result from differences in
389 the experimental conditions used, for example, in previous studies, endothelial cells were treated with
390 higher concentrations of curcumin ($\geq 5 \mu$ M) for 1 hour prior to TNF- α activation [12-14].
391 Nevertheless, the chronic consumption of curcumin has been associated *in vivo* with a decrease in the
392 expression of genes coding for ICAM-1 and VCAM-1 in the aorta of atherosclerotic rabbits [9].
393 Tethering of monocytes with endothelial cells activates ICAM-1 and VCAM-1 clustering in lipid raft
394 domain of the endothelial membrane [37-39] resulting in the firm adhesion of monocytes to the
395 endothelial cells prior to their extravasation [39-41]. Wójciak-Stothard et al. [42] have demonstrated
396 that inhibition of adhesion molecule clustering reduced the adhesion of monocytes to activated
397 endothelial cells. Moreover, it has been reported that curcumin, at 5 to 10 μ M, may affect
398 delocalization of integrin and the formation of a signaling complex at a lipid raft, both of which are
399 required for cell mobility [43, 44]. Taken together, these results suggest that the decrease in monocyte
400 adhesion to endothelial cells could result from the capacity of curcumin to affect the subcellular
401 movement of adhesion molecules required for monocyte adhesion and diapedesis.

402 Following the firm adhesion to endothelial cells, monocytes undergo diapedesis. For the first
403 time, we showed that curcumin can also reduce monocyte transendothelial migration. Extravasation of
404 monocytes across the endothelium requires regulation of endothelial junctional permeability [18]. In
405 addition, we demonstrated that 1 μ M curcumin reduced the TNF- α -induced increase in permeability
406 of the confluent monolayer of HUVECs. In agreement with these results, Li et al. previously reported
407 that oral supplementation with curcumin protects against the alteration in permeability of the coronary
408 artery as measured in a rat model of coronary disease [22]. Moreover, in western-diet induced

409 atherosclerotic mice, curcumin has been previously shown to reduce intestinal epithelium permeability
410 by acting on tight junction proteins involved in the selective barrier function such ZO-1 and claudin 1
411 [10, 18]. Endothelial permeability is controlled not only by the regulation of cell-cell junctions but
412 also by cytoskeleton dynamics [45]. Indeed, re-organization of actin into contractile stress fibers or
413 microtubule disassembly increases endothelial contraction, resulting in interendothelial gap formation
414 and consequently an increase in permeability. Interestingly, we observed that endothelial cells pre-
415 exposed to curcumin were less sensitive to TNF- α induced modification of the cell surface, which is
416 dependent on the actin cytoskeleton. All together, these results suggest that curcumin may have an
417 impact on endothelial permeability by acting on the regulation of junction proteins and on the
418 dynamics of the actin cytoskeleton. This hypothesis is in agreement with the lower number of
419 monocytes within the atheromatous lesion observed in curcumin-supplemented ApoE^{-/-} mice [6].

420 It is worth noting that endothelial cells in vessels are exposed to the luminal surface of blood flow
421 that induces a physical strain called shear stress [46, 47]. This physiological shear stress is known to
422 activate endothelial mechanoreceptors resulting in the induction of the expression of transcription
423 factors such as Krüppel-like factor 2 (KLF2) and nuclear factor (erythroid-derived 2)-like 2 (NRF2),
424 among others [48]. The activation of these factors contributes to the preservation of endothelial
425 function by inducing vasodilatory, antioxidant, antithrombotic, antiadherent and antiangiogenic
426 responses. Here, we demonstrated for the first time that the exposure of endothelial cells to low
427 concentrations of curcumin under physiological shear stress could reduce monocyte adhesion and
428 extravasation. To the best of our knowledge, only one other study, that of Chacko et al., demonstrated
429 a similar observation under flow in response to 1 μ M of isoflavone, another subfamily of polyphenols
430 [49, 50]. In addition, we observed that the magnitude of the effect of curcumin on adhesion and TEM
431 in the condition of shear stress was markedly higher (+44% and +37%, respectively) than those
432 observed in the static condition. Hence, one can speculate that curcumin may potentiate the beneficial
433 effect of physiological shear stress on endothelial function.

434 To decipher the underlying mechanisms of action of curcumin, we also screened for the
435 expression of 93 genes coding for adhesion and permeability proteins in response to curcumin
436 exposure in endothelial cells for the first time. Among the genes significantly modulated by the

437 exposure to curcumin, our results revealed the overexpression of PXN, VIM and VCL, coding for
438 paxilin, vimentin and vinculin, respectively, which are three proteins involved in the focal adhesion of
439 endothelial cells to extracellular matrix [19]. Down-regulation of such proteins was associated to an
440 increase in endothelial barrier permeability [48, 51]. Similarly, the expression of genes TJP2 and
441 CASK, coding the tight junction protein 2 (also called ZO-2) and the Ca²⁺/Calmodulin dependent
442 serine protein kinase (CASK), both involved in endothelial cell-cell junction [45], were shown to
443 increase in response to curcumin. Such change in the expression of these genes has been described to
444 be associated to a reduction in endothelial permeability [52]. Our gene expression assay also showed
445 that curcumin affects the expression of caveolin-1 (CAV1), which plays an important role in the
446 organization of protein interactions between cells and the extracellular matrix that contributes to
447 control endothelial permeability [53]. Moreover, we observed that curcumin modulated the expression
448 of two Rho GTPases (ARPC2 and CDC42), which are known to control microtubule dynamics and
449 actin-rich surface projections involved in TEM process [54], and the expression of the PDPK1 kinase,
450 which regulates cell migration [55]. In addition, we observed the modulation of PAK4 expression by
451 curcumin. PAK4 has been described to activate the downstream target RHOA involved in the
452 regulation of the assembly of focal adhesions and actin stress fibers [19]. The expression of caldesmon
453 (CALD1), a binding protein that plays a role in the stabilization of actin filament structure [56], was
454 also significantly upregulated in curcumin-exposed HUVECs. Taken together, the changes in gene
455 expression that we observed suggest that curcumin could maintain endothelium integrity by
456 reinforcing cell-cell and cell-matrix interactions, and by stabilizing endothelial cell cytoskeleton
457 through nutrigenomic effect. This hypothesis fits with the observed impact of curcumin on endothelial
458 cell function and surface area.

459 The molecular mechanisms of curcumin involve a large range of targets including signaling
460 pathways and transcription factors [16]. In a previous animal study, we demonstrated that a dietary
461 supplementation with 0.2% curcumin inhibits atherosclerosis by affecting NF-κB-transcriptional
462 activity [6]. In the present *in vitro* study, we have stimulated endothelial cells with TNF-α, an
463 activator of the NF-κB signaling pathway [57], and we showed that 1 μM of curcumin significantly

464 reduced the phosphorylation of NF- κ B p65 (serine 536). Others have previously described such an
465 inhibition with curcumin in a similar range of concentrations [12, 13, 21]. In contrast to previous
466 observations [12-14], in our experimental conditions, inhibition of NF- κ B by curcumin did not result
467 in a lowering of the expression of NF- κ B target genes such as ICAM and VCAM. However, Cobb et
468 al. [58] reported that induction of VCAM1 can be NF- κ B-independent and that proteasome can
469 regulate VCAM-1 and ICAM-1 expression in endothelial cells without affecting NF- κ B activation.
470 Recently, Hasima et al. [59] suggested that curcumin can modulate proteasomal pathways in a dose-
471 dependent manner. Thus, it can be speculated that curcumin may regulate the expression of adhesion
472 molecules in a different way than the NF- κ B pathway, potentially by acting on proteasomal activity.

473 The inhibition of the NF- κ B pathway observed in response to exposure of endothelial cells to
474 curcumin is also supported by our results obtained *in silico* that revealed favorable binding of
475 curcumin to some kinases, namely, TAK1, PDK1 and AKT. TAK1 and PDK1 have been reported to
476 activate the NF- κ B canonical pathway by phosphorylating the I κ B Kinase β (IKK β) [60]. AKT is
477 known to be activated by TNF- α stimulation [61] and to regulate the activity of NF- κ B by inducing
478 phosphorylation and subsequent degradation of inhibitor of NF- κ B (I κ B) [62]. Regarding the binding
479 mode of curcumin in the active domain of these kinases, the occupancy and the interactions we
480 observed mimic those of a heterocyclic core of known inhibitors of kinases that have been shown to
481 attenuate NF- κ B activation [63-65]. Similarly, Gupta et al. reported that curcumin might interact
482 directly with a large range of targets including signaling proteins and protein kinases, leading in their
483 inhibition [66]. In addition, *in silico* structural evaluation highlighted that quercetin, another
484 polyphenol, may interact and therefore inhibit serine/threonine kinases such as AKT [67].
485 Collectively, our *in silico* analysis suggests that curcumin can bind to cell-signaling proteins and affect
486 their activity, which consequently results in modulation of the expression of genes as we observed.

487 Another possible mechanism of action by which curcumin could modulate adhesion and
488 monocyte transendothelial migration in endothelial cells may involve the modulation of miRNAs.
489 miRNA are noncoding single-strand RNAs of 22 nucleotides that constitute a class of gene regulators
490 at the posttranscriptional level [68]. Among the miRNAs potentially modulated by curcumin and

491 involved in the regulation of the 15 aforementioned genes shown as differentially expressed in
492 response to curcumin in the present study, 4 were previously detected as modulated by dietary
493 curcumin in murine ApoE^{-/-} liver (miR-137, miR-188-5p, miR-324-5p and miR-335-5p) [69]. MiR-
494 137 has been described as a regulator of Cdc42 expression and a Rho GTPase [70], and miR-335-5p is
495 involved in cytoskeleton dynamics [71]. In addition, Howell et al. have recently described the ability
496 of curcumin to modulate 29 miRNAs in epithelial cell line ARPE-19 [72]. Nine of these miRNAs
497 were reported to regulate the expression of cell-cell junction proteins and adhesion molecules
498 (miR181a, miR18a, miR150 miR101, miR126, miR181b, miR27a, miR155, and miR20A) [73]. These
499 findings were supported by bioinformatic analyses suggesting that the miRNAs detected as modulated
500 by curcumin in murine ApoE^{-/-} liver [69] and in murine melanoma [74] may regulate several pathways
501 influencing cell communication and focal adhesion. Therefore, further investigation is needed to
502 determine the impact of curcumin on miRNAs as post-transcriptional mode of regulation of monocyte
503 TEM in endothelial cells.

504 In conclusion, this *in vitro* study provides new mechanistic insights into the ability of dietary
505 curcumin to preserve vascular health during inflammation, as summarized in fig. 8. Our findings
506 clearly support that, in physiologically relevant conditions, exposure of endothelial cells to curcumin
507 can reduce endothelial permeability and monocyte adhesion and transmigration, ultimately promoting
508 vascular integrity. This effect seems to be related to a complex and multimodal action of curcumin on
509 the intracellular dynamics of endothelial cells. However, these mechanisms warrant further
510 investigation to be fully understood.

511

512 **Statement of authorship**

513 The authors' responsibilities were as follows: LEM designed methods and experiments, carried out the
514 experiments, analyzed the data and wrote the paper; SM carried out cell experiments and flow
515 cytometry analysis, and DB performed the gene expression assay; RT performed molecular docking
516 analysis; NBC participated in discussion and revision of the article; DM & CM carried out study
517 design, data interpretation and manuscript preparation. All authors have contributed to read and
518 approved the manuscript.

519

520

521 **Conflict of interest**

522 None of the authors has any conflict of interest to declare.

523

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526 variation in response to consumption of plant food bioactives and determinants involved).

527

528

529 **Legends**

530

531 **Figure 1: Effect of curcumin on monocyte adhesion to endothelial cells and their**
532 **transendothelial migration (TEM) in the static condition of culture.** (A) Monocyte adhesion to
533 TNF- α activated HUVECs after a 3-hour exposure of endothelial cells to curcumin. In the absence of
534 curcumin, HUVECs were exposed to vehicle (0.2% DMSO). The results are expressed as a percentage
535 of monocyte adhesion to 'vehicle+TNF- α ' treated HUVECs that is normalized at 100%. (B) TEM of
536 monocytes in response to MCP-1 across HUVEC monolayer exposed to curcumin or vehicle for 3
537 hours. The results are expressed as the percentage of migration measured in the vehicle+MCP-1
538 condition that is normalized at 100%. Bars represent the mean \pm SEM of 3-4 independent
539 experiments. Significances: * $p \leq 0.05$, ** $p \leq 0.01$ *** $p \leq 0.001$.

540

541 **Figure 2: Effect of curcumin on endothelium integrity.** (A) Permeability of the HUVEC
542 monolayer to FITC-dextran (40 kDa) after 3-hour exposure to vehicle or curcumin (0.5 and 1 μ M)
543 followed by 4-hour TNF- α activation. The results are expressed as a percentage of permeability
544 measured in 'vehicle+TNF- α ' treated HUVECs that is normalized at 100% (n=4). (B, C) Impact of
545 curcumin exposition on mean HUVEC surface area measured after monocyte/TNF- α -activated
546 endothelial cells interaction. (B) Representative immunostainings of the actin cytoskeleton of
547 HUVECs after 3-hour exposure to vehicle or curcumin (1 μ M) followed by 4-hour TNF- α activation
548 and co-incubation with monocytes (Magnitude: 20x). (C) Quantification of the mean HUVEC surface
549 area. The results are expressed in comparison to the mean area measured at steady state (n=3).
550 Significances: * $p \leq 0.05$, *** $p \leq 0.001$.

551

552 **Figure 3: Chemoattractive potency of TNF- α -activated endothelial cells in response to**
553 **curcumin.** (A) Transmigrated monocytes stimulated by the conditioned media harvested from
554 vehicle/-TNF- α -, vehicle/+TNF- α - and Cur 1 μ M/+TNF- α -HUVECs were assessed using a transwell

555 assay. Bars represent means \pm SEM (n=4). (B) Relative expression of gene coding the
556 chemoattractive protein MCP-1 normalized first to the expression of the housekeeping gene GAPDH
557 and then to the relative expression detected in vehicle/+TNF- α -treated HUVECs. Values indicate fold-
558 changes expressed as the mean \pm SEM (n=4). Significances: *p \leq 0.05, **p \leq 0.01 ***p \leq 0.001.

559 **Figure 4: Effect of curcumin on monocyte adhesion to endothelial cells and their**
560 **transendothelial migration under shear stress.** (A) Monocyte adhesion to TNF- α activated
561 HUVECs after a 3-hour exposure of endothelial cells to curcumin under 1 Hz pulsatile and 7 dyne/cm²
562 shear stress. The results are expressed as a percentage of monocyte adhesion to 'vehicle/+TNF- α '
563 treated HUVECs that normalized at 100%. (B) Transmigrated monocytes accumulated throughout the
564 entire 10-minute duration of the adhesion assay. The results are expressed as a percentage of
565 transmigrated monocytes to 'vehicle/+TNF- α ' treated HUVECs that is normalized at 100%.
566 Significances: *p \leq 0.05, **p \leq 0.01 ***p \leq 0.001.

567
568 **Figure 5: Gene expression in curcumin-exposed HUVECs.** (A) Expression of 93 genes coding
569 proteins involving TEM quantified by TLDA. Values (mean \pm SEM, n=4) indicate gene expression
570 fold change measured in 1 μ M Cur/+TNF- α HUVECs compared to Vehicle/+TNF- α -exposed
571 HUVECs. (B) ~~Expression of endothelial adhesion molecules (ICAM and VCAM) at protein level.~~
572 *p \leq 0.05 in comparison to vehicle/-TNF- α group.

573
574 **Figure 6: Impact of curcumin on endothelial adhesion molecules and on NF- κ B signaling**
575 **pathway proteins in HUVECs.** (A) Representative images of a western blot showing the detection of
576 adhesion molecules, -total NF- κ B and phosphor-ser536, total I κ B α and phosphor-ser532, and GAPDH
577 in HUVECs exposed to curcumin (0.5 μ M and 1 μ M) or vehicle and stimulated by 1 ng/mL TNF- α
578 for 4h (vCAM and ICAM) or 15 min (NF- κ B and I κ B α). (B, C) Quantification of the expression of
579 endothelial adhesion molecules (vCAM and ICAM respectively). (D, E) Ratios of
580 phosphorylated/total NF- κ B and I κ B α respectively. protein are expressed as the means Graphs
581 represent means \pm SEM (n=3). *p \leq 0.05.

582

583 **Figure 7: 3D structures from *in silico* docking of curcumin to cell proteins of the NF- κ B**
584 **signaling pathway.**

585

586 **Figure 8: Overview of the demonstrated and putative mechanisms by which curcumin can**
587 **modulate endothelial cell activation.**

588

589 **Table 1: Binding score of curcumin interplay with cell-signaling proteins.** (N/A: none
590 adapted)

591

592

593 **Supplementary data**

594 **Suppl. Movie 1:** Monocyte rolling, tethering, adhesion and transmigration on TNF- α activated
595 HUVECs under physiological shear stress.

596 **Suppl. Table 1:** List of mRNAs with their fold-change in HUVECs exposed to 1 μ M curcumin and
597 activated by TNF- α compared to expression in TNF- α stimulated HUVECs. Significant differential
598 expressions are in blot.

599 **Suppl. Table 2:** Virtual screening of curcumin against selected targets involved in NF- κ B signaling
600 pathway

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819

820

Table 1

Target	Binding Affinity of curcumin (Kcal/mol)	Number of H-Bonds	Location of H-Bonds
PDK1	-6,5	1	Tyr126 of the active site
AKT1	-7.3	0	N/A
AKT2	-6.3	0	N/A
TAK1	-7.8	1	Lys227 (coil) of the protein kinase domain, blocking the access at the ATP binding site and interaction with MAPK8IP1

Figure 1

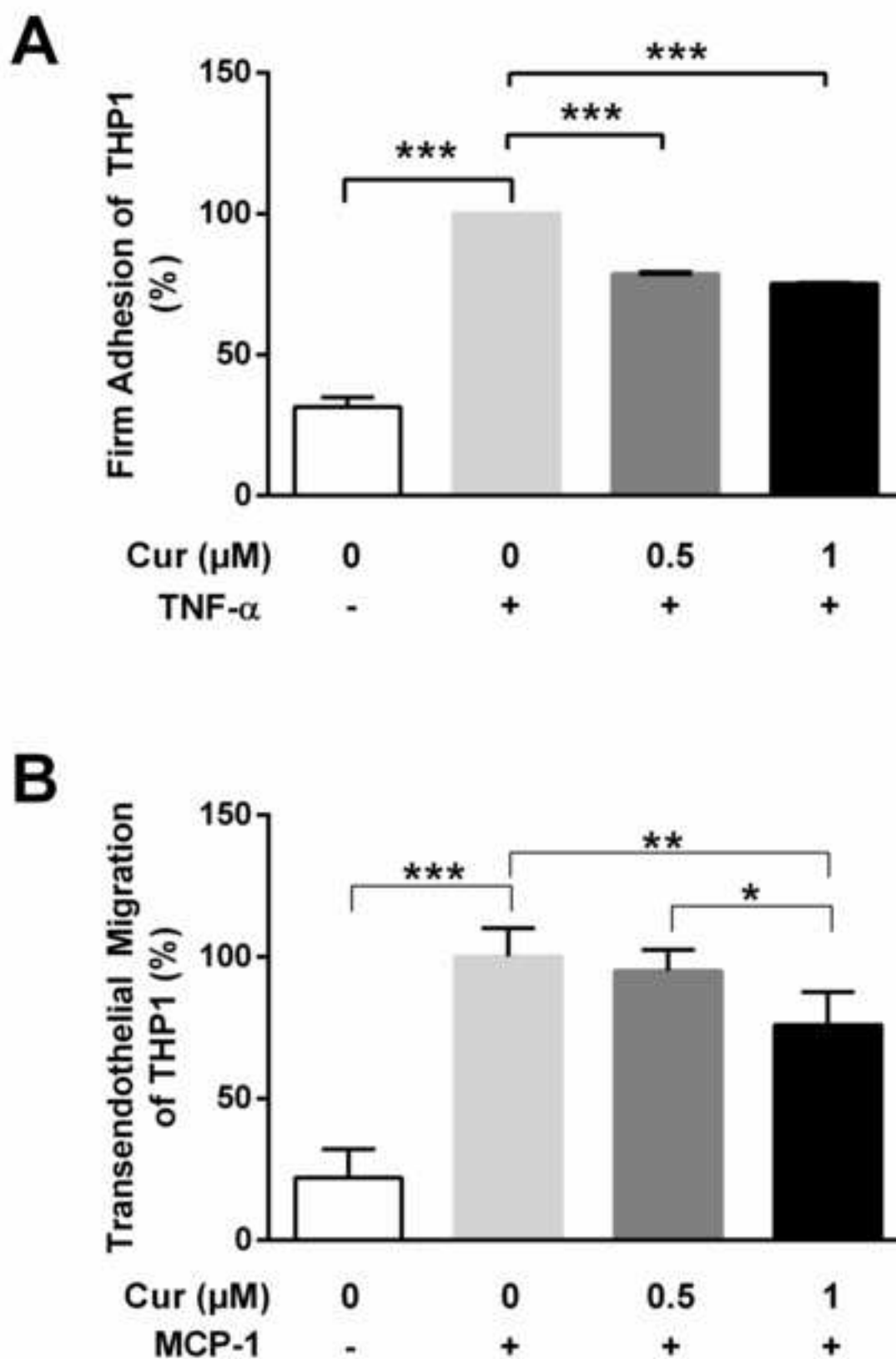


Figure 2

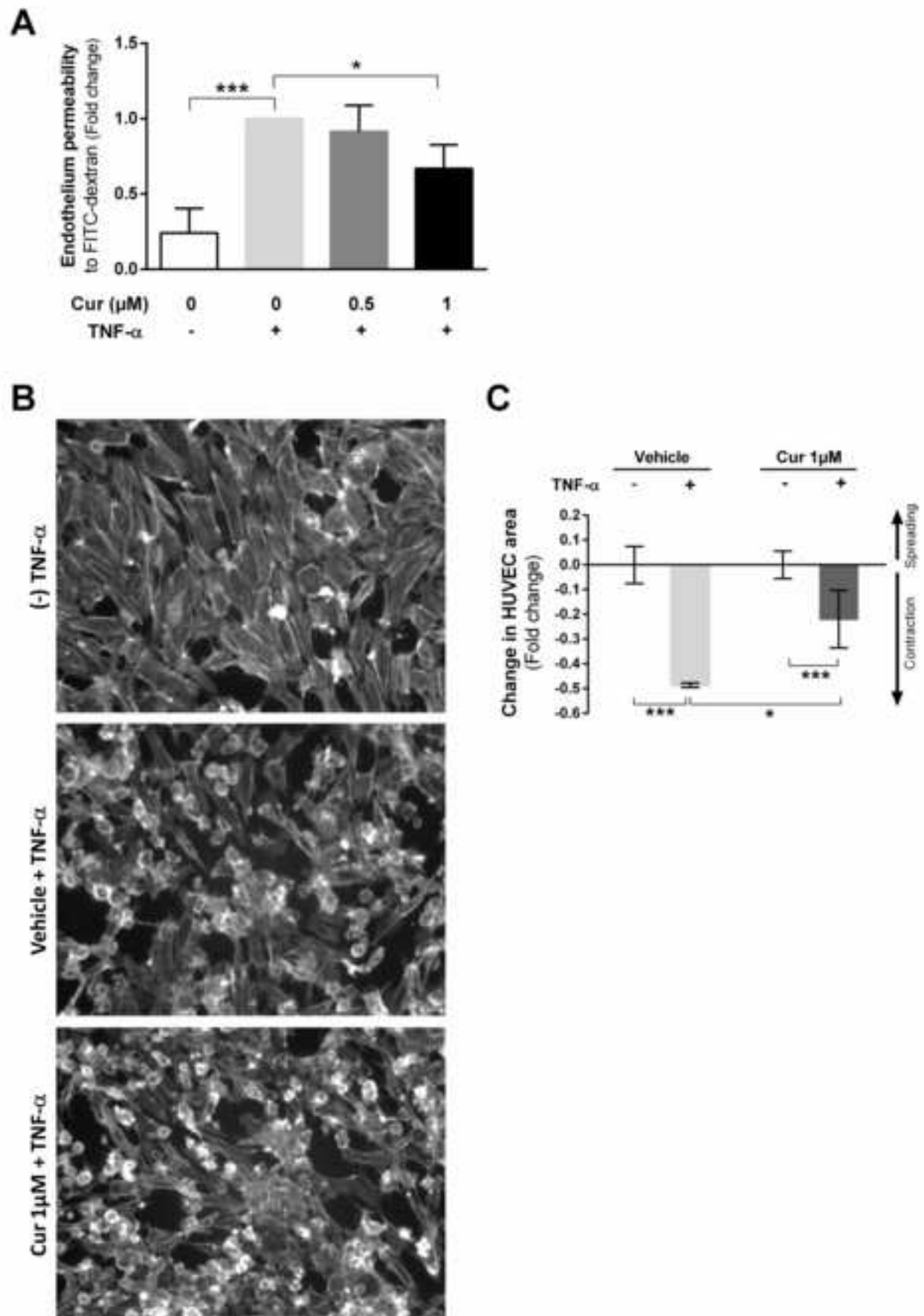


Figure 3

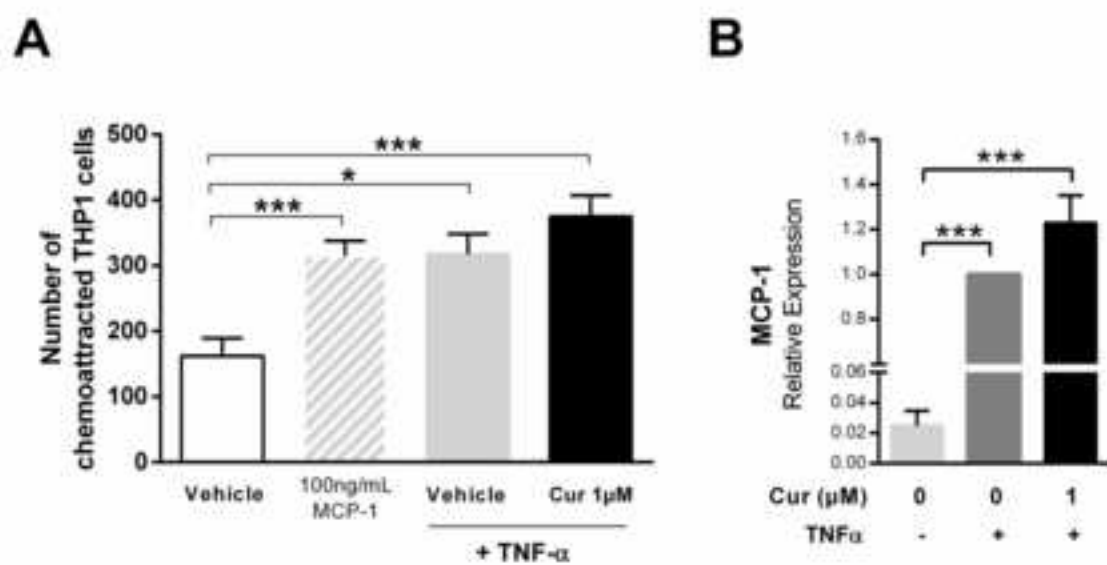


Figure 4

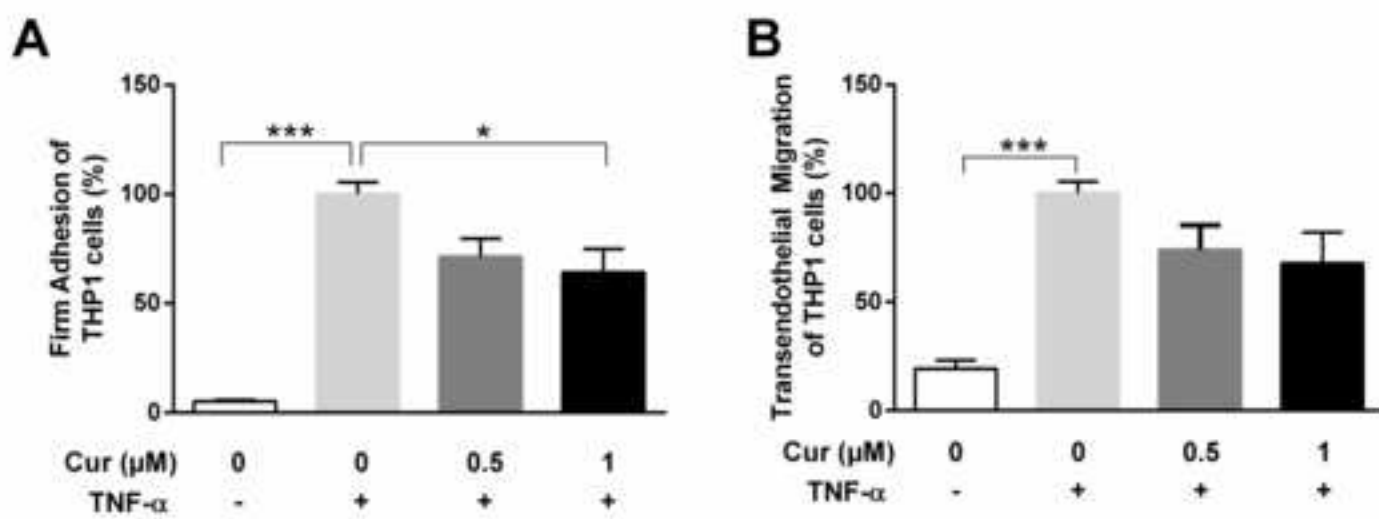


Figure 5

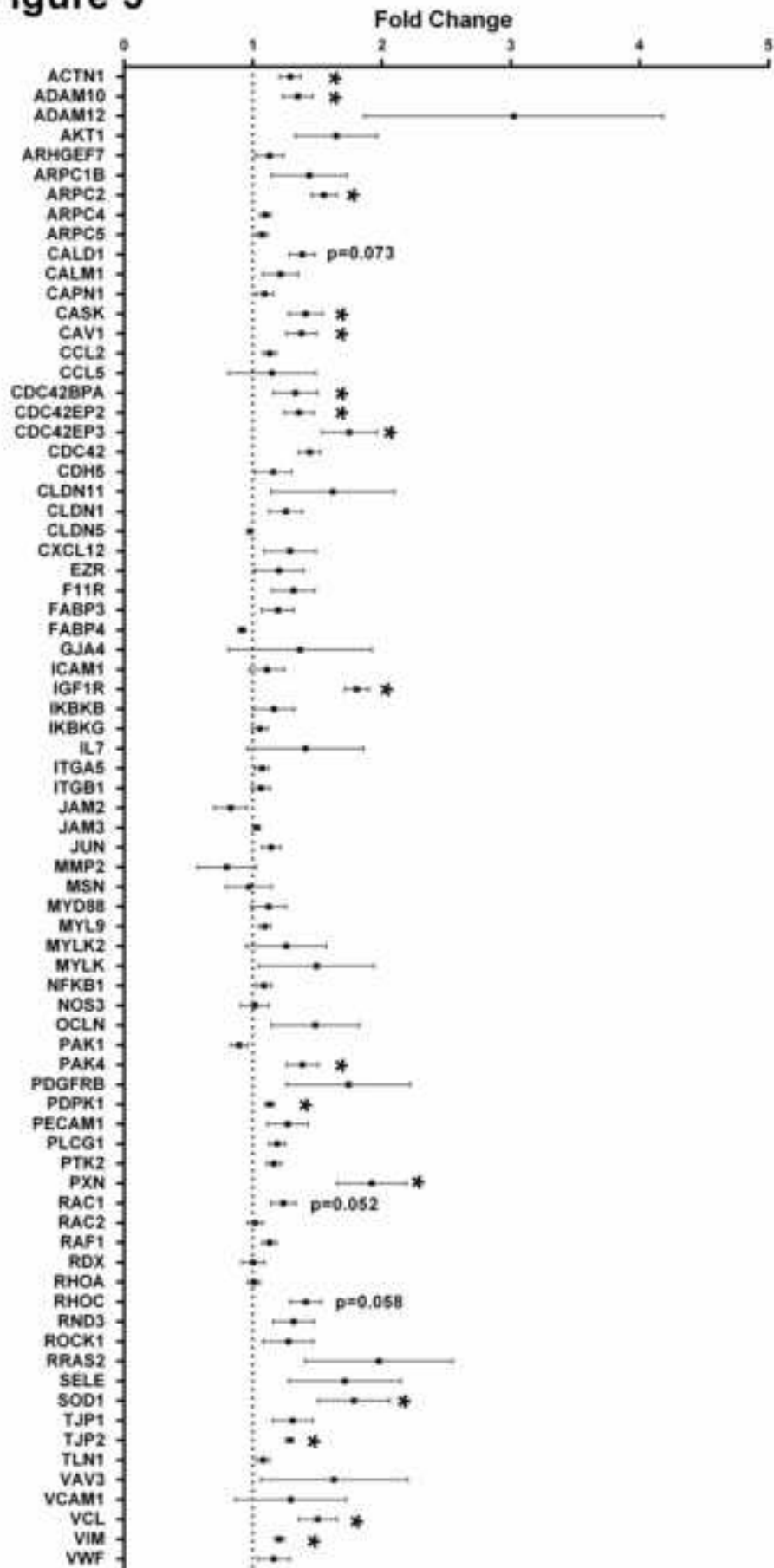
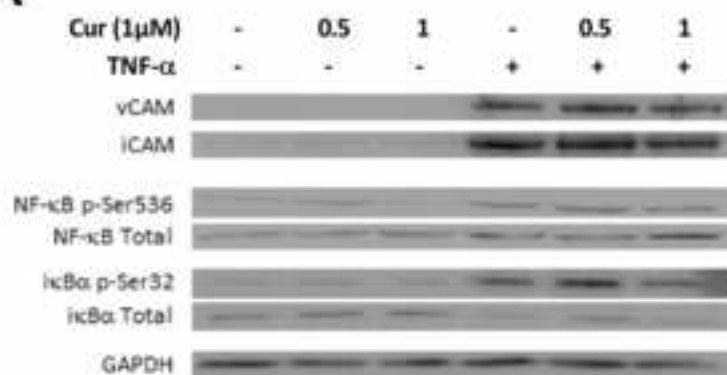
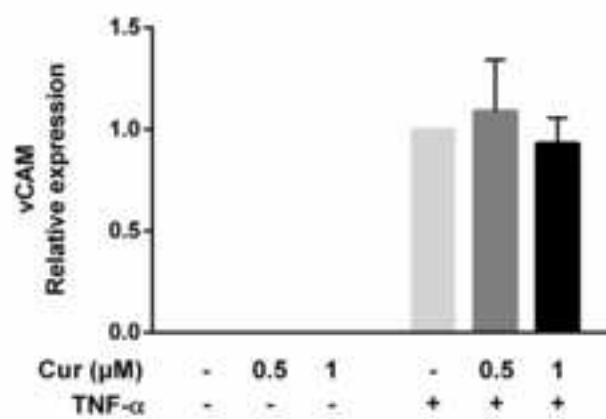


Figure 6

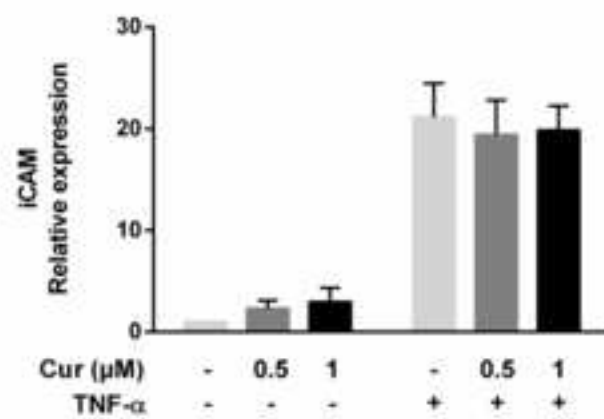
A



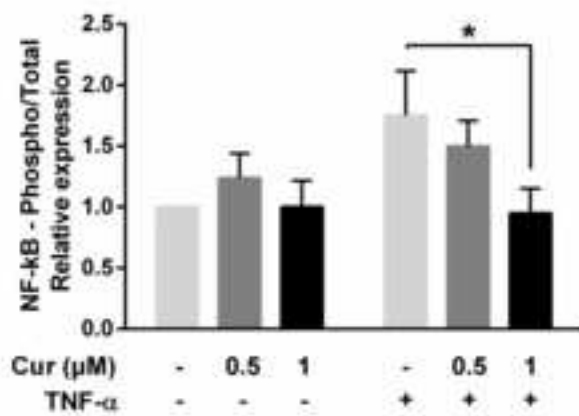
B



C



D



E

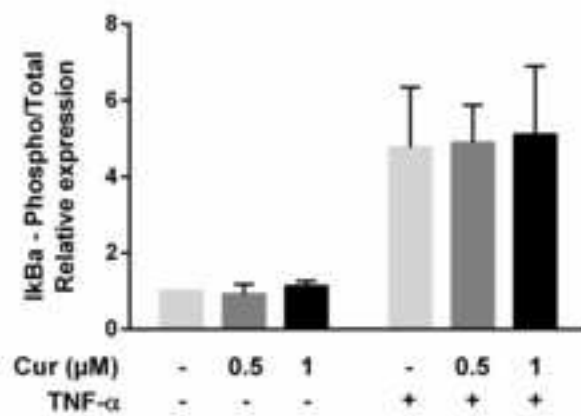
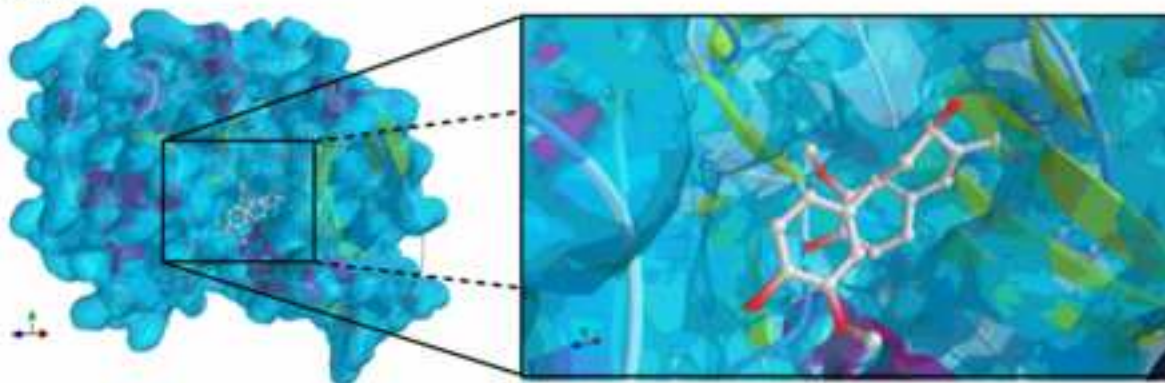
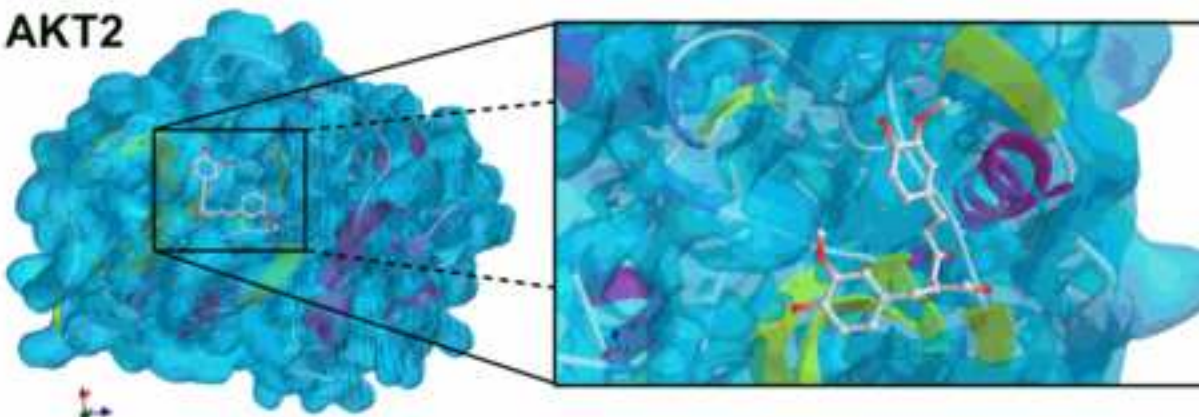


Figure 7

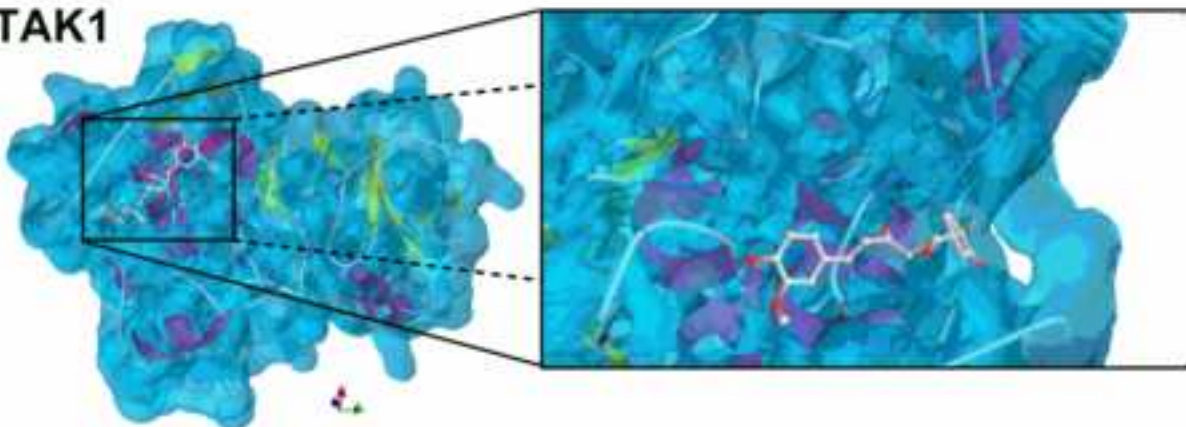
AKT1



AKT2



TAK1



PDK1

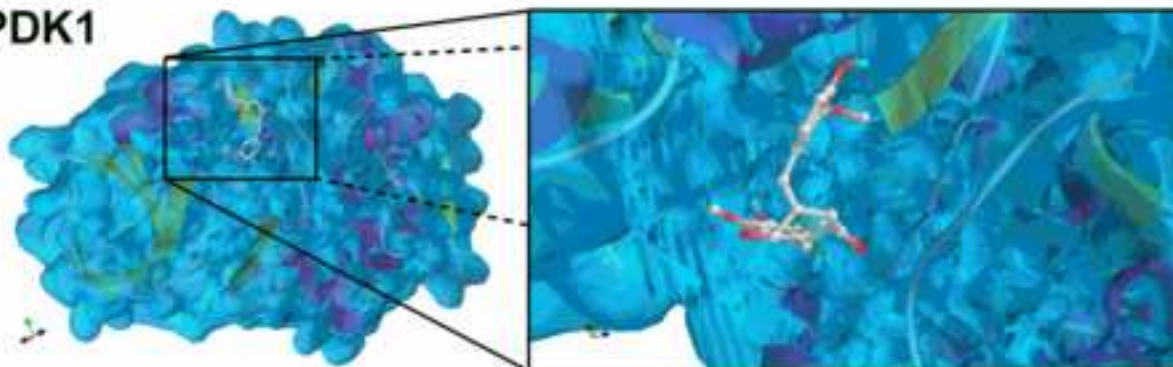
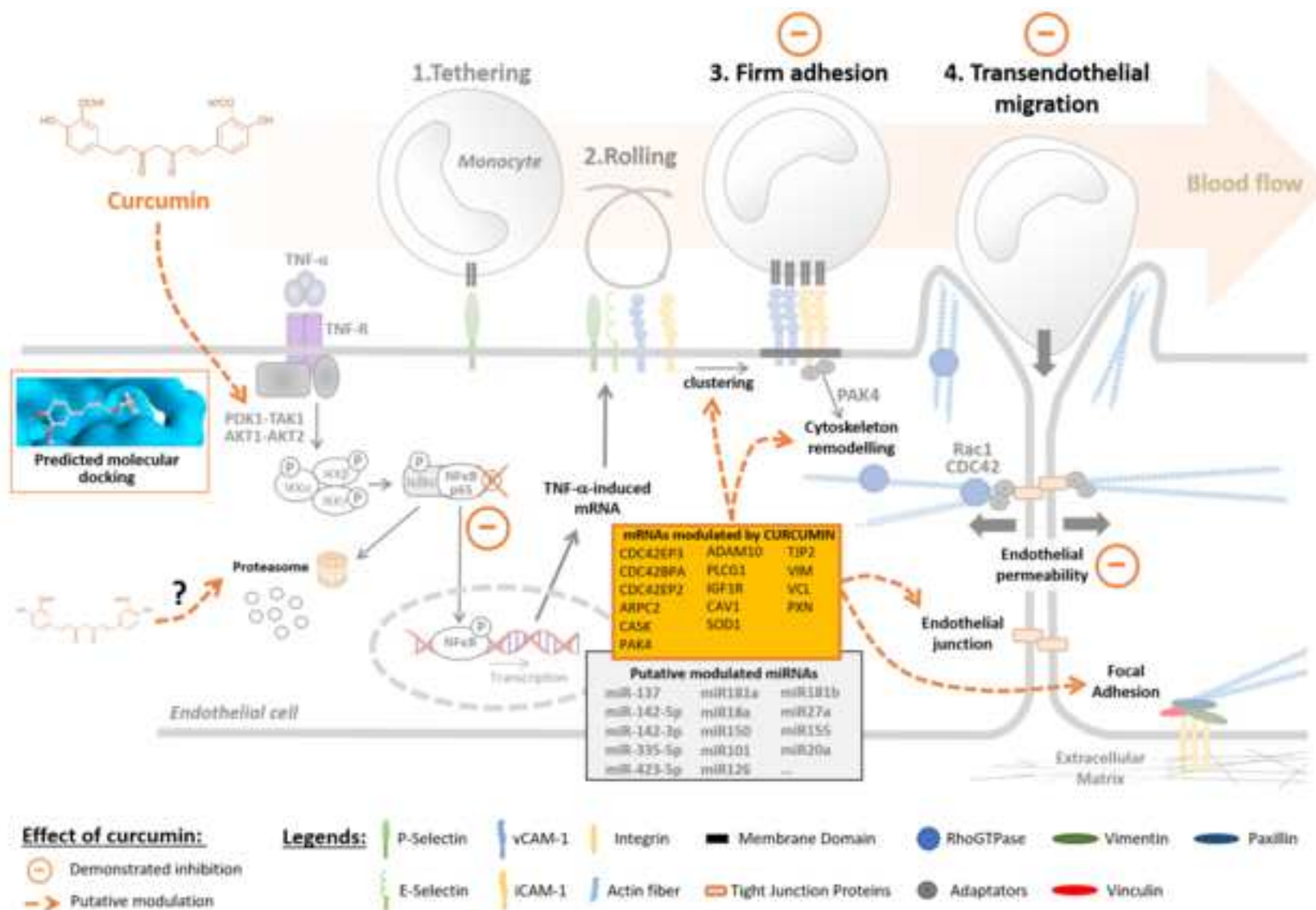


Figure8

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Suppl Table 1

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Suppl Table 2

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