

1 **Inhibition of histone deacetylase 1 or 2 reduces microglia activation through a gene**
2 **expression independent mechanism.**

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24

25 **Abstract**

26 Histone deacetylase (HDAC) inhibitors prevent neural cell death in *in vivo* models of cerebral
27 ischaemia, brain injury and neurodegenerative disease. One mechanism by which HDAC
28 inhibitors may do this is by suppressing the excessive inflammatory response of chronically
29 activated microglia. However, the molecular mechanisms underlying this anti-inflammatory
30 effect and the specific HDAC responsible are not fully understood. Recent data from *in vivo*
31 rodent studies has shown that inhibition of class I HDACs suppresses neuroinflammation and
32 is neuroprotective. In our study we have identified that selective HDAC inhibition with
33 inhibitors apicidin, MS-275 or MI-192, or specific knockdown of HDAC1 or 2 using siRNA,
34 suppresses the expression of cytokines interleukin-6 (IL-6) and tumour necrosis factor-alpha
35 (TNF- α) in BV2 murine microglia activated with lipopolysaccharide (LPS). Furthermore, we
36 found that in the absence of HDAC1, HDAC2 is upregulated and these increased levels are
37 compensatory, suggesting these two HDACs have redundancy in regulating the inflammatory
38 response of microglia. Investigating the possible underlying anti-inflammatory mechanisms
39 suggests an increase in protein expression is not important. Taken together, this study supports
40 the idea that inhibitors selective towards HDAC1 or HDAC2, may be therapeutically useful for
41 targeting neuroinflammation in brain injuries and neurodegenerative disease.

42

43 **Significance Statement**

44 The number of patients suffering a stroke or a neurodegenerative disease, such as Alzheimer's
45 is increasing. These conditions are severely debilitating and are leading causes of mortality,
46 with neural cell death and loss of brain tissue being a major feature. A number of mechanisms
47 contribute to neuronal death, including inflammation in the brain, but we still lack clinical
48 therapies to inhibit this. The work presented here provides further insight into potential
49 molecular therapeutic targets called histone deacetylases (HDACs), which are thought to

50 contribute to neural cell death by promoting inflammation. We show that down regulation of
51 HDAC1 and 2 is sufficient to reduce this inflammatory response. Our findings have clinical
52 relevance because they identify HDAC1 and 2 as promising targets for therapy.

53

54 **INTRODUCTION**

55

56 Microglia are the innate immune cells of the brain that are responsible for the excessive and
57 chronic neuroinflammatory response known to contribute to the pathogenesis of brain injury
58 and disease (Block et al., 2007; Glass et al., 2010). Inhibitors of histone deacetylases (HDACs)
59 reduce the inflammatory response of isolated microglia to stimulants such as
60 lipopolysaccharide (LPS) (Suuronen et al., 2003; Peng et al., 2005; Chen et al., 2007; Faraco
61 et al., 2009; Suh et al., 2010; Kannan et al., 2013). When delivered *in vivo*, HDAC inhibitors
62 (HDACi) reduce neuroinflammation, promote neuroprotection and improve functional
63 outcomes in models of cerebral ischaemia (Kim et al., 2007; Sinn et al., 2007; Xuan et al.,
64 2012; Kim and Chuang, 2014), traumatic brain injury (Zhang et al., 2008; Shein and Shohami,
65 2011) and encephalomyelitis (Camelo et al., 2005; Zhang et al., 2010). Therapies for treating
66 brain injury and disease are lacking, but these studies highlight a role for HDACs in
67 neuroinflammation and suggest they are appropriate targets to inhibit.

68

69 The mechanism by which HDAC inhibition is anti-inflammatory is not understood, but we
70 know HDACs remove acetyl groups from lysine residues on proteins including, histones
71 (Strahl and Allis, 2000), enzymes and transcription factors (Glozak et al., 2005; Yao and Yang,
72 2011). As a consequence, deacetylation of histones promotes a compact chromatin structure
73 and reduces gene expression, and deacetylation of specific lysine residues on transcription
74 factors can modulate their activity (Gu and Roeder, 1997; Boyes et al., 1998). The identity of

75 which acetylated proteins are responsible for the anti-inflammatory responses observed when
76 using HDAC inhibitors is unclear.

77

78 There are 18 mammalian HDAC isoforms (class I HDACs (1, 2, 3 and 8), class II HDACs (4-
79 7, 9 and 10), class III sirtuins (1-7) and the class IV HDAC11) and the majority of studies to
80 date have focused on using non-selective HDAC inhibitors such as suberoylanilide hydroxamic
81 acid (SAHA), trichostatin-A (TSA) and valproic acid (VPA) which inhibit the majority of these
82 isoforms. As a result, we do not know which HDACs modulate the microglial inflammatory
83 response and how inhibition of these leads to the acetylation of specific proteins to reduce
84 neuroinflammation. Recent studies have begun to address these questions and have shown that
85 selective inhibition of class I HDACs 1, 2 and 3 with the HDAC inhibitor MS-275 (Hu et al.,
86 2003; Simonini et al., 2006; Beckers et al., 2007; Khan et al., 2008) can reduce
87 neuroinflammation in a mouse model of Alzheimer's disease (Zhang and Schluesener, 2013)
88 and the inflammatory response of macrophages to the inflammatory stimulant LPS (Jeong et
89 al., 2014).

90

91 In our study, we have used selective HDAC inhibitors and siRNA mediated knockdown to
92 identify HDAC1 and HDAC2 as the key HDACs involved in the neuroinflammatory response
93 of microglia. We show that selective class I HDAC inhibitors and siRNA to specifically
94 knockdown HDAC1 and 2, both suppressed the expression of cytokines in BV2 murine
95 microglia. Knockdown of HDAC1 alone resulted in a compensatory increase in the levels of
96 HDAC2 and did not suppress cytokine expression, showing these two enzymes have
97 redundancy in the neuroinflammatory response. We show that the HDACi are effective in the
98 absence of new protein synthesis suggesting that the anti-inflammatory mechanism of HDACi
99 does not involve increased protein expression. This identification suggests that HDAC

100 selective inhibitors may be therapeutically useful for targeting microglia and
101 neuroinflammation, in brain injury and disease by modulating the acetylation levels and
102 function of non-histone protein(s).

103 MATERIALS & METHODS

104

105 CELL CULTURE

106

107 BV2 murine microglia were cultured in Dulbecco's Modified Eagle's Medium (DMEM) high
108 glucose AQmedia™ (Sigma-Aldrich) supplemented with 10% v/v foetal bovine serum (FBS,
109 PAA Cell Culture Company) and 100U penicillin/100 µg streptomycin (Sigma-Aldrich). Cells
110 were seeded into 6-well plates at either 350,000 or 500,000 cells/well and 24-well plates at
111 175,000 cells/well. The cells were cultured for 24 hours before treatment. BV2 cells were
112 treated with vehicle control, or HDAC inhibitor with or without 500 ng/mL lipopolysaccharide
113 (LPS, Sigma) and cells harvested after 6 or 24 hours. Inhibitors used were; apicidin (Sigma-
114 Aldrich), MI192 (School of Chemistry, University of Leeds), MS-275 (Cayman Chemicals),
115 SAHA (Cayman Chemicals) all dissolved in DMSO and valproic acid (VPA, Sigma-Aldrich)
116 dissolved in phosphate buffered saline (PBS, Oxoid). For pre-treatment experiments, BV2 cells
117 were treated with vehicle control or appropriate drugs for 24 hours before the addition of 500
118 ng/mL LPS for a further 6 hours.

119

120 CELL TRANSFECTION

121

122 BV2 microglia seeded into 6-well plates were washed with 1 mL PBS and maintained in 1 mL
123 Opti-MEM® (Gibco) throughout the transfection procedure. Cells were transfected with 50
124 pmoles of Silencer® Select Negative Control siRNA (Ambion) or Silencer® Select Pre-
125 designed siRNA targeted against HDAC1 (id: s119557, Ambion) or HDAC2 (id: s67417,
126 Ambion) as follows. For each well, 3 µL of Lipofectamine™ 2000 (Invitrogen) was dissolved
127 in 100 µL of Opti-MEM® and 1 µL of 50 µM siRNA was dissolved in 100 µL of Opti-Mem®,

128 these were incubated for 5 minutes before combining and incubating for a further 20 minutes
129 at room temperature. Afterwards, 200 μ L of this mix was added to the well, followed by
130 incubation at 37°C in a humid atmosphere with 5% CO₂ for 4 hours. This was then removed
131 and the cells were cultured in 3 mL DMEM high glucose AQmedia™ supplemented with 1%
132 v/v FBS and 100U penicillin/100 μ g streptomycin for 24 hours. Medium was then changed and
133 the cells were cultured for a further 24 hours before treating with 500 ng/mL LPS for 6 hours.

134

135 WHOLE CELL PROTEIN EXTRACTION

136

137 BV2 cells seeded into 6-well plates were washed with 1 mL PBS and scraped into 250 μ L ice-
138 cold RIPA Buffer [10 mM Tris-HCL pH 8.0, 1 mM EDTA, 1% Triton X-100, 0.1% sodium
139 deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 140 mM NaCl, 1 mM PMSF (all from
140 Sigma-Aldrich)], then incubated for 30 minutes on ice. The lysate was clarified by
141 centrifugation at 13400 \times g for 20 minutes at 4°C and the supernatant containing proteins was
142 collected and the concentration determined using the Bicinchoninic Acid (BCA) protein assay
143 (Sigma-Aldrich).

144

145 HISTONE PROTEIN EXTRACTION

146

147 Histone proteins were extracted from BV2 microglia cultured in 6-well plates. Cells were
148 washed with 1 mL PBS then scraped into 1 mL ice-cold PBS and pelleted by centrifugation at
149 400 \times g for 5 minutes at room temperature. The cell pellet was resuspended in 400 μ L of Triton
150 Lysis Buffer per 1 \times 10⁷ cells [0.5% v/v Triton X-100, 2 mM PMSF, 0.02% w/v NaN₃ (all from
151 Sigma-Aldrich) and PBS] and incubated on ice for 10 minutes. Lysed cells were centrifuged at
152 6600 \times g for 10 minutes at 4°C. The pellet was resuspended in half the volume of Triton Lysis

153 Buffer used earlier then centrifuged at 6600 × g for 10 minutes at 4°C. The nuclei pellet was
154 resuspended in 50 µL of 200 mM HCl (Acros Organics) and histone proteins were extracted
155 overnight at 4°C. The samples were centrifuged at 6600 × g for 10 minutes at 4°C and the
156 supernatant containing histone proteins was collected and the protein concentration determined
157 using the Bradford protein assay (Sigma-Aldrich).

158

159 WESTERN BLOTTING

160

161 Protein samples (10 µg) were separated by sodium dodecyl sulfate polyacrylamide gel
162 electrophoresis (SDS-PAGE) and wet-transferred onto PVDF membrane at 30V for 30
163 minutes. The membranes were blocked overnight at 4°C with blocking solution [5% w/v nonfat
164 dried milk powder, 0.1% v/v Tween® 20 (both from Sigma-Aldrich) and PBS] and then
165 incubated with either; Anti-HDAC1 [1:2000, rabbit polyclonal, Abcam], Anti-HDAC2
166 [1:2000, rabbit polyclonal, Abcam], Anti-HDAC3 [1:2000, rabbit polyclonal, Abcam], Anti-
167 β-actin [1:10,000, mouse monoclonal, Sigma], Anti-Histone H3 [1:1000, mouse monoclonal,
168 Cell Signaling], Anti-acetyl Histone H3 Lysine 9 [1:1000, rabbit polyclonal, Millipore] or Anti-
169 acetyl Histone H4 Pan-lysine [1:10,000, rabbit polyclonal, Millipore] (all dissolved in blocking
170 solution) for 1 hour at room temperature. Membranes were washed with PBS-0.1% v/v
171 Tween® 20 and incubated with an appropriate secondary antibody; Goat-Anti-Rabbit IgG-
172 horseradish peroxidase (HRP) linked [1:2000, Cell Signaling] or Goat-Anti-Mouse IgG-HRP
173 linked [1:2000, Cell Signaling] for 1 hour at room temperature followed by washing as before.
174 Membranes were incubated with an enhanced chemiluminescence (ECL) substrate
175 (Amersham) and exposed to photographic film, a Fujifilm LAS-3000 imaging system or a
176 cDigit® Scanner (LICOR). The intensity of each band was quantified and normalized to the β-
177 actin loading control.

178

179 RNA EXTRACTION, REVERSE TRANSCRIPTION AND QUANTITATIVE RT-PCR

180

181 Total RNA was extracted from BV2 microglia cells using TRI Reagent® (Sigma-Aldrich) as per

182 manufacturer's instructions, resuspended in Tris-EDTA (TE), pH 7.5 and concentrations

183 determined using a NanoDrop 2000c (Thermo Scientific). RNA (2.5 µg) was primed for reverse

184 transcription at 65°C for 5 minutes with 1.25 µL of Oligo(dT)15 primers (0.5 µg/µL, Promega),

185 1.25 µL of Random primers (0.5 µg/µL, Promega) in a final volume of 32.5 µL. cDNA was

186 synthesised at 37°C for 60 min using M-MLV Reverse Transcriptase, RNase H Minus (200 U/µL

187 and RNasin® Plus, Promega) with 2 mM dNTP (Bioline) in a final reaction volume of 50 µL.

188 Quantitative PCR (qPCR) reactions were carried out in duplicate using a Rotor Gene 6000 PCR

189 Analyzer (Corbett) using SensiMix™ SYBR® & Fluorescein (Bioline). Each reaction comprised

190 of 50 or 100 ng of sample cDNA, 300 nM of each gene primer, in a final volume of 20 µL.

191 Primers used were: IL-6 5'-CCCAACTTCCAATGCTCTCC and 5'-ACATGGGATTCCA-

192 CAAAC, TNF-α 5'-TGAACTTCGGGGTGATCG and 5'-GGGCTTGTCACCTCGAGTTTT ,

193 U6 5'- CCGCTTCGGCAGCACA and 5'-AACGCTTCACGAATTTGCGT, HDAC1 5'- GA-

194 CCGCAAGTGTGTGG and 5'-GAGCAACATTCCGGATGGTG, HDAC2 5'- CAACAGAT-

195 CGCGTGATGACC and 5'-CCCTTTCCAGCACCAATATCC, HDAC3 5'-GACGTGCAT-

196 CGTGCTCCAGT and 5'-ACATTCCCCATGTCCTCGAAT. A RNA control (no reverse

197 transcription) and a no template control were also run. PCR conditions were: 95°C for 10 min

198 followed by 45 cycles of 95°C for 10 s, 60°C for 15 s and 72°C for 30 s. Relative quantitation of

199 transcript levels was performed using the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001) and the

200 house-keeping gene U6. Data are presented either as mean percentage expression of the vehicle

201 control ± standard error of the mean (SEM) or mean percentage expression of LPS +vehicle

202 condition ± SEM. Statistical analysis for each experimental condition vs. the vehicle condition

203 was performed using a one-way repeated measures ANOVA followed by the Dunnet *post hoc*
204 test (Lew, 2007) at the 5% significance level.

205

206 ENZYME LINKED IMMUNOSORBANT ASSAYS (ELISAs)

207

208 Cell culture supernatants were taken from BV2 microglia cells cultured in 24-well plates. The
209 culture medium was removed and centrifuged for 30 seconds at 16000 $\times g$ to pellet any
210 detached cells then 950 μL was removed for analysis. The concentration of mouse IL-6 protein
211 was determined in triplicate by 96-well plate format ELISAs (Invitrogen) following
212 manufacturer's instructions. Data is presented as mean protein concentration \pm standard
213 deviation (SD). Statistical analysis comparing the absolute absorbance values for each
214 experimental condition vs. the vehicle control +LPS condition was performed using a
215 Student's unpaired t-test assuming equal variances at the 5% significance level.

216

217 INHIBITION OF PROTEIN SYNTHESIS

218

219 Cells were treated to 500 ng/mL LPS and either vehicle control, 1 μM SAHA or 500 nM
220 apicidin, in the presence or absence of 1 $\mu\text{g/mL}$ cycloheximide (CHX) for 3 hours. Protein
221 synthesis was assessed in the cells using a Click-iT[®] Plus O-propargyl-puromycin (OPP)
222 Protein Synthesis Assay Kit (Molecular Probes, Life Technologies). Briefly, after 2.5 hours of
223 drug treatments, Click-iT[®] OPP was added directly to each well to give a final concentration
224 of 20 μM . The cells were incubated for a further 30 minutes at 37°C. The culture medium was
225 removed, the cells washed with PBS, followed by fixation with 100 μL of 4% w/v
226 paraformaldehyde (Sigma-Aldrich) for 15 minutes at room temperature. The cells were then
227 permeabilised with 100 μL of 0.5% v/v Triton X-100 (Sigma-Aldrich) for 15 minutes at room

228 temperature, washed twice with PBS and processed for imaging following the manufacturer's
229 instructions. Imaging of labelled cells was carried out using the IncuCyte™ FLR with a 10×
230 objective lens. Nine non-overlapping images were taken in phase-contrast and green-
231 fluorescence (excitation wavelength of 450-490 nm) per well. RNA was extracted from parallel
232 treated cultures for qRT-PCR analysis as described above.

233

234 **RESULTS**

235

236 HDAC INHIBITION SUPPRESSES CYTOKINE EXPRESSION IN BV2 MICROGLIA

237

238 Previous studies using isolated murine microglia report that non-selective HDAC inhibitors
239 (SAHA, TSA and VPA) suppress LPS induced inflammation as measured by a reduction in
240 LPS induced cytokine expression (tumour necrosis factor- α , TNF- α and interleukin-6, IL-
241 6) (Suh et al., 2010; Kannan et al., 2013). The specific HDAC(s) important in microglia and
242 neuroinflammation are still largely unknown though identification of the specific HDAC is a
243 requisite for the development of any targeted therapy. We first tested the response of LPS
244 activated BV2 microglia cells to the classical HDAC inhibitors SAHA and VPA (for a
245 discussion of this model system see; (Bocchini et al., 1992; Horvath et al., 2008; Henn et al.,
246 2009; Gresa-Arribas et al., 2012; Stansley et al., 2012)). Six hours after LPS stimulation, IL-
247 6 and TNF- α mRNA expression was increased by $3384 \pm 271\%$ and $50990 \pm 5190\%$
248 respectively (Fig 1A), and after 24 hours IL-6 protein secretion was increased by $5406 \pm 439\%$
249 (Fig 1F). BV2 cells express the class I HDACs, 1, 2 and 3 with highest levels of HDAC1 and
250 lowest levels of HDAC3 (Fig 1B, C). Treatment with the HDAC inhibitors, SAHA and VPA
251 produced an increase in the level of Histone H4 acetylation levels within 1 hr which was stable
252 over a period of 24 hr (Fig 1D) suggesting these inhibitors provide rapid and stable HDAC

253 inhibition. Activation by LPS in the presence of either 1 μ M SAHA or 10 mM of VPA,
254 produced a significantly reduced response in IL-6 mRNA expression by $84.1 \pm 2.8\%$ ($P =$
255 0.004) and $89.7 \pm 1.6\%$ respectively and TNF- α mRNA expression by $59.7 \pm 3.2\%$ and $77.9 \pm$
256 2.5% respectively (Fig 1E). Furthermore, SAHA significantly suppressed the LPS induced
257 increase in IL-6 protein secretion by $85.6 \pm 2.5\%$ (Fig 1F).

258

259 In order to understand the role of specific HDACs in microglia and neuroinflammation, we
260 tested HDAC inhibitors that show some selectivity towards specific HDAC isoforms in vitro.
261 We treated LPS induced cells with 5 μ M MS-275 (which has reported selectivity for HDAC 1
262 (Bradner et al., 2010)), 500 nM apicidin (which has reported selectivity for HDAC1, 2 and 3
263 (Bradner et al., 2010)), or 1 μ M MI-192, (which has reported selectivity for HDAC2 and 3
264 (Boissinot et al., 2012)). Treatment of BV2 cells with these inhibitors showed a rapid and stable
265 increase in acetylated Histone H4 for apicidin but a gradual increase for MS-275 and MI-192
266 over a 24 hour period (Fig 2A). Quantification of histone acetylation levels showed that
267 apicidin produced a similar rate of increase to SAHA while MS-275 and MI-192 required
268 longer incubation periods to induce high levels of histone acetylation (Fig 2B). MS-275 and
269 MI-192 are members of the benzamide class of HDAC inhibitors which have previously been
270 shown to bind HDACs with a slower association rate compared to hydroxamic acid inhibitors
271 such as SAHA (Lauffer et al., 2013). Consistent with these data, co-treatment of LPS with
272 apicidin was sufficient to reduce the induction of IL-6 and TNF- α expression by $82.7 \pm 2.3\%$
273 and $50.3 \pm 4.5\%$ but co-treatment of MS-275 or MI-192 was not, (not shown). Pre-treatment
274 of BV2 cells, to mitigate the slow kinetics of inhibition, with either apicidin, MI-912 or MS-
275 275 for 24 hr prior to LPS stimulation significantly reduced the LPS stimulated expression of
276 IL-6 and TNF- α (Fig 2C). Together these data suggest that HDAC1 and HDAC2/3 contribute
277 to the inflammatory response in microglia.

278

279 KNOCKDOWN OF HDAC1 OR HDAC2 SUPPRESSES CYTOKINE EXPRESSION IN
280 BV2 MICROGLIA

281

282 Although the HDAC inhibitors show selectivity, they are not isoform specific, therefore we
283 used an siRNA approach to specifically knockdown HDAC1 and HDAC2 to determine their
284 involvement in the inflammatory response of microglia. We were able to significantly knock-
285 down HDAC1 protein expression by $62.6 \pm 4.5\%$ (Fig 3A) and HDAC2 protein expression by
286 $68.8 \pm 7.7\%$ (Fig 3A). Knockdown of HDAC1 resulted in an increased expression of HDAC2
287 with no change in HDAC3 (Fig 3A), while knockdown of HDAC2 did not result in any change
288 of expression of HDACs 1 or 3 (Fig 3A). Following knockdown, we treated cells to 500 ng/mL
289 LPS for 6 hours and assessed the expression of IL-6 and TNF- α mRNA. We found, that cells
290 in which HDAC1 was knocked down, there was no change in the response to LPS compared
291 to control cells (not shown) but cells in which HDAC2 was knocked down showed a reduced
292 induction of IL-6 (by $48.2 \pm 13\%$) and TNF- α (by $22.0 \pm 3.6\%$) expression in response to LPS
293 (Fig 3B). To determine if the increase in HDAC2 expression, as a result of HDAC1
294 knockdown, was acting as a compensatory mechanism we used HDAC1 siRNA to knockdown
295 HDAC1 in combination with a titrated amount of HDAC2 siRNA to reduce HDAC2 to levels
296 seen in control cells (Fig. 3, HDAC1 + 2). Using this approach we were able to significantly
297 reduce HDAC1 levels by $63.5 \pm 2.4\%$, while maintaining the level of HDAC2 to that seen in
298 control cells ($89.7 \pm 6.2\%$, Fig 3A HDAC1 + 2). The expression of HDAC3 was not
299 significantly altered ($116 \pm 5\%$ of Scr siRNA). Cells in which HDAC1 levels are reduced but
300 HDAC2 levels are unchanged showed a reduced response to LPS with with IL-6 and TNF- α
301 mRNA levels of $34.8 \pm 3.0\%$ and $35.7 \pm 4.8\%$ respectively compared with control cells (Fig
302 3B).

303 In summary our data identify HDAC1 and 2 activities as important contributors to the
304 neuroinflammatory response of microglia. Furthermore, they show redundancy in this function
305 with increased HDAC2 levels being compensatory for reduced HDAC1.

306

307 HDAC INHIBITION IS EFFECTIVE IN REDUCING THE INFLAMMATORY RESPONSE
308 IN THE ABSENCE OF NEW PROTEIN SYNTHESIS

309

310 The mechanism by which HDAC inhibitors exert their effects is often assumed to involve
311 increase in gene expression – indeed HDAC inhibitors do result in increased histone acetylation
312 (e.g. Fig 1D) and the association of increased acetylation with increased gene expression was
313 first identified nearly 30 years ago (Hebbes et al., 1988). However recent proteomic data has
314 identified in excess of 4000 proteins that are modified by acetylation (Choudhary et al., 2009;
315 Lundby et al., 2012; Liu et al., 2014), a number comparable to targets of phosphorylation,
316 suggesting that acetylation is involved in many more processes than gene regulation alone. To
317 determine if the anti-inflammatory action of HDAC inhibition results from changes in gene
318 expression we blocked new protein synthesis using cycloheximide and tested the effectiveness
319 of HDAC inhibitors to block IL-6 and TNF- α stimulation by LPS. Incubation of BV2 cells with
320 cycloheximide for 1 or 3 hours completely blocked new protein synthesis as measured by O-
321 propargyl-puromycin incorporation and protein synthesis was blocked under all conditions
322 used to quantify gene expression levels (Fig 4A). Continued exposure to cycloheximide for 6
323 hr led to cell death (not shown) though cells were still healthy after 3 hour exposure. The
324 presence of cycloheximide did not affect the induction of IL-6 mRNA expression by LPS (Fig
325 4B, compare left two bars) and did not prevent either SAHA or apicidin inhibiting this response
326 (Fig 4B, right two bars). Thus these data indicate that the mechanism by which HDAC
327 inhibition reduces the inflammatory response in microglia is manifest within 3 hours and does

328 not require new protein synthesis. Together this suggests that increased gene expression
329 resulting from enhanced histone acetylation is not important for ability of HDAC inhibitors to
330 reduce microglia activation and future work should aim to identify the important molecular
331 targets.

332 DISCUSSION

333

334 It has been previously shown that inhibitors of HDACs can reduce the inflammatory response
335 in activated microglia (Chen and Greene, 2004; Faraco et al., 2009; Suh et al., 2010; Kannan
336 et al., 2013) and by suppressing microglia activation show neuroprotective effects following
337 transient ischaemia *in vivo* (Kim et al., 2007; Sinn et al., 2007; Xuan et al., 2012; Kim and
338 Chuang, 2014). However, the identity of the important HDACs involved has not been
339 uncovered and the mechanism by which HDAC inhibition is beneficial is yet to be elucidated.
340 Here we have shown that the function of both HDAC1 and HDAC2 contribute to the
341 inflammatory response in microglia and that in the absence of HDAC1, increased HDAC2
342 levels compensate suggesting that these two HDACs show redundancy in this function.
343 Furthermore the effectiveness of HDAC inhibition in the absence of new protein synthesis
344 suggests that the HDACs are promoting the inflammatory response by regulating the
345 acetylation levels of a non-histone protein rather than increasing levels of gene expression as a
346 result of increased histone acetylation.

347

348 Microglia are often referred to as the immune cells of the brain and recently, selective inhibition
349 and genetic knockdown of class I HDACs, was shown to reduce the production of cytokines in
350 the inflammatory response of macrophages (Jeong et al., 2014). In macrophages, knockdown
351 of either HDAC1 or 2 resulted in increased expression of the other and only a combined
352 knockdown of HDACs 1, 2 and 3 resulted in reduced inflammatory response to LPS (Jeong et
353 al., 2014). In T-lymphocytes deletion of HDAC1 resulted in an increase in HDAC2 protein
354 levels but deletion of HDAC2 had no effect on HDAC1 (Dovey et al., 2013). Here we show
355 that in microglial cells, HDAC1 is the most highly expressed class I HDAC and knockdown
356 of HDAC1 resulted in a compensatory increase in the levels of HDAC2 (Fig 3A). Likewise,

357 we did not observe any compensatory increase in the levels of HDAC1 protein upon
358 knockdown of HDAC2 (Fig 3A) however this contrasts to observations made using
359 macrophages (Jeong et al., 2014). The mechanisms resulting in a compensatory increase in one
360 HDAC upon loss of another are not known though HDAC1 does regulate its own promoter
361 (Schuettengruber et al., 2003) and may also repress expression of other HDACs. One prediction
362 of such a model would be that chemical inhibition of HDAC activity would also result in such
363 compensatory increase. However, we did not observe any compensatory changes in HDAC
364 expression in cells treated with HDAC inhibitors (not shown), suggesting it is not brought about
365 by loss of HDAC enzyme activity but is potentially a mechanism involving the absence of the
366 protein itself. In the absence of HDAC1 in T-lymphocytes the levels of SIN3 and MTA2 are
367 reduced which may indicate that incomplete co-repressor complexes are turned over quickly
368 (Dovey et al., 2013). This structural, rather than enzymatic, requirement for HDAC1 may
369 underlie the reason that knockdown of HDAC1, but not inhibition results in a compensatory
370 increase in HDAC2. Additionally, compensatory changes in HDAC protein levels have been
371 observed in the absence of changes in mRNA levels, suggesting the mechanism involves
372 enhanced translation or protein stability (Jurkin et al., 2011).

373

374 HDAC1 and 2 do not exist in the cell as isolated enzymes but are components of three
375 independent co-repressor complexes; Sin3, NuRD and CoREST (for a review see (Kelly and
376 Cowley, 2013)). Each co-repressor complex contains two molecules of HDAC which may
377 consist of two molecules of HDAC1, two molecules of HDAC2 or one of each. Others have
378 observed that upon a loss of HDAC1, HDAC2 can become incorporated into the Sin3, NuRD
379 and CoREST multi-protein complexes in its place (Dovey et al., 2013). The compensatory
380 effect of HDAC2 in the inflammatory response may be explained by such a mechanism.
381 Following a loss of HDAC1, HDAC2 is upregulated and this HDAC is incorporated into a

382 specific complex in place of HDAC1. This complex, specifically targets a protein (which
383 regulates the inflammatory response) for deacetylation. Regardless of the HDAC composition,
384 be it two molecules of HDAC1, HDAC2 or one of each, the specificity for the substrate to be
385 deacetylated comes from the complex itself rather than the HDACs. This hypothesis would
386 suggest it doesn't matter which of the two HDAC isoforms is inhibited, an anti-inflammatory
387 effect depends on a reduction in the number and activity of this specific functional multi-
388 protein complex. Similarly, the compensatory effect of HDAC3 when HDAC1 and 2 are both
389 lost in macrophages (Jeong et al., 2014) may be explained by HDAC3 being in a specific
390 complex that targets the same substrate as the complex with either HDAC1 or 2. Further
391 research is now needed to investigate these hypotheses and identify the complexes (and
392 composition of them) that when inhibited is responsible for the suppression of pro-
393 inflammatory mediator expression in BV2 microglia.

394

395 What is the important target of HDAC1 and 2 that promotes the inflammatory response? Our
396 data identify that new protein synthesis is not required for the HDAC inhibitor response.
397 Formally, we cannot rule out a transcriptional response involving increased miRNA expression
398 and subsequent down regulation of a protein targeted by the miRNA(s), however the ability of
399 the inhibitors to show effectiveness within 3 hours makes such a mechanism unlikely. HDAC
400 enzymes were originally characterised by their ability to deacetylate histone proteins, however
401 these are not their only target and the acetylome may contain on the order of 4000 proteins
402 (Choudhary et al., 2009; Liu et al., 2014). Additionally, the original idea, that HDAC inhibition
403 leads to increased histone acetylation and increased gene expression is likely too simplistic
404 because as many genes are repressed as are activated upon HDAC inhibition by SAHA (Peart
405 et al., 2005). The specific HDAC target(s) important for the microglial response has not been
406 unequivocally identified though a number of potential target proteins can be implicated based

407 on a correlation of their acetylation with microglial activation. Perhaps the most studied non-
408 histone protein involved in the inflammatory response and regulated by acetylation is the
409 transcription factor NF- κ B (Greene and Chen, 2004). Quiescent NF- κ B is restricted to the
410 cytoplasm via its inhibitory binding partner I κ B but upon cell stimulation becomes dissociated
411 and moves into the nucleus where it activates target gene expression. Initially, it was proposed
412 that deacetylation of NF- κ B enhanced its interaction with I κ B and removal from the nucleus
413 however NF- κ B can be acetylated at multiple sites and more recent data suggests that
414 deacetylation at specific residues can result in activation of a subset of NF- κ B targets
415 (Rothgiesser et al., 2010), thus inhibition of HDACs may enhance the level of acetylated NF-
416 κ B, reducing its activity. In support of this idea, Furumai et al, 2011 showed that inhibition of
417 HDACs in HeLa cells, with TSA, caused a reduction in the recruitment of NF- κ B, and RNA
418 polymerase II to the promoter of IL-8, which in turn caused a reduction in IL-8 expression
419 (Furumai et al., 2011). Another candidate protein is MKP-1, a member of the MAPK
420 inflammatory signalling pathway and negative regulator of the inflammatory response. In
421 macrophages MKP-1 activity is reduced when it is deacetylated by HDAC1, 2 or 3 (Jeong et
422 al., 2014). MKP-1 is not just expressed in macrophages but also in microglia (Eljaschewitsch
423 et al., 2006) making this another attractive candidate for the functional response observed here.

424

425 In summary our new data here highlight a role for HDAC1 and 2 in regulating microglia
426 activation and suggest the mechanism by which they do so involves acetylation of proteins
427 other than histones. Future studies should now be aimed toward identifying which proteins are
428 the important targets. Although HDAC inhibitors have been approved clinically in the
429 treatment of some cancers they are not without side effects and a more complete understanding
430 of their mechanism of action would open doors to more specific therapeutic targets.

431

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581 **Figure 1. Histone deacetylase (HDAC) inhibition suppress activation of cytokine**
582 **expression in murine microglia.** A) BV2 microglia were stimulated with 500 ng/mL
583 lipopolysaccharide (LPS) and mRNA expression of interleukin-6 (IL-6) and tumor necrosis
584 factor- α (TNF- α) was determined using qPCR. Relative transcript levels were normalized to
585 the U6 gene and expressed as a percentage of the expression in control cells treated with
586 vehicle. Shown are mean \pm SEM, $n=3$. B) Quantitative RT-PCR of RNA extracted from BV2
587 cells. Expression levels expressed as a percentage of the U6 gene. Shown are mean \pm SEM,
588 $n=3$ C) Western blot analysis of protein extracted from BV2 cells and analysed using anti-
589 HDAC1, HDAC2, HDAC3 and β -actin. Representative blots are shown and positions of
590 molecular weight markers are identified on the left. D) Histone proteins were extracted from
591 control cells and cells treated with 1 μ M SAHA or 10 mM VPA for 1, 2, 4, 6, or 24hr. Proteins
592 underwent western blotting for acetylated histone H4 (Ac-H4) and total histone H3.
593 Representative blots are shown. E) BV2 microglia were treated with 500 ng/mL LPS \pm 1 mM
594 SAHA or 5 mM valproic acid (VPA) for 6 hours and the mRNA expression of IL-6 and TNF-
595 α was determined using qPCR, normalized to the U6 house-keeping gene and expressed as a
596 percentage of the expression in control cells treated LPS and vehicle shown are mean \pm sem,
597 $n=3$. F) BV2 microglia were treated with 500 ng/mL LPS \pm 1 mM SAHA for 24 hours and the
598 changes in IL-6 protein secretion was determined by ELISA. Shown are mean protein
599 concentration \pm SEM, $n=3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

600 **Figure 2. Selective Histone deacetylase (HDAC) inhibitors suppress activation of cytokine**
601 **expression in murine microglia.** A) Histone proteins were extracted from control cells and
602 cells treated with 500nM mM Apicidin, 1 μ M MI-192 or 5 μ M MS-275 for 1, 2, 4, 6, or 24hr.
603 Proteins underwent western blotting for acetylated histone H4 (Ac-H4) and total histone H3.
604 Representative blots are shown. B) Quantification of acetylated histone H4 levels, normalised
605 to Histone H3 and expressed relative to acetylation levels at 24hr. C) BV2 microglia were

606 pretreated with HDACi for 24 hours and then stimulated with 500 ng/mL lipopolysaccharide
607 (LPS) for 6 hours. mRNA expression of interleukin-6 (IL-6) and tumor necrosis factor- α (TNF-
608 α) was determined using qPCR. Relative transcript levels were normalized to the U6 house-
609 keeping gene and expressed as a percentage of the expression in control cells treated with
610 vehicle. Shown are mean \pm SEM, $n=3$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to LPS
611 stimulated cells.

612 **Figure 3. HDAC1 and HDAC2 are involved in the inflammatory response in microglia.**

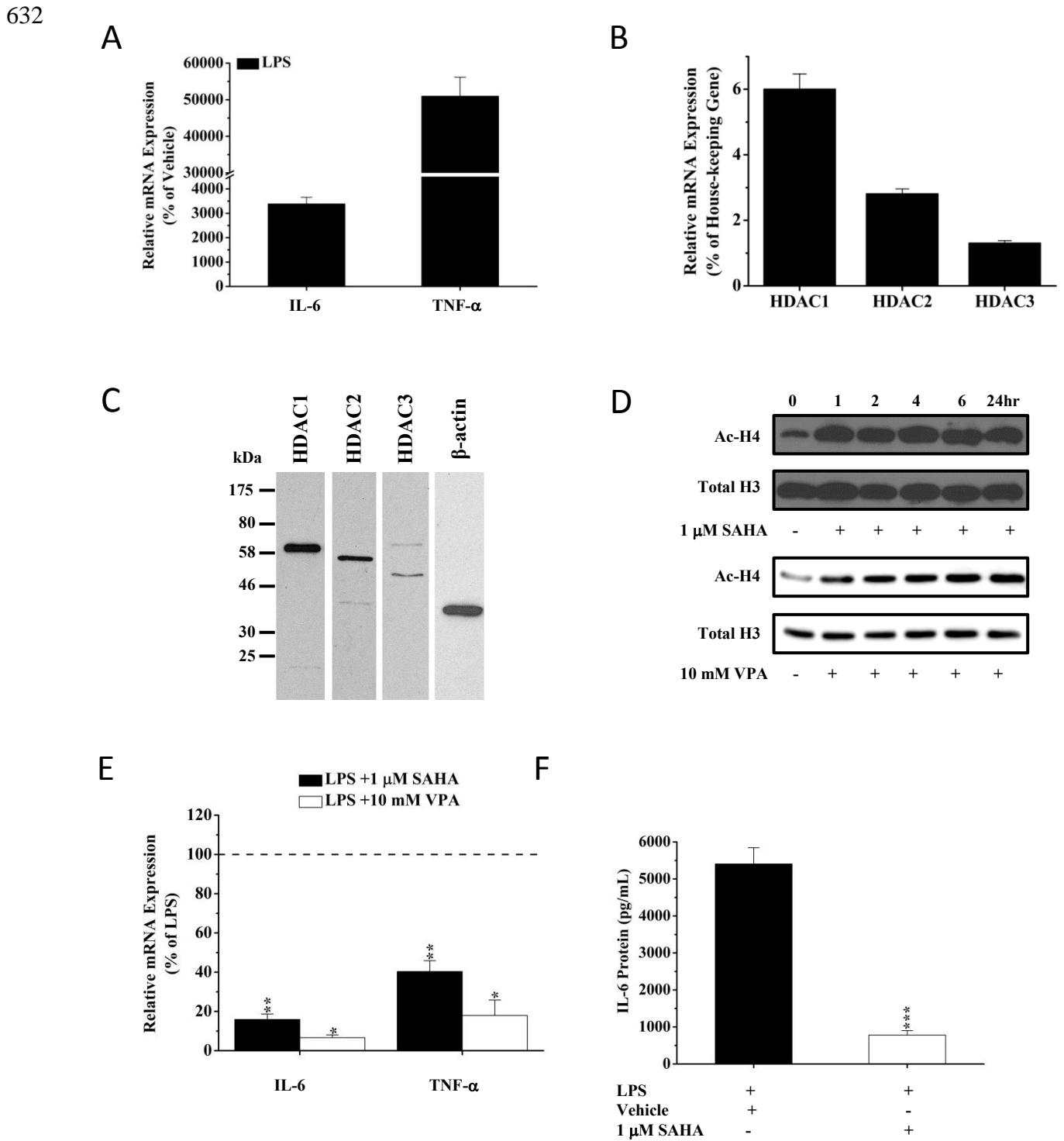
613 A) BV2 microglia were transfected with either scrambled (Scr) siRNA or HDAC1, HDAC2 or
614 HDACs1 and 2 siRNAs. Levels of HDAC1, 2 and 3 and Beta-actin were quantified by
615 immunoblotting. Shown are a representative blots (left) and quantification of protein levels
616 (right) expressed relative to levels in cells treated with Scrambled siRNA. Shown are mean \pm
617 SEM, $n=3$ * $p < 0.05$ compared to scrambled siRNA. B) BV2 microglia transfected with Scr,
618 HDAC1, HDAC2 or HDAC1 and 2 siRNAs were stimulated with LPS and changes in IL-6
619 and TNF- α mRNA expression was determined using qPCR. Relative transcript levels in each
620 treatment were normalized to the U6 gene and expressed relative to the expression in the Scr
621 siRNA +LPS. Shown are mean \pm SEM, $n=3$. * $P < 0.05$

622 **Figure 4: Protein synthesis is not required for HDAC inhibitor efficacy.** A) BV2 microglia

623 were treated for 1 or 3 hours with or without cyclohexamide and incorporation of O-propargyl-
624 puromycin (green, New protein) identified new protein synthesis. Bottom shows phase contrast
625 images, scale bar 50 μ m. B) Expression of the IL-6 was measured by quantitative PCR in cells
626 exposed to LPS and treated with vehicle, SAHA or apicidin in the presence of cyclohexamide.
627 Transcript levels for each treatment were normalised to U6 and data shown are mean mRNA
628 expression levels expressed as a percentage of the expression in LPS +vehicle \pm SEM, $n=3$,
629 *** $P < 0.001$ vs. LPS +vehicle

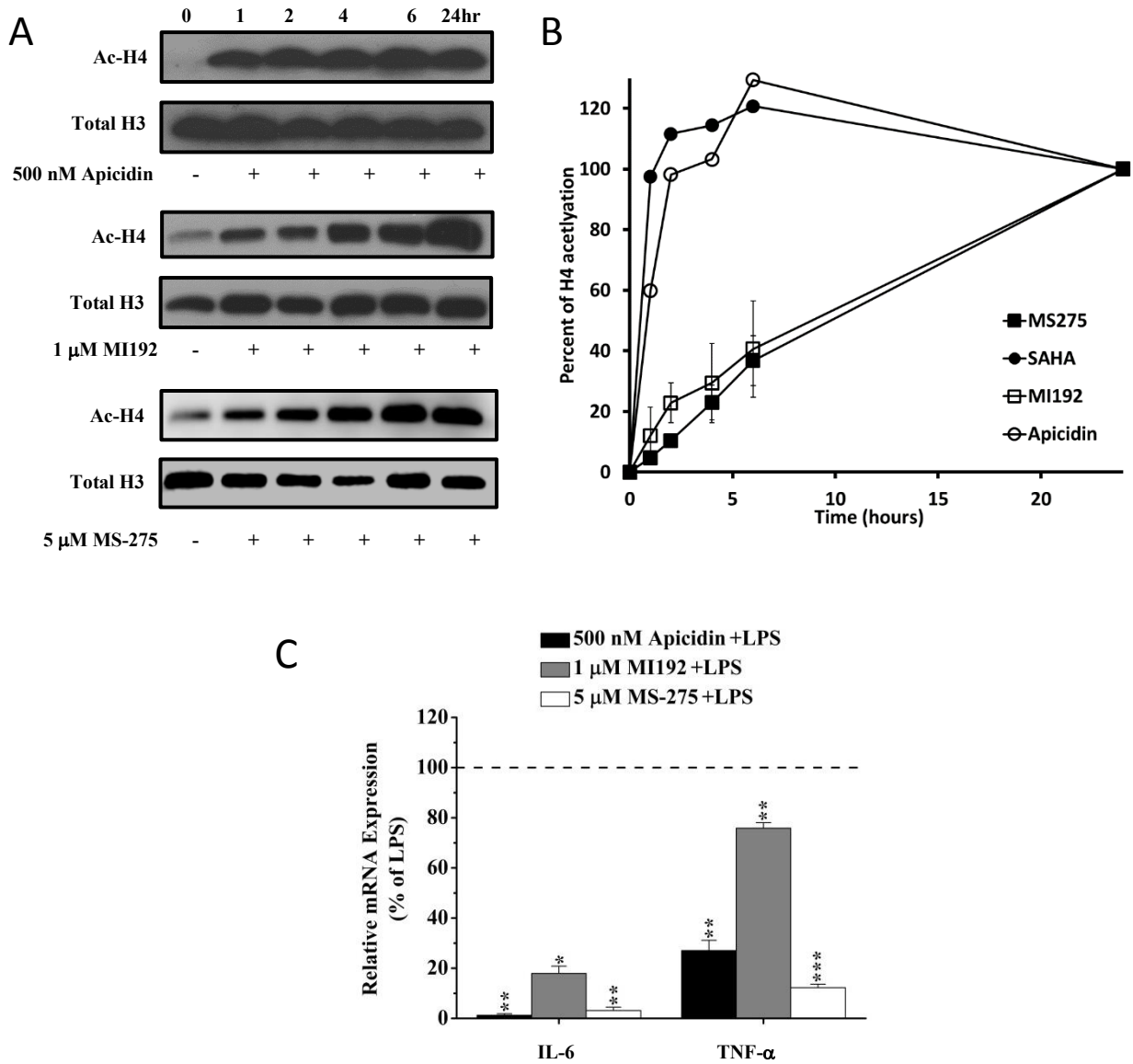
630

631 **Figure 1**



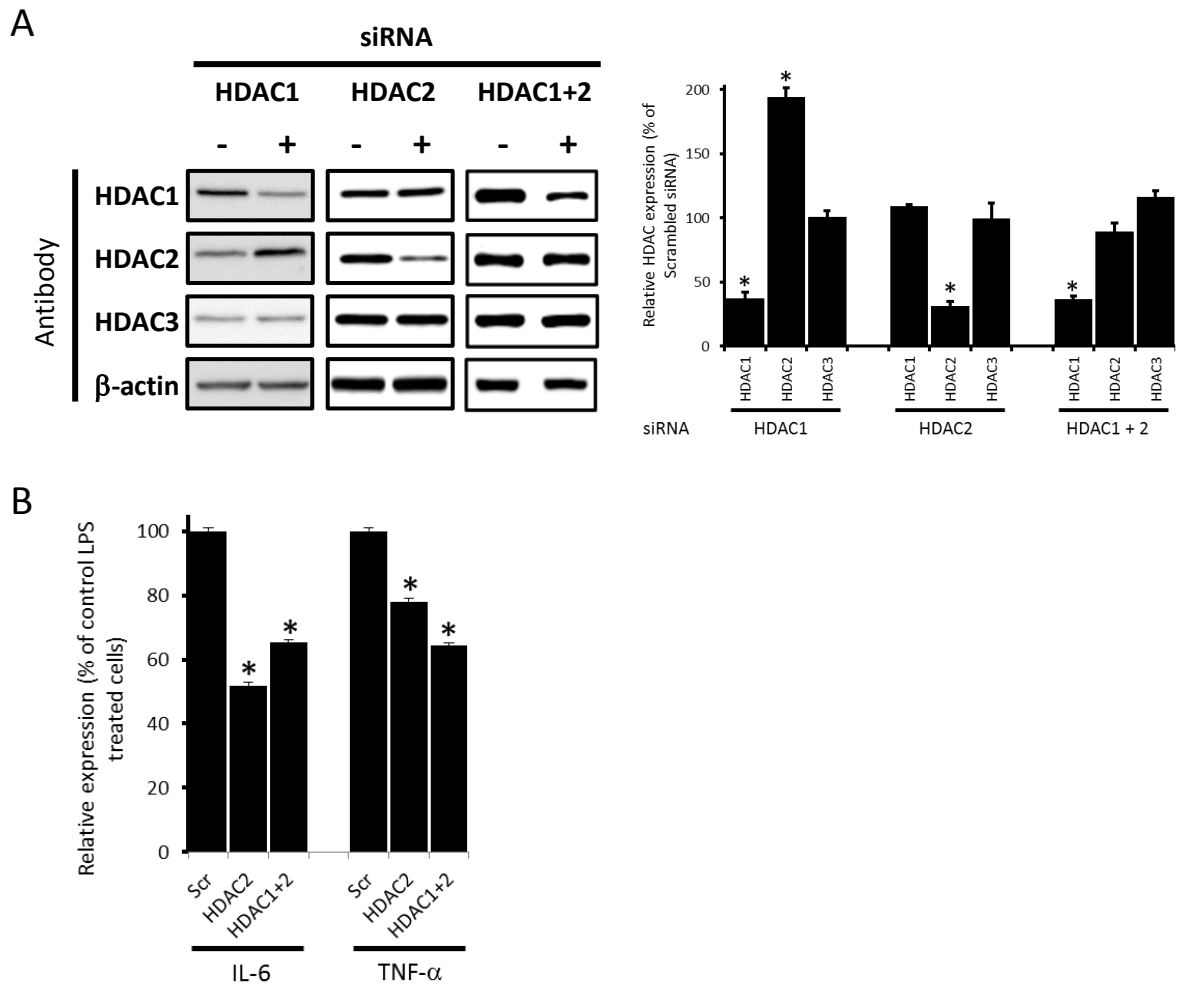
633 **Figure 2**

634



635 **Figure 3**

636



637 **Figure 4**

