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► **To cite this version:**

Périne Millot, Carine San, Evangeline Bennana, Baptiste Porte, Nicolas Vignal, et al.. STAT3 inhibition protects against neuroinflammation and BACE1 upregulation induced by systemic inflammation. *Immunology Letters*, 2020, 228, pp.129 - 134. 10.1016/j.imlet.2020.10.004 . hal-03492809

HAL Id: hal-03492809

<https://hal.science/hal-03492809>

Submitted on 21 Nov 2022

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STAT3 inhibition protects against neuroinflammation and BACE1 upregulation induced by systemic inflammation

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ABSTRACT

Abnormal activation of the transcriptional factor STAT3 (signal transducer and activator of transcription 3) was recently associated with Alzheimer Disease (AD). STAT3 phosphorylation is critical for cytokine secretion linked to neuroinflammation. Moreover, STAT3 may act as a transcriptional regulator of BACE1 (β -APP cleaving enzyme-1), the key enzyme in amyloid β ($A\beta$) production. We have previously shown that neuroinflammation and increased brain BACE1 levels triggered by LPS-induced systemic inflammation in wild-type mice are associated with an enhanced STAT3 activation. Using this LPS model, the goal of this study was to investigate if a STAT3 inhibitor administration could be protective against neuroinflammation and abnormal BACE1 regulation. Our results show that intraperitoneal injection of Stattic, a molecule that selectively inhibits the activation of STAT3, decreases LPS-induced microglial activation in the hippocampus. In addition, STAT3 inhibition reduced brain levels of cytokines IL-6, IL-1 β and TNF- α triggered by LPS systemic administration. A significant reduction of BACE1 levels was observed in the hippocampus of mice treated with LPS and Stattic compared to those exposed to LPS alone. Taking together, our results show that Stattic can protect hippocampus against two pathological hallmarks of AD, and pave the way for further explorations of the therapeutic potential of STAT3 inhibition in AD.

Keywords: STAT3, Neuroinflammation, microglial activation, LPS, Stattic, BACE1

ABBREVIATIONS

AD: Alzheimer Disease

A β : Amyloid- β

APP: Amyloid precursor protein

BACE1: β -APP cleaving enzyme-1

CA: Cornu Ammonis

DG: Dentate gyrus

eIF-2 α : eukaryotic initiation factor 2 alpha

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

IBA-1: ionized calcium-binding adapter molecule 1

IL: Interleukin

IFN- γ : Interferon γ

I.p.: Intraperitoneal

JAK: Janus kinase

LPS: Lipopolysaccharide

PKR: Protein kinase R

STAT3: Signal transducer and activator of transcription 3

TNF- α : Tumor necrosis factor

WT: Wild type

1 1. INTRODUCTION

2
3 Interleukin 6 (IL-6)/Janus kinase (JAK)/signal transducer and activator of transcription 3
4 (STAT3) signaling pathway is involved in various physiological processes, including immune
5 reaction, cell growth and hematopoiesis. JAK/STAT3 pathway is activated by cytokines such
6 as IL-6 and intercellular mediators [1]. In non-stimulated cells, STAT3, like other STATs
7 proteins, remains in an inactive cytoplasmic conformation. Then, once turned on, STAT3
8 translocates into the nucleus where it behaves as a transcription factor. This transcriptional
9 activation of STAT3 is induced by phosphorylation on a critical tyrosine residue (Tyr₇₀₅) and
10 dimerization of the protein [2].

11
12 Abnormal activation of STAT3 signaling was also associated with neurodegenerative
13 disorders such as Huntington disease or Alzheimer Disease (AD) [3]. The pathology of AD is
14 characterized by the presence of extracellular amyloid- β (A β) aggregates, forming A β
15 plaques, due to an increase of the expression of β -APP cleaving enzyme-1 (BACE1), the
16 enzyme responsible for the first cleavage of the amyloid precursor protein (APP) [9, 10].
17 Other features include neurofibrillary tangles, neuroinflammation and neuronal loss [4]. In
18 AD patients, neuropathological analyses revealed an elevation of STAT3 Tyr₇₀₅
19 phosphorylation in the hippocampus [3, 5] and phosphor-STAT3 is strongly increased in
20 hippocampus of APP/PS1 transgenic mice [5]. In this model, STAT3 activation may especially
21 act as an inducer of astrocyte reactivity [3] and its selective down-regulation in astrocytes
22 improved reduces memory decline [6, 7]. Neuroinflammation is marked by increasing
23 proinflammatory cytokines production and microglial cells overactivation, in particular
24 around A β plaques [8]. STAT3 can interfere with BACE1 levels and neuroinflammation in AD.
25 The translation of BACE1 is controlled by a pro-apoptotic kinase involved in several
26 pathological hallmarks of AD: the Protein kinase R (PKR) [9] inducing the phosphorylation of
27 eukaryotic initiation factor 2 alpha (eIF-2 α) [10]. BACE1 is also regulated at the
28 transcriptional level, notably by STAT3 activation [11-13]. STAT3 phosphorylation plays a role
29 in cytokines secretion, involved in AD neuroinflammation in AD, such as IL-1 β or tumor
30 necrosis factor (TNF- α) [14].

31 We have previously shown that systemic inflammation induced by intraperitoneal (i.p.)
32 lipopolysaccharide (LPS) administration can trigger neuroinflammation and increase brain A β

33 production in wild type (WT) mice [11]. In the same study, the genetic invalidation of PKR,
34 led to the reduction of neuroinflammation and A β accumulation associated with a reduction
35 of STAT3 activation [11].

36
37 Inhibition of STAT3 is an emerging field of research for cancer therapy [15-18]. Several
38 inhibitors have been developed and are currently tested in clinical trials for solid and
39 hematopoietic tumors [19, 20]. For this study, we selected Stattic, a small molecule that
40 selectively inhibits activation, dimerization, and nuclear translocation of STAT3 [21, 22] and
41 used the LPS model to assess the consequences of a chemical STAT3 inhibition on
42 neuroinflammation and hippocampal BACE1 levels.

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65 2. MATERIAL & METHODS

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67 2.1. *Animals and treatments*

68 All experimental procedures were approved by the Ethical Committee on Animal
69 Experiments of Paris Nord (CEEA n°121). Eight-week-old C57BL/6 male mice were obtained
70 from Janvier Labs (Le Genest-Saint-Isle, France). Mice were housed in cages with alternative
71 periods of light and dark (12h/12h) with water and food *ad libitum*. Efforts were made to
72 minimize the number of mice used in accordance with European guidelines for care of
73 laboratory animal's 2010/63/UE by using the same brain for immunohistochemistry and
74 protein quantification. Every day, i.p. injection of 1 mg/kg of LPS from *Salmonella enterica*
75 *serotype typhimurium* (Sigma Aldrich, L'Isle d'Abeau Chesnes, France) or equivalent volume
76 of saline was performed for 3 days with a syringe. In addition, mice were injected i.p with 20
77 mg/kg Stattic (solubilized in 2% DMSO+30% polyethylene glycol) or vehicle (containing 2%
78 DMSO+30% polyethylene glycol) at the same time and following the same procedure. The
79 concentration of Stattic used in this study was determined based on several publications
80 using this dosage in in vivo models [23, 24]. Four groups of mice were explored: saline
81 controls, Stattic, LPS+Saline and LPS+Stattic. The number of mice used for each group is
82 detailed in the figure legend corresponding to each experiment. 24 hours after the last
83 injection, mice were anesthetized with lethal dose of ketamine/xylazine. Brains were
84 extracted and sliced on the middle of the sagittal plan. One hemisphere was immersed in
85 paraformaldehyde (PFA 4%) for immunohistochemistry. The second part was dissected.
86 Hippocampus were separated on ice and frozen in liquid nitrogen for biochemical analyses.

87

88 2.2. *Immunohistochemistry*

89 Hemispheres were successively placed in 0.4% PFA (5 days), 30% sucrose (24h), frozen in
90 isopentane and then sectioned using a cryostat. Sagittal sections (30 µm) were chosen
91 according to the Mouse Brain Atlas at 0.84 to 1.32 lateral positions. After PBS rinsing, brain
92 sections were blocked with 5% BSA (bovine serum albumin, Sigma Aldrich) and 0.4% Triton
93 (Janssen Chemical, Beerse, Belgium) for 1 hour. The brain sections were then incubated with
94 the primary antibody for IBA-1 (1:1000, Wako, Richmond, VA), MAC-1/CD11b (1:200, Bio-
95 Rad/AbD Serotec) or NeuN (Merck-Millipore, Darmstadt, Germany) overnight at 4°C. The
96 sections were washed and incubated for 2 hours with secondary antibodies goat anti-rabbit

97 or goat anti-rat AlexaFluor 488 (ThermoFisher Scientific, Waltham, MA). Sections were
98 mounted on blades with mounting medium containing DAPI (Vectashield, Vector Laboratory,
99 Burlingame, CA) and imaged using Dmi8 microscope confocal (Leica, Wetzlar, Germany) and
100 LASX (Leica).

101 Microglial morphological analyses were performed by combining several ImageJ plugins [25]
102 including AnalyzeSkeleton (2D/3D) [26] and FracLac [27]. NeuN Immunoreactive cells were
103 counted in each hippocampal sub-regions by an experimenter blind to group identity.
104 Anatomical regions were determined as per Franklin and Paxinos (2007). CA1 and CA2 were
105 combined (CA1/2) due to the relatively amorphous boundary separating these two regions.

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108 *2.3. Immunoblotting and ELISA*

109 Proteins were extracted from hippocampus by sonication with Bioruptor (Diagenode,
110 Denville, NJ) until homogenization in RIPA lysis buffer containing phosphatase and protease
111 inhibitors. Proteins quantification was determined by a colorimetric method (BCA, Thermo
112 scientific) and absorbance was measured using Victor technology (PerkinElmer, Wellesley,
113 MA). After denaturation, 50 µg of protein lysate were separated on a gradient gel 4-20%
114 polyacrylamide Bis-Tris (Bio-Rad). Blots were transferred on nitrocellulose membranes
115 (BioRad, Hercules, CA). Membranes were blocked 1h in 5% milk (Fisher BioReagent, Bio-Rad)
116 and then incubated with primary antibody overnight at 4°C. After TBST rinses, membranes
117 were incubated with a secondary antibody (HRP Goat Anti-Rabbit IgG, Jackson
118 ImmunoResearch, Cambridgeshire, UK). Chemiluminescence signals were detected by
119 placing blots in Clarity Western ECL (Bio-Rad) and using an imaging system (Chemi-doc XRS,
120 Bio-Rad). Results were normalized with GAPDH (Glyceraldehyde 3-phosphate
121 dehydrogenase) and analyzed on Multigauge software (Fuji-Film, Tokyo, Japan).
122 Quantification of cytokines, TNF- α , IL-1 β , IL-6, IFN- γ (Interferon γ), IL-10 and IL-17- α , was
123 performed using Luminex multiplexing system (Bio-Rad) according to the manufacturer's
124 protocols.

125

126 *2.4. Statistical analyses*

127 Statistical analyses were performed with Prism 6 (GraphPad Software). Pooled results are
128 expressed as means \pm SEM. Two-way ANOVA was used to analyze the effect of treatments

129 (LPS, Static). Unpaired Student's *t* tests (unless the data were shown not to be normally
130 distributed) or Mann and Whitney tests were used for other comparisons. For all tests, *p*
131 values <0.05 were considered statistically significant.

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135 **3. RESULTS**

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137 **3.1. In the hippocampus, Stattic reverses STAT3 phosphorylation induced by systemic** 138 **injection of LPS**

139 We first evaluated the impact of Stattic on STAT3 phosphorylation (Figure 1A).
140 Administration of LPS increases the phosphorylation of STAT3 on Tyrosine 705 in the
141 hippocampus (+187%) of LPS-treated mice compared to controls. Peripheral injection of
142 Stattic in LPS-treated mice lead to a significant decrease of phosphoSTAT3_{Tyr705}/STAT3 ratio
143 (-34.6%) compared with LPS-treated only animals. No significant difference is seen between
144 saline-injected mice and saline + Stattic-treated animals. (Figure 1B)

145

146 **3.2. STAT3 inhibition decreases LPS-induced microglial activation in the hippocampus.**

147 To assess the impact of Stattic on neuroinflammation, we performed an
148 immunohistochemistry using two markers of microglial cells/ IBA-1 (ionized calcium-binding
149 adapter molecule 1) (Figure 2A-D) and MAC-1 (CD11b) (Supplementary Figure 1A). We
150 observe a strong increase (+139%) of IBA-1 expression in LPS-treated mice hippocampus
151 compared to untreated mice (Figure 2A, 2C and 2F). Quantification of MAC-1 staining reveal
152 a +284% increased expression (Supplementary Figure 1B). The enhanced intensity of these
153 two markers is associated with morphological changes typical of activated microglia, such as
154 enlarged amoeboid cell bodies with shorter ramified projections (see right boxes under
155 Figure 2A-D). By combining several ImageJ plugins [25], we have precisely quantified
156 microglial ramification length. In the LPS condition, we found a 49% reduction of cell process
157 length (Figure 2G). Stattic injections in LPS treated mice reduce both IBA-1 and MAC-1 levels
158 (respectively -39% and -44%) (Figure 2C-D, F and Supplementary Figure 1B) and microglial
159 morphological switch from ramified to amoeboid state (+64% for cell ramification length)
160 compared to LPS only treated mice (Figure 2G). No statistical difference is observed between
161 the saline group and the saline+Stattic group for the two microglial markers (Figure 2A-B, 2F
162 and Supplementary Figure 1B). To assess the potential impact of STAT3 inhibition on
163 neuronal death, we perform an immunohistochemistry with the neuronal marker, NeuN
164 (Supplementary Figure 2A-D). Quantification of NeuN immunoreactive cells in the different

165 regions of the hippocampus, *Cornu Ammonis* (CA)1/2, CA3 and Dentate gyrus (DG), did not
166 reveal any difference between the 4 groups of our study (Supplementary Figure 2E)

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170 **3.3. Stattic reduces cytokines production triggered by LPS administration.**

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172 To determine the impact of Stattic on cytokines production, we measured using Luminex six
173 mediators known for their role in brain inflammation: TNF- α , IL-1 β , IL-6, IFN- γ , IL-10 and IL-
174 17 α . IL-1 β , TNF- α and IL-6 are significantly increased in LPS-injected mice hippocampus in
175 comparison to saline treated animals, respectively +104% (Figure 3A), +50% (Figure 3B), and
176 +44% (Figure 3C). These level changes are drastically reduced following Stattic injections (-
177 34% for IL-1 β , -39% for TNF- α and -35% for IL-6). IFN- γ production is also significantly
178 increased following LPS production and Stattic injections reverse this augmentation (Figure
179 3D). The administration of Stattic alone does not affect cytokines production (Figure 3A-D).
180 LPS or Stattic exposure does not modify the levels of the two other tested cytokines, IL-10
181 and IL-17 α (data not shown).

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183 **3.4. Stattic reduces hippocampal BACE1 levels in LPS-treated mice**

184

185 BACE1 protein levels are assessed with immunoblot analyses in hippocampal extracts from
186 saline, saline+Stattic, LPS and LPS+Stattic treated mice (Figure 4A). We observe a 33%
187 increase of BACE1 levels in the hippocampus of LPS-treated mice compared to controls.
188 Stattic injections in LPS-treated mice reduce BACE1 concentrations to control levels. No
189 significant difference between saline-group and saline-Stattic-treated group is detected
190 (Figure 4B).

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201 **4. DISCUSSION**

202 Our results show that peripheral injections of Stattic, a selective STAT3 inhibitor, protect *in*
203 *vivo* against, (i) neuroinflammation by reducing microglial activation and pro-inflammatory
204 cytokines production, and (ii) excess hippocampal BACE1 protein levels induced by LPS.

205 In this study, we used a mouse model previously developed by our group showing that
206 systemic inflammation produced by LPS administration can induce neuroinflammation and
207 BACE1 upregulation in WT mice, two pathophysiological hallmarks involved in AD [11].
208 Although LPS models are commonly used to study aberrant innate immune activation in AD
209 models [28, 29], few of them also induce deregulation of A β metabolism [29].

210 Preliminary works of our group has demonstrated the involvement of PKR, a pro-apoptotic
211 kinase involved in several pathways leading to AD brain lesions [9, 30-32], in the severity of
212 hippocampal neuroinflammation as in the regulation of BACE1 expression [11]. Aberrant
213 phosphorylation of STAT3 seems to be the key mechanism linking phosphorylated PKR,
214 neuroinflammation and BACE1 deregulation. The group of Kroemer has proposed that PKR
215 and STAT3 could directly interact by forming a protein complex that may modulate
216 autophagy [33, 34]. The existence of a similar interaction in microglia and neurons remains
217 to be explored.

218 STAT3 is already a therapeutic target, particularly in certain cancers, and numerous analyzes
219 on the side effects and toxicity of STAT3 inhibitors have already been carried out [17, 35].
220 Among STAT3 inhibitors, we chose Stattic for two reasons: (i) the demonstration in a
221 previous study that the i.p. injection of Stattic decreased the phosphorylation of STAT3 in
222 the brain of mice suggesting that Stattic crosses the blood-brain barrier; (ii) the existence of
223 numerous pre-clinical models using this molecule [22].

224 Our results showed that STAT3 could mitigate microglia activation state and their pro-
225 inflammatory cytokines production, IL-1 β , IL-6, IFN- γ and TNF- α . If several previous works

226 carried out in mouse models of AD support a role of STAT3 activation in astrocytes reactivity
227 [3, 6, 7], its roles in microglial activation in the AD pathological context have been poorly
228 explored. Nevertheless, inhibition of the JAK-STAT3 pathway in a Parkinson disease rat
229 model abolished neuroinflammation by suppressing microglial activation, macrophage
230 infiltration and production of proinflammatory cytokines. These findings using a different
231 STAT3 inhibitor and our results suggest a potential protective effect of STAT3 inhibition in
232 neurodegeneration [36].

233 Finally, we have shown that Stattic could modulate A β metabolism by decreasing BACE1
234 protein level induced by systemic LPS administration (Figure 4C). This result confirms
235 previous observations revealing a role of STAT3 in A β -induced neuronal death [5]. Previous
236 works suggest that the regulation of BACE1 by STAT3 may occur at the transcriptional level
237 and it was shown that phosphorylated STAT3 act as a transcription factor binding mRNA of
238 BACE1 [12]. To deepen this study, other STAT3 inhibitors should be tested in our model.
239 Moreover, an evaluation of beneficial effects of Stattic on AD hallmarks (amyloid plaques
240 and tau phosphorylation) should be assessed in AD mouse models, such as 3xTg-AD or APP/
241 PS1 mice [37, 38].

242 Targeting neuroinflammation is a promising field of pharmaceutical drug development in AD
243 [39]. Recently, molecules inhibiting inflammatory pathway, such as the protein kinase p38
244 MAPK (p38 MAPK α), or the NLRP3 inflammasome have shown promising results for reduce
245 neuroinflammation and slow cognitive decline in AD patients [40-43]. With its powerful anti-
246 inflammatory action [44], STAT3 inhibition may be considered as an interesting candidate to
247 normalize brain inflammation in AD context.

248 In conclusion, this study provides a proof of concept of the beneficial effects of STAT3
249 inhibition on neuroinflammation and BACE1 metabolism and paves the way for future
250 explorations of Stattic in animal models of AD.

251

252 **ACKNOWLEDGMENTS**

253 The authors thank Fortune Hontonou, Calypso Nepost, Cindie Courtin, Luana Spano, Marion
254 Brault, Bruno Saubaméa and Véronique Cochois for their technical help.

255

256 **FUNDINGS**

257 This work was partially supported by University of Paris, Institut national de la santé et de la
258 recherche médicale (INSERM), and Assistante Publique des Hôpitaux de Paris (APHP).

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262 **AUTHORS' CONTRIBUTIONS**

263 FML and BH designed the study. FML, CP and JH analyzed the results. FML and PM wrote the
264 manuscript with feedback from all coauthors. CS and NV performed experimental
265 procedures on mouse model. PM, EB and BP performed biochemical and molecular biology
266 experiments.

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428 **FIGURE LEGENDS**

429 **Figure 1. Stattic injections reduce LPS-induced phosphorylation of STAT3 in mice**
430 **hippocampus. A.** Immunoblot of hippocampal protein extracts revealed by different
431 antibodies specific for phospho-STAT3, STAT3 full and GAPDH. **B.** Corresponding
432 quantification of phospho-STAT3 /STAT3 ratio after normalization on GAPDH level in the
433 four groups of mice non-exposed to LPS and Stattic (Saline, n=9), treated with Stattic
434 (Saline+Stattic, n=7) or LPS only (LPS, n=8) and injected in parallel with LPS and Stattic
435 (LPS+Stattic, n=6). The error bars indicate the \pm SEM. AU: Arbitrary unit. ***p<0.001

436

437 **Figure 2. Effect of Stattic on microglial activation in the hippocampus after LPS exposure.**

438 **A-D.** Double-labeling in immunofluorescence of DAPI (blue) and IBA1 (green) in
439 hippocampus sagittal sections of WT mice treated with saline (**A**), saline+Stattic (**B**), LPS (**C**)
440 and LPS+Stattic (**D**). Left boxes: "MICROGLIAL CELL". Higher magnification of hippocampal
441 microglial cells (boxed indicated by white arrow and enlarged on the respective bottoms).
442 Cellular contours of microglial cells (white line) are obtained by image J analyses. Right
443 boxes: "SKELETAL ANALYSIS". Higher magnification of microglial cells after skeletal and
444 fractal analyses using Image J software. Microglial processes are highlighted in red. **E.**
445 Schematic sagittal representation of hippocampal sections used for immunohistochemistry.
446 **F.** Relative IOD (integrated optical density) of IBA1 immunoreactivity in activated microglial
447 cells. **G.** Microglial processes length determined using Image J software. n=8 for each group.

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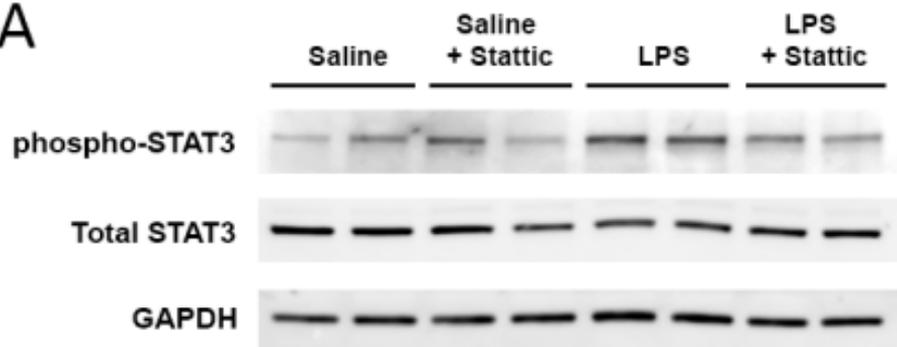
449 **Figure 3. Protective role of Stattic injections on cytokines production in mice hippocampus**

450 **after LPS treatment.** Hippocampus protein extracts analyzed with Luminex technology to
451 determine cytokines levels of respectively IL-1 β (**A**), TNF- α (**B**), IL-6 (**C**) and IFN- γ (**D**) in saline
452 and LPS-treated mice with or without Stattic injection. n is comprised for each group
453 between 5 and 8 animals (IL-1 β), 8 and 15 animals (TNF- α , IL-6 and IFN- γ). The error bars
454 indicate the \pm SEM (standard error of the mean). AU: Arbitrary unit. *p<0.05, **p<0.01,
455 ***p<0.001

456

457 **Figure 4. Inhibition of STAT3 decreases BACE1 levels and in mice hippocampus after LPS**

458 **systemic challenge. A.** Immunoblot analysis of mature BACE1 protein. **B.** Corresponding
459 quantification after normalization on GAPDH level in the four groups: untreated mice
460 (Saline, n=9), mice treated with Stattic (Saline+Stattic, n =7) or LPS only (LPS, n=7) and mice
461 injected in parallel with LPS and Stattic (LPS+Stattic, n=7). AU: Arbitrary unit. The error bars
462 indicate the \pm SEM. **p<0.01. **C.** Schematic representation of Stattic protective effects on
463 neuroinflammation and BACE1 levels. Peripheral inflammation and Stattic communicates
464 with the brain through the blood-brain barrier to induce microglial activation and pro-
465 inflammatory mediator's production involving STAT3 activation. Cytokines, including TNF- α
466 and IL-6, induce STAT3 nuclear translocation in hippocampal neurons leading to an increase
467 of BACE1 mRNA transcription.

A**B**