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1 **Phosphatase PP2A is essential for T_H17 differentiation**

2 Short title: PP2A is essential for T_H17 differentiation

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23 **Abstract**

24 Phosphatase PP2A expression levels are positively correlated to the clinical severity of systemic lupus
25 erythematosus (SLE) and IL17A cytokine overproduction, indicating a potential role of PP2A in
26 controlling T_H17 differentiation and inflammation. By generating a mouse strain with ablation of the
27 catalytic subunit α of PP2A in peripheral mature T cells (PP2A cKO), we demonstrate that the PP2A
28 complex is essential for T_H17 differentiation. These PP2A cKO mice had reduced T_H17 cell numbers
29 and less severe disease in an experimental autoimmune encephalomyelitis (EAE) model. PP2A
30 deficiency also ablated C-terminal phosphorylation of SMAD2 but increased C-terminal
31 phosphorylation of SMAD3. By regulating the activity of ROR γ t *via* binding, the changes in the
32 phosphorylation status of these R-SMADs reduced *Il17a* gene transcription. Finally, PP2A inhibitors
33 showed similar effects on T_H17 cells as were observed in PP2A cKO mice, i.e., decreased T_H17
34 differentiation and relative protection of mice from EAE. Taken together, these data demonstrate that
35 phosphatase PP2A is essential for T_H17 differentiation, and that inhibition of PP2A could be a possible
36 therapeutic approach to controlling T_H17-driven autoimmune diseases.

37 **Significance statement**

38 By using a gene knockout that leads to T-cell specific deletion, we reveal the essential role of Ser/Thr
39 phosphatase PP2A in T_H17 differentiation. We also show that this works through the regulation of
40 SMAD2/3 phosphorylation status, which elucidates molecular pathways by which PP2A modulates the
41 expression of T_H17 phenotypes. This finding extends our understanding of the close relationship
42 between PP2A overexpression and inflammatory disease. PP2A is the first Ser/Thr phosphatase shown
43 to be capable of controlling T_H17 differentiation *via* modulating R-SMADs activity. We also
44 demonstrate the translational potential of these findings by showing a therapeutic effect of PP2A
45 inhibitors in controlling autoimmune disease in the EAE model.

46 **Keywords:** T_H17, PP2A, TGF β

47

48 **/body Introduction.**

49 T-helper type 17 (T_H17) cells, a subset of CD4⁺ T cells defined by IL17, IL22 and IL21 production, are
50 essential for control and clearance of extracellular bacterial and fungi (1, 2). However, excessive T_H17
51 responses are involved in chronic inflammation and development of many human autoimmune diseases
52 (3). Upon encountering antigen in the context of a local cytokine milieu including transforming growth
53 factor β (TGF β) and IL6, naïve CD4⁺ T cells undergo differentiation into effective T_H17 cells. TGF β is
54 the principal, essential factor promoting the differentiation of T_H17 cells (4, 5).

55 Through two related transmembrane Ser/Thr kinase receptors, TGF β induces Ser/Thr signal cascades in
56 activated T cells. Recent work including work from our lab has revealed the regulatory roles of some
57 other Ser/Thr kinases in this process. For example, both MEKK2/3 and MINK1 suppress T_H17
58 differentiation through direct phosphorylation of the TGF β signaling components SMAD2 and SMAD3
59 (6, 7). Precise regulation of SMAD2/3 Ser/Thr phosphorylation status is thus important in driving T_H17
60 differentiation (6-8). Dephosphorylation of SMAD2/3 is equally critical in this process but the specific
61 phosphatases that catalyze SMAD2/3 dephosphorylation remain unknown.

62 As one of the major Ser/Thr phosphatases in eukaryotes, phosphatase PP2A is critical for many cellular
63 functions including cell survival, proliferation, activation and differentiation (9). It has been reported
64 that elevated PP2A expression levels are linked to the upregulation of IL17A production by CD4⁺ T
65 cells in human systemic lupus erythematosus patients (10). Studies in the PP2A^c transgenic mouse
66 model also demonstrated the relationship and mechanism linking of PP2A and *Il17*-dependent
67 immunopathology (11, 12). PP2A is composed of three polypeptide chains, the structural A, the
68 regulatory B and the catalytic C subunits (13). The heterodimer of the A subunit and the C subunit
69 (PP2A_A-PP2A_C) forms the PP2A core enzyme that associates with one regulatory B subunit, thus
70 determining the substrate specificity of the holoenzyme complex (13).

71 In TGF β signaling, two related regulatory B subunits, B α (*Ppp2r2a*) and B δ (*Ppp2r2d*), opposingly
72 modulate TGF β /Activin/Nodal signaling (14), while carboxy terminal phosphorylation of MAD (the
73 SMAD homolog protein in *Drosophila*) is negatively regulated by the PP2A inhibitor Okadaic Acid
74 (15). By analogy, these observations suggest that PP2A might be a Ser/Thr phosphorylation modulator
75 involved in controlling T_H17 differentiation.

76 Here, we present data showing that T_H17 cell polarization was largely impaired when *Ppp2ca* was

77 ablated in mature T cells and rendered resistance towards MOG-induced experimental autoimmune
78 encephalomyelitis (EAE). We also show that PP2A knockout leads to altered activation of R-SMADs
79 (specifically decreasing SMAD2 activation and increasing SMAD3 activation). This synergistically
80 inhibited ROR γ t mediated *Il17a* transcription. This work thus reveals specific role of PP2A in
81 regulating the canonical TGF β -R-SMAD-ROR γ t signaling process during T_H17 differentiation and
82 indicates a possible therapeutic approach for controlling T_H17 driven autoimmune diseases *via*
83 inhibition of PP2A.
84

85 **Results.**

86 **Normal T cell development in PP2A cKO mice.** To explore the function of PP2A in peripheral T cells,
87 we deleted the dominant PP2A C α isoform of PP2A catalytic subunit (PP2Ac) in T cell by crossing
88 ^{dis}-Lck (dLck) Cre with *Ppp2ca*^{fl/fl} mice (in which exons 3-5 of *Ppp2ca* are *loxP* flanked) (16) to
89 generate *Ppp2ca*^{fl/fl} dLck-Cre (termed PP2A cKO mice) and *Ppp2ca*^{fl/+} dLck-Cre or *Ppp2ca*^{+/+}
90 dLck-Cre mice (collectively called PP2A WT mice here) (*SI Appendix*, Fig. S1A and B). The dLck-Cre
91 was driven by the distal promoter of the lymphocyte protein tyrosine kinase (*Lck*) gene, enabling
92 investigation of the *Ppp2ca* deletion after positive selection in T cells (17).

93 To assess deletion efficiency, *Ppp2ca* mRNA and protein levels were measured and showed clear
94 reduction in peripheral T cells in PP2A cKO mice, while remaining normal in thymic subsets and
95 splenic B cells (*SI Appendix*, Fig. S1C and D). The catalytic subunit of PP2A has two isoforms, C α and
96 C β (encoded by *Ppp2ca* and *Ppp2cb* respectively). Notably we didn't observe compensatory
97 overexpression of *Ppp2cb* (*SI Appendix*, Fig. S1C). PP2A activity in cKO CD4⁺ T cells was reduced to
98 half of that measured in WT controls (*SI Appendix*, Fig. S1E).

99 Analysis of the numbers and frequencies of different T cell subsets in these mice showed that cKO
100 mice exhibited normal T cell development in thymus (*SI Appendix*, Fig. S2A-C) as well as in peripheral
101 lymphoid organs (*SI Appendix*, Fig. S2A, D and E). The proportions of naïve/effective T cells in spleen
102 and mesenteric lymph nodes (MLN) were also similar between WT and cKO littermates (*SI Appendix*,
103 Fig. S2F and G). The normal development of peripheral lymphocytes in PP2A cKO mice allowed
104 further investigation of the role of PP2A in T cell differentiation.

105 **T_H17 cell numbers are reduced in PP2A cKO mice.** To clarify whether PP2A is involved in T helper
106 cell lineage commitment, we analyzed the populations of T helper subsets *in vivo*. Interestingly, CD4⁺
107 T cells from PP2A cKO mice only contained half the number of T_H17 cells comparing to their WT
108 littermates (*SI Appendix*, Fig. S3A and B), while the numbers of T_H1 and Treg CD4⁺ T cells were not
109 affected in the peripheral lymphoid organs (*SI Appendix*, Fig. S3A, C, D and F). The frequency of
110 Foxp3⁺ regulatory T cells in the thymus was also comparable between PP2A WT and cKO mice (*SI*
111 *Appendix*, Fig. S3E and F). Similarly, subsets analysis of the lamina propria also showed a consistent
112 reduction of T_H17 cells (*SI Appendix*, Fig. S3G and I). These data demonstrate that PP2A is involved in
113 maintaining T_H17 cell composition, while other T cell subsets, including Treg and T_H1, appear
114 unaffected.

115 **PP2A deletion impairs T_H17 differentiation *in vitro*.** To investigate whether reduced levels of T_H17
116 cells in PP2A cKO mice result from impaired T_H17 differentiation, we sorted naïve CD4⁺ T cells from
117 both WT and cKO cells and polarized them under T_H1, T_H2, T_H17 and iTreg conditions to compare
118 their differentiation efficiencies. The results showed that only the generation of T_H17 cells notably
119 declined with the PP2A deficiency, while other T helper subsets were not affected (Fig. 1A and B and
120 *SI Appendix*, Fig. S4A and B). The expression levels of *Ppp2ca* mRNA and PP2A C α protein were more
121 abundant in T_H17 cells than in other T helper subsets (*SI Appendix*, Fig. S4G and H).

122 p-PP2Ac (Y307) levels, a negative indicator for PP2A activity, are lower in T_H17 and T_H1 than in the
123 other subsets, indicating higher PP2A activity in these two subsets (*SI Appendix*, Fig. S4I). Cytokine
124 production showed similar results, confirming that PP2A deficiency specifically reduced T_H17
125 differentiation (Fig. 1C and *SI Appendix*, Fig. S4C). We further tested the expression of several key
126 T_H17 signature genes and found notably decreased *Il17a* and *Il17f* expression and slightly reduced
127 expression of *Rora* and *Il21*, while the expression of *Rorc*, *Il22* and *Il23r* were not significantly
128 affected (Fig. 1D). The alteration of the expression pattern of T_H17 signature genes (including *Il17a*
129 and *Il17f*) induced by PP2A deficiency was also confirmed by an RNAseq analysis (*SI Appendix*, Fig.
130 S5A and B and Dataset 1-3).

131 The proliferation capacity of WT and cKO T cells was measured under T_H17 conditions and were
132 comparable 2 and 5 days after stimulation (*SI Appendix*, Fig. S4D). Upon PI and Annexin staining, WT
133 and cKO cells showed comparable apoptotic rates (*SI Appendix*, Fig. S4E). Thus, reduced IL17A⁺
134 CD4⁺ T cell levels and IL17A production were not due to either impaired proliferation or increased
135 apoptosis in cKO cells. Furthermore, we did not observe an increased portion of IFN γ ⁺ or Foxp3⁺ CD4⁺
136 T cells in cKO T cells under T_H17 conditions, which rules out the likelihood of T_H17 cells converting to
137 other cell subsets in these circumstances (Fig. 1A and *SI Appendix*, Fig. S4F). These findings therefore
138 demonstrate a T-cell intrinsic impairment of the T_H17 polarization program upon PP2A deficiency
139 which is independent of proliferation, apoptosis or subset conversion.

140 **Reduced severity of EAE in mice with PP2A deficiency.** Given the required role of PP2A in inducing
141 normal T_H17 polarization, the question of whether defective T_H17 PP2A cKO cells also influence
142 T_H17-driven autoimmune disease was investigated *in vivo* using an experimental autoimmune
143 encephalomyelitis (EAE) model. We therefore immunized PP2A WT and cKO mice with myelin

144 oligodendrocyte glycoprotein peptide of amino acids 35–55 (MOG₃₅₋₅₅) to induce EAE.

145 Clinical scoring showed that PP2A deficiency alleviated symptoms of autoimmunity (Fig. 2A).

146 Histological examination showed significantly less mononuclear cell infiltration and demyelination in

147 the cerebral and spinal cord of the cKO mice (Fig. 2B). Inflammatory cell infiltration into the central

148 nervous system (CNS) was also greatly diminished in the cKO group (Fig. 2C). Although the

149 proportion of CD4⁺ T cells was unchanged in the CNS of cKO mice, the total numbers of infiltrating

150 CD4⁺ and CD8⁺ cells were significantly reduced (Fig. 2C). IL17A producing CD4⁺ T cells were also

151 present in significantly decreased numbers in both the CNS and draining lymph nodes (DLN), whereas

152 the frequencies of IFN γ producing CD4⁺ T cells were unchanged. (Fig. 2D-F). These data indicate that

153 reduced IL17A production upon PP2A cKO results in less severe MOG-induced CNS inflammation.

154 Indeed, *in vitro* MOG recall analysis also showed reduced IL17A production by PP2A cKO splenocytes,

155 while IFN γ production was unaffected (Fig. 2G). Intriguingly, Foxp3⁺ CD4⁺ T cell proportions were

156 also reduced in the CNS but not in the DLN (Fig. 2D-F). This might be explained by markedly milder

157 inflammation in the CNS which consequently recruited less regulatory cells. PP2A cKO mice are thus

158 resistant to EAE and this is strongly correlated with an observed significant reduction in T_H17 cell

159 induction.

160 **Intact TCR and IL6 signaling in PP2A cKO CD4⁺ T cells.** We next investigated how PP2A

161 deficiency affected intracellular signaling in controlling T_H17 cell differentiation. Intact TCR signaling

162 is indispensable for T_H17 commitment (18). However, our results showed that PP2A deficiency did not

163 affect normal CD69 upregulation (*SI Appendix*, Fig. S6A), cell proliferation (*SI Appendix*, Fig. S6B) or

164 production of IL2 or IFN γ (*SI Appendix*, Fig. S6C) following anti-CD3 stimulation. Interestingly,

165 Western Blot analysis showed upregulation of pERK and p38 in PP2A deficient cells (*SI Appendix*,

166 Fig. S6D) and these two pathways are known to play opposing roles in T_H17 differentiation. Inhibition

167 of MEK-ERK signaling enhances T_H17 differentiation while activation of P38 is critical for optimal

168 T_H17 polarization (19-21). We found that use of an MEK inhibitor (inhibiting ERK, U0126) failed to

169 restore T_H17 differentiation due to PP2A deficiency (*SI Appendix*, Fig. S6E). Thus, subtle changes in

170 MAPKs activation are not correlated with decreased T_H17 differentiation due to PP2A deficiency.

171 PP2A overexpression has been previously reported to upregulate *Il17a* gene transcription by enhancing

172 IRF4 activity (11). However, the observed mRNA, protein and IRF4 activity did not support

173 involvement of IRF4 in leading to diminished *Il17a* transcription (*SI Appendix*, Fig. S5C-E).

174 By using the PP2A inhibitor OA, previous work has suggested a possible regulatory role of PP2A in
175 regulating IL6 signaling, including promoting the stability of IL6 receptor gp130 (CD130) (22) and
176 modulating STAT3 phosphorylation (23). However, our experiments did not show any alterations in
177 either IL6 receptor expression (*SI Appendix*, Fig. S7A and B) or STAT3 phosphorylation (Y705 and
178 S727) after IL6 stimulation in cKO T cells (*SI Appendix*, Fig. S7C) thus excluding the possibility that
179 the inhibition of T_H17 differentiation upon PP2A deletion is due to TCR or IL6 signaling changes.

180 **Differential modulation of SMAD2/3 activity by PP2A deficiency restrains T_H17 differentiation.**

181 In TGFβ pathway, TGFβ RI and RII, which are reported to be opposingly regulated by two different
182 PP2A B subunits (14), were similarly expressed in PP2A cKO T cells (Fig. 3A and B). Meanwhile, we
183 found that pSMAD2 (Ser465/467) level was decreased but pSMAD3 (Ser423/425) level was increased
184 after TGFβ stimulation in PP2A cKO cells (Fig. 3C). In accordance, SMAD2 hyper-phosphorylation
185 and SMAD3 under-phosphorylation was observed in PP2A Cα overexpressed 293FT cells after TGFβ
186 stimulation (*SI Appendix*, Fig. S8A and B). We next performed the Co-IP assay in Jurkat cells to
187 explore the binding of R-SMADs to PP2Ac. We found that PP2Ac can form stable complex with
188 SMAD2 and SMAD3, which does not depend on either TCR or TGFβ stimulation (*SI Appendix*, Fig.
189 S8C and D).

190 In addition, *in vitro* dephosphorylation assay showed that PP2A can directly dephosphorylate pSMAD3
191 (*SI Appendix*, Fig. S8E-G). Studies on SMAD2 and SMAD3 conditional knockout mice have revealed
192 the opposite functions of these two molecules in inducing T_H17 cells (24-27). We thus suspected that
193 the altered activation of SMAD2/3 in PP2A cKO cells might serve as the major contributor towards
194 decreased *Il17a* transcription. Indeed, when we expressed different activation forms of SMAD2/3 (WT,
195 dominant negative form 2SA, constitutive active mutants 2SD) in naïve CD4⁺ T cells and analyzed cell
196 differentiation in T_H17 condition, the results clearly showed that insufficient activation of SMAD2
197 caused defective T_H17 differentiation, while constitutively activated SMAD2 promoted optimal T_H17
198 priming (Fig. 3D and E). On the contrary, ectopic expression of SMAD3 dramatically repressed T_H17
199 polarization. Interestingly, a SMAD3-2SA mutant also showed inhibitory activity to T_H17 polarization
200 although to a lesser degree (Fig. 3F and G), indicating that suppression of T_H17 differentiation by
201 SMAD3 depends on both SMAD3 activation and on its overall expression level. To rule out alterations

202 in iTreg cell skewing condition in the previous experiments, we also used a series of TGF β doses for
203 suboptimal iTreg priming but observed no difference between the PP2A cKO and WT groups (*SI*
204 *Appendix*, Fig. S7D).

205 **Changes in SMAD2/3 activity synergistically downregulate *Il17a* transcription via reducing**
206 **ROR γ t activity.** We next asked how the shift of SMAD2/3 phosphorylation status affected *Il17a*
207 transcription. We found that the protein expression levels of ROR γ t and ROR α were not different in
208 PP2A WT and cKO T_H17 cells (Fig. 4A and B). More importantly, retrovirus-mediated ectopic
209 expression of ROR γ t could not completely restore T_H17 potentiation in cKO T cells (Fig. 4C and D).
210 These data supported the hypothesis that PP2A controlled T_H17 differentiation is independent of ROR γ t
211 protein expression. To address whether overactivation of SMAD3 and insufficient activation of
212 SMAD2 could work cooperatively to suppress ROR γ t activity, ChIP analysis of ROR γ t occupancy of
213 *Il17a* gene region was carried out. The result confirmed our hypothesis that with equal ROR γ t
214 expression, its activity was largely reduced due to PP2A deficiency (Fig. 4E). Phosphorylation changes
215 of SMAD2/3 affected their binding ability with ROR γ t. In cKO T_H17 cells, ROR γ t binded more
216 SMAD3 and less SMAD2 than observed in WT T_H17 cells (Fig. 4F).

217 Different activation forms of SMAD2/3 were then co-transfected with ROR γ t in the 293FT cell line.
218 These experiments showed that the constitutively active SMAD2/3 preferentially interacts with ROR γ t
219 over the inactive forms, and that SMAD3 is more accessible to bind ROR γ t than SMAD2 (Fig. 4G).
220 These results suggest that phosphorylation changes in SMAD2/3 may inhibit ROR γ t activity by
221 affecting the capacity of SMAD2/3 to form complexes with ROR γ t. Based on this hypothesis, we
222 performed rescue experiments with SMAD2-2SD transfection or SMAD3 knockdown. Both
223 approaches significantly improved T_H17 polarization in cKO naïve CD4⁺ T cells (Fig. 4H-K). SMAD3
224 knockdown efficiency by siRNA was verified by Western Blot and RT-PCR (*SI Appendix*, Fig. S9A and
225 B).

226 **PP2A inhibitors phenocopy phosphorylation changes of SMAD2/3 and restrain T_H17 polarization**
227 ***in vitro*.** We observed that administration of PP2A inhibitors in 293FT cells phenocopied the SMAD2/3
228 activation changes in a dose dependent manner (Fig. 5A). Further, the PP2A inhibitor Cantharidin
229 (CAN) restrained ROR γ t mediated *Il17a* promoter activation (Fig. 5B). More importantly, when
230 administrating PP2A inhibitor Cantharidin in T_H17 culture medium, we also observed a dose dependent

231 inhibitory role at a concentration that had no effect on cell proliferation (Fig. 5C-E). Another two PP2A
232 inhibitors OA and Fostriecin (FOS) also showed similar effects upon T_H17 differentiation (*SI Appendix*,
233 Fig. S10A-E). In addition, PP2A cKO T cells did not display suppression effects by PP2A inhibitors,
234 ruling out the off-target effects might result in T_H17 depression (Fig. 5F and *SI Appendix*, Fig. S10F).

235 **A PP2A inhibitor protects mice from EAE.** We examined whether Cantharidin can block T_H17 cell
236 mediated inflammation in EAE. A significant reduction of disease severity was observed in Cantharidin
237 treated PP2A WT mice compared to PBS treated WT mice (Fig. 6A). Histological examination also
238 showed significantly less mononuclear cells infiltration and demyelination in the spinal cord of the
239 Cantharidin treated WT mice (Fig. 6B). Meanwhile, there was no observable aggravation of clinical
240 symptoms by applying Cantharidin in PP2A cKO mice, suggesting that Cantharidin dose was within a
241 safe range (Fig. 6A). Fewer mononuclear lymphocytes and CD4⁺ T cells infiltrated into the CNS of
242 Cantharidin treated WT mice than of the PBS WT group at the onset or peak of EAE, while the CD8⁺
243 and myeloid population was not significantly changed (Fig. 6C-D). Additionally, the ratio of T_H17 cells
244 was lower in Cantharidin treated WT mice than in PBS treated WT mice (p=0.08) (Fig. 6E). The
245 number of infiltrated T_H17 cells was also significantly lower in the CNS. Treg cells and IFN γ
246 producing cells were slightly decreased (Fig. 6F). Furthermore, Cantharidin treatment in PP2A cKO
247 groups did not alter the number of inflammatory lymphocytes infiltrated into CNS (Fig. 6G-H).
248 Cantharidin treated cKO groups showed no effects to EAE symptoms, suggesting the specific targeting
249 of Cantharidin to PP2A in T cells in this experimental setting. However, whether Cantharidin can also
250 act on other cells and contribute to the therapeutic effects is unclear. Collectively, data above showed
251 that PP2A inhibitor Cantharidin can limit EAE development principally by reducing T_H17
252 differentiation.

253

254 **Discussion.**

255 By using peripheral T cell specific KO mice, we have established the positive regulatory role of PP2A
256 in T_H17 differentiation as well as in inflammatory autoimmune diseases. We have also proved that
257 underactivated SMAD2 and overactivated SMAD3 downstream of TGFβ signaling collectively
258 reduced RORγt mediated *Il17a* transcription in PP2A cKO mice. PP2A inhibitors also reduced T_H17
259 polarization, indicating a promising therapeutic avenue for treating T_H17 cell mediated autoimmune
260 diseases.

261 PP2A is one of the most abundant phosphatases and crucial for many key cellular events. A total
262 knockout of the PP2A catalytic subunit *a* in mice is lethal (16). To our surprise, PP2A deficiency did
263 not lead to fundamental changes in basic processes such as cell survival and proliferation. This is either
264 because of the residual PP2A expression in our cKO mice or the stage and cell specific functions of
265 PP2A.

266 As shown by RNAseq and RT-PCR analysis, the transcriptional changes of T_H17 signature genes
267 induced in PP2A cKO were limited. Obvious changes were observed in *Il17a* and *Il17f*, but not in *Rorc*.
268 Expression of RORγt was intact, which exclude the possibility that PP2A is involved in the pathways
269 leading to RORγt induction. Therefore, intracellular signaling events downstream of RORγt and closely
270 related to *Il17a* transcription might be the candidate targets of PP2A mediated inhibition of T_H17
271 differentiation.

272 SMAD2, SMAD3 and SMAD4 are all critical for TGFβ signaling and participate in T_H17 or iTreg cell
273 priming to induce balanced expression of Foxp3 and RORγt (24-26, 28-30). It is intriguing that
274 SMAD2 knockout mice show reduced T_H17 cell differentiation and ameliorated EAE, while a
275 deficiency in SMAD3 has the opposite effects (24, 26-28). Furthermore, overexpression of SMAD2
276 and RORγt augments the differentiation of T_H17 cells. However, SMAD3 binds to RORγt in Co-IP
277 experiments and decreases its transcriptional activity (26). Moreover, R-SMAD activation is mainly *via*
278 the phosphorylation of the C-terminal SSXS motif, which is critical for R-SMAD function. The active
279 form of SMAD3 might enhance its binding affinity with RORγt and it is known that SMAD3 can
280 compete with SMAD2 for binding with RORγt (27). These results suggest that active SMAD2 plays a
281 positive role and active SMAD3 plays a negative role in T_H17 cell differentiation likely *via* dynamic
282 interaction with RORγt (26, 27).

283 Our study confirmed that altered SMAD2/3 activation by PP2A affects T_H17 differentiation, resulting

284 in decreased *Ill7a* transcription *via* forming complex with ROR γ t and reducing its activity and thus
285 affecting T_H17 differentiation. Importantly, this is the first study to identify PP2A as the critical
286 phosphatase responsible for Ser/Thr dephosphorylation on R-SMADs and necessary for efficient T_H17
287 differentiation.

288 It is important to know the precise dephosphorylation site on R-SMAD and the responsible kinases.
289 Our previous work has elucidated the importance of threonine residue T324 in the α -helix 1 region of
290 SMAD2 for regulation during T_H17 differentiation (6). It is thus likely that PP2A upregulates SMAD2
291 C-terminal phosphorylation *via* modulating MINK1 activity. However, the function of PP2A appears to
292 be broader and more dominant than this single phosphorylation event, since we also observed increased
293 SMAD3 phosphorylation in cKO CD4⁺ T cells, which also contributes to decreased T_H17 differentiation.
294 This observation is in accordance with a previous finding that the PP2A structural subunit PR65 could
295 interact with SMAD3 (31).

296 It is also likely that PP2A directly regulates the phosphorylation status of ROR γ t. Recent study showed
297 that two functional phosphorylation sites identified on ROR γ t played opposite roles in T_H17
298 polarization. IKK α was discovered as upper stream regulator for the phosphorylation change (32).
299 Whether PP2A participates in the interaction with IKK α or ROR γ t in regulating ROR γ t activity remains
300 to be elucidated.

301 The classical TGF β pathway is also critical for iTreg differentiation (33). SMAD2 and SMAD3 double
302 knockout mice showed dramatic loss of Foxp3 induction (28). Then why is iTreg differentiation not
303 affected by PP2A deficiency? It is most likely because of the redundant roles of SMAD2 and SMAD3
304 in TGF β induced iTreg plasticity (28). Overactivated SMAD3 may compensate for insufficient
305 activation of SMAD2 in Foxp3 induction. In a recent study, PP2A was reported to be indispensable for
306 the maintenance of the suppressive function of Treg cells *via* regulating the activity of the mTORC1
307 complex. Specific ablation of PP2A in Treg cells by Foxp3-YFP-Cre leads to autoimmunity with
308 similar clinical features of scurfy mice (34). We also observed the same Treg phenotype in dLck-driven
309 PP2A cKO mice, but the overall outcome of PP2A defect in peripheral T cells is dominated by T_H17
310 differentiation impairment, which is demonstrated by their reduced susceptibility to autoimmune
311 diseases induction.

312 Finally, in addition of finding the importance of PP2A to T_H17 differentiation, we demonstrated the

313 translational potential of this pathway by showing the therapeutic effect of PP2A inhibitor in
314 controlling autoimmune diseases in the EAE model.
315

316 **Materials and Methods.**

317 **Mice.** *Ppp2ca* floxed mice were provided by X. Gao, Model Animal Research Center of Nanjing University. Mice with
318 Cre recombinase driven by the distal promoter of the gene encoding the kinase Lck were bought from the Jackson Laboratory.
319 The experimental protocols were approved by the Review Committee of Zhejiang University School of Medicine and followed
320 institutional guidelines.

321 **EAE Induction.** EAE was induced as described previously (35). Briefly, mice aged 6-8 weeks were immunized with 200 mg
322 MOG₃₅₋₅₅ (Sangon, MEVGWYRSPFSRVVHLYRNGK) in an equal amount of Complete Freund's Adjuvant (Chondrex, Inc.) and
323 received 200 ng pertussis toxin (List Biochemicals) intravenously on days 0 and 2 post-induction. Clinical evaluation was
324 assigned daily using a 5-point scale: 1, flaccid tail; 2, impaired righting reflex and hindlimb weakness; 3, hindlimb paralysis; 4,
325 hindlimb and forelimb paralysis; 5, moribund. Detailed materials and methods are presented fully in SI Appendix, SI Materials
326 and Methods.

327 **Statistical Analysis.** Statistical analysis was performed using GraphPad Prism. The data were analyzed by Student's t-test. All P
328 value less than 0.05 was considered significant (P < 0.05 = *; P < 0.01 = **; P < 0.001 = ***).

329

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339 Q.X., X.J., M.Z., D.R., G.F. and Z.W. performed the research; Q.X., X.J. and L.L. analyzed the data;
340 Q.X., X.J., R.S., L.W., H.H. and L.L. wrote the paper.

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451 **Figure legends**

452 **Fig 1. PP2A deficiency specifically limits T_H17 differentiation *in vitro*.**

453 (A, B) Flow cytometry (A) and quantification (B) of IL17A staining in naïve CD4⁺ T cells from PP2A
454 WT and cKO mice differentiated under T_H17 polarizing condition for 5 days. (C) Enzyme-linked
455 immunosorbent assay (ELISA) of IL17A in the culture medium of each polarizing condition (n=3
456 technical replicates). (D) RT-PCR analysis of T_H17 signature genes (n=3 technical replicates). Each
457 symbol represents an individual mouse (n=8), error bars show mean ± SEM. Data are representative of
458 at least three independent experiments with similar results.

459 **Fig 2. Loss of PP2A protects mice from EAE by repressing IL17A production.**

460 (A) Mean clinical scores for EAE from each group. (B) Representative histology of the brain and spinal
461 cord (hematoxylin and eosin (H&E) on the left and luxol fast blue (LFB) on the right) of mice after
462 EAE induction (day 19). Arrowheads indicate inflammatory infiltration (left) and demyelination (right).
463 Scale bars represent 100 µm. (C) Number and frequency of mononuclear cells or CD4⁺ or CD8⁺ T cells
464 infiltrated into central nervous system. (D) Flow cytometry of IL17A, IFNγ and Foxp3 staining from
465 CNS (left panel) or DLN (right panel) CD4⁺ T cells. (E, F) Quantification of IL17A⁺, IFNγ⁺ and
466 Foxp3⁺ CD4⁺ T cells in CNS (E) or DLN (F). (G) Splenocytes were rechallenged with MOG peptide
467 (5µg/ml) or control vehicle for 3 days, and cytokine production was measured by ELISA. Each symbol
468 represents an individual mouse (n=4-6); error bars show mean ± SEM. Data are representative of three
469 independent experiments with similar results.

470 **Fig 3. Insufficient SMAD2 activation and overactivated SMAD3 under TGFβ pathway restrains**
471 **T_H17 differentiation.**

472 (A, B) Histograms (A) and mean fluorescence intensity (MFI) quantification (B) of TGFβ receptor I
473 and II staining on CD4 gated cells from splenocytes of PP2A WT and cKO mice. (C) Enriched CD4⁺ T
474 cells from PP2A WT and cKO were stimulated with 10 ng/ml TGFβ as indicated, whole cell lysates
475 were probed with the indicated antibodies in the immunoblots. (D, E) Flow cytometry (D) and
476 quantification (E) of T_H17 polarization with ectopic expression of Vector, WT, 2SA and 2SD of
477 SMAD2 in WT naïve CD4⁺ T cells. GFP expressing cells were gated for analysis on day 3. (F, G) Flow
478 cytometry (F) and quantification (G) of T_H17 polarization with ectopic expression of Vector, WT, 2SA
479 and 2SD of SMAD3 in WT naïve CD4⁺ T cells. GFP expressing cells were gated for analysis on day 3.
480 Each symbol represents an individual mouse (n=4). Error bars show mean ± SEM. Data are
481 representative of three independent experiments (C) or two independent experiments with two
482 replicates (D, F).

483 **Fig 4. Differential modulation of SMAD2/3 inhibits RORγt mediated *Il17a* transcription by**
484 **forming complex with RORγt.**

485 (A) Flow cytometry of RORγt staining from naïve CD4⁺ T cells primed under T_H17 polarizing
486 condition for 2 days. (B) RORα was immunoblotted with whole cell lysate of T_H17 cells. (C-D) Flow
487 cytometry (C) and quantification (D) of T_H17 polarization with ectopic expression of Vector or RORγt

488 in PP2A WT and cKO naïve CD4⁺ T cells. GFP expressing cells were gated for analysis on day 3. (E)
489 RORγt binding to the sites of the *Il17a* gene promoter was analyzed by using chromatin
490 immunoprecipitation (ChIP) assay with RT-PCR. (F) Co-IP analysis of binding ability with RORγt
491 among SMAD2/3 in WT and cKO T_H17 cells. (G) Co-IP analysis of binding ability with RORγt among
492 WT, 2SA and 2SD mutant of SMAD2/3 in 293FT cells. (H) Flow cytometry of T_H17 polarization with
493 ectopic expression of Vector or SMAD2-2SD in PP2A WT and cKO naïve CD4⁺ T cells. (I) Ratio of
494 IL17A frequency comparing VEC-cKO or SMAD2-2SD-cKO to VEC-WT. (J) Flow cytometry of T_H17
495 polarization after suppressing SMAD3 expression by siRNA in naïve CD4⁺ T cells. (K) The ratio of
496 IL17A frequency in cKO cells transfected with siRNA-SMAD3 or control to the frequency of WT cells
497 transfected with control. Error bars show mean ± SEM. Data are representative of three (A, B, G, H, J)
498 or two (C, D, E, F) independent experiments.

499 **Fig 5. PP2A inhibitors lead the same change of SMAD2/3 activation under TGFβ pathway and**
500 **limit T_H17 priming *in vitro*.**

501 (A) SMAD2/3 was overexpressed in the 293FT cells and PP2A inhibitors were added in serum free
502 medium for 2 hours followed by TGFβ (3 ng/ml) stimulation for 3 hours. Western Blot analysis using
503 phospho-specific SMAD2/3 (p-SMAD2/3) and FLAG-tag (total-SMAD2/3) antibodies. (B) *Il17a*
504 promoter and SMAD2/3 with or without RORγt was transfected into 293FT cells and then treated with
505 PP2A inhibitors and TGFβ as Figure (A). Luciferase activity was measured and normalized based on
506 Renilla luciferase gene. (C) Sorted naïve CD4⁺ T cells were polarized under T_H17 priming condition
507 with the indicated concentration of Cantharidin for 3 days and IL17A⁺ population was analyzed using
508 flow cytometry on day 5. (D) The concentration of IL17A in the culture supernatant was measured by
509 ELISA. (E) Histogram of CFSE fluorescence staining of cells in Figure (C). (F) WT naïve CD4⁺ T cells
510 cultured with Cantharidin (5μM) for the first 3 days in T_H17 priming condition and analyzed IL17A⁺
511 population by flow cytometry on day 5. Error bars show mean ± SEM. Data are representative of two
512 independent experiments with similar results.

513 **Fig 6. A PP2A inhibitor suppresses EAE development.**

514 (A) PP2A WT and cKO mice were immunized with MOG₍₃₅₋₅₅₎ peptide. PP2A inhibitor Cantharidin
515 (0.6 μg/g) was administered intraperitoneally daily from day 10 to day 12 and then was given once
516 every two days. Mean clinical scores for EAE from each group. (B) Representative histology of the
517 spinal cord (H&E left and LFB right) of mice after EAE induction (day 19). Arrowheads indicate
518 inflammatory infiltration (left) and demyelination (right). Scale bars represent 100 μm. (C)
519 Quantification of total mononuclear cells, CD4⁺ T cells, CD8⁺ T cells and myeloid cells that infiltrated
520 into the CNS of WT mice at peak of the disease. (D) Ratio of CD4⁺ and CD8⁺ T cells (gated at CD45^{hi}
521 CD11b⁻) in the CNS of WT groups. (E, F) Ratio (E) and number (F) of IL17A⁺ or Foxp3⁺ or IFNγ⁺
522 CD4⁺ cells in the CNS of the WT groups at disease peak. (G) Quantification of total mononuclear cells,
523 CD4⁺ T cells, CD8⁺ T cells and myeloid cells that infiltrated into the CNS of the cKO groups. (H) Ratio
524 of CD4⁺ and CD8⁺ T cells (gated at CD45^{hi} CD11b⁻) that infiltrated into the CNS of the cKO groups.
525 Each symbol represents an individual mouse (n=5-7 per genotype); error bars show mean ± SEM. Data
526 are representative of two independent experiments with similar results.











