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ITK signaling regulates a switch between T helper 17 and T regulatory cell lineages via a calcium-mediated pathway

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29 **Abstract:**

30 The balance of pro-inflammatory T helper type 17 (Th17) and anti-inflammatory T
31 regulatory (Treg) cells is crucial in maintaining immune homeostasis in health and disease
32 conditions. Differentiation of naïve CD4⁺ T cells into Th17/Treg cells is dependent upon
33 T cell receptor (TCR) activation and cytokine signaling, which includes the kinase ITK.
34 Signals from ITK can regulate the differentiation of Th17 and Treg cell fate choice,
35 however, the mechanism remains to be fully understood. We report here that in the
36 absence of ITK activity, instead of developing into Th17 cells under Th17 conditions,
37 naïve CD4⁺ T cells switch to cells expressing the Treg marker Foxp3 (Forkhead box P3).
38 These switched Foxp3⁺ Treg like cells retain suppressive function and resemble
39 differentiated induced Tregs in their transcriptomic profile, although their chromatin
40 accessibility profiles are intermediate between Th17 and induced Tregs cells. Generation
41 of the switched Foxp3⁺ Treg like cells was associated with reduced expression of
42 molecules involved in mitochondrial oxidative phosphorylation and glycolysis, with
43 reduced activation of the mTOR signaling pathway, and reduced expression of BATF.
44 This ITK dependent switch between Th17 and Treg cells was reversed by increasing
45 intracellular calcium. These findings suggest potential strategies for fine tune the TCR
46 signal strength via ITK to regulate the balance of Th17/Treg cells.

47

48 INTRODUCTION

49 Immune function mediated by T helper (Th) cells is critical for the development of effective
50 barriers to pathogens and environmental toxins. Distinct functions are mediated by
51 specific lineages of T helper cells that in general have pro-inflammatory function such as
52 that mediated by Th1, Th2, Th9 and Th17 lineages, and anti-inflammatory function such
53 as that mediated by the T regulatory (Treg) and T regulatory type 1 (Tr1) lineages. These
54 distinct T helper cells lineages are characterized by expression of lineage specific
55 transcription factors and the effector cytokines they produce that mediate immune
56 response (1). Th17 cells are characterized by expression of the transcription factor Ror γ
57 (RAR-related orphan receptor gamma), and produce effector cytokines IL17A, IL17F,
58 IL21, IL22 in response to infection with extracellular bacteria, parasites and allergens (1).
59 Tregs are characterized by expression of the transcription factor Foxp3 (Forkhead box
60 P3) and produce effector cytokines such as IL10 and TGF β , among other effectors that
61 can suppress function of Th1, Th2, Th9 and Th17 cells among other immune cells (1).

62 Commitment of naïve CD4⁺ T cells to generally pro-inflammatory Th17 effector
63 fates, or to generally anti-inflammatory Foxp3⁺ Treg fates, have been shown to be
64 dependent on the strength of TCR signaling and the cytokines present in the
65 microenvironment (2-6). Key events include triggering of TCR upon interaction with
66 antigen/MHC complexes and subsequent recruitment of the Src family of kinases
67 Lck/Fyn, and Syk family of kinase ZAP-70 (zeta chain associated kinase), which
68 phosphorylates adaptor proteins LAT (linker for activation of T cell) and SLP76 (SH3
69 containing lymphocyte protein) (7, 8). This is followed by assembly of a proximal signaling
70 complex that includes the recruitment of ITK (IL-2 inducible T cell kinase) and SLP76 (7,

71 8). ITK functions in part by activating PLC γ (phospholipase C γ) to generate effector
72 molecules IP3 (inositol triphosphate) and DAG (diacylglycerol) (7-9). This allows
73 activation of MAPK (mitogen activated protein kinase) cascades, enhanced cytosolic
74 calcium concentrations required to activate NFAT (nuclear factor of activated T cells) and
75 initiation of Akt dependent mTOR (mammalian target of rapamycin) (8). TCR signal
76 strength has been shown to be regulated by ITK (8). While the initial stages of T cell
77 activation is dependent on TCR activation and presence of select cytokines in the
78 microenvironment, once activated the T cells undergo further metabolic changes unique
79 to each T cell subset (10). These metabolic changes allow maintenance and function as
80 select T cell subsets, for example Th17 cells are highly dependent on mitochondrial
81 oxidative phosphorylation and glycolysis (11, 12). These metabolic changes in turn
82 regulate expression of key molecules that allow function of these T cell subsets as pro-
83 inflammatory Th17 or anti-inflammatory Treg cells (10).

84 Given the critical role of ITK in regulating development and function of T cell
85 lineages, several studies have investigated the function of ITK in the development of
86 difference immune responses. We and others have also shown that in naïve CD4⁺ T cells,
87 the absence of ITK, or ITK activity, impairs differentiation into Th2, Th17 and Tr1 lineages
88 while enhancing differentiation into Th1 and Treg lineages (13-18). In particular, TCR
89 signal strength traveling via ITK has been shown to play an important role determining
90 Th17 versus Treg lineage commitment (14, 17, 19). However, whether inhibition of ITK
91 results a similar regulation and the mechanisms by which this dichotomy is controlled is
92 unclear.

93 Here, we investigate this by utilizing ITK inhibitors and allele sensitive ITK (ITKas)
94 expressing mice allowing selective inhibition by ITKas selective inhibitor compounds (16,
95 20-23). We show that under conditions that promote Th17 differentiation, inhibition of ITK
96 suppresses naïve CD4⁺ T cells differentiation to Th17 lineage, and instead leads to the
97 generation of Foxp3⁺ Treg-like cells. The resultant population of Foxp3⁺ Treg-like cells
98 express markers associated with Tregs, effectively retain expression of Treg transcription
99 factor Foxp3, and demonstrate effective suppression of responding T cell proliferation.
100 Furthermore, the switched Foxp3⁺ Treg-like cells have Treg-like transcriptomic and
101 chromatin accessibility profile. The resultant Foxp3⁺ Tregs resemble iTregs by displaying
102 reduced expression of key markers involved in mitochondrial oxidative phosphorylation
103 and the glycolytic pathways. This overall reduction in oxidative phosphorylation was
104 associated with reduced phosphorylation of mTOR and downstream substrate S6K
105 (ribosomal S6 kinase), which may act to prevent expression of the Th17 pioneer
106 transcription factor BATF (basic leucine zipper ATF-like transcription factor). Finally, we
107 show that bypassing the TCR signal to increase calcium signaling prevents this ITK
108 dependent switch response. Our results indicate that ITK signals can tune the balance
109 between Th17 cells and Tregs.

110

111 **RESULTS**

112 **ITK controls a switch between Th17 differentiation and Foxp3⁺ Treg-like cells when**
113 **naïve CD4⁺ T cells are activated under Th17 differentiation conditions.**

114 In the absence of ITK activity, Th17 differentiation is inhibited, and in *Itk*^{-/-} T cells, the
115 absence of ITK results in reduced Th17 differentiation, and the appearance of Foxp3⁺
116 Treg cells (14-17). However, whether this switch from Th17 to Treg differentiation occurs
117 when ITK kinase activity is inhibited is unclear. To determine the role of ITK activity in the
118 development of Foxp3⁺ T regulatory cells under Th17 conditions, we stimulated naïve
119 WT, *Itk*^{-/-} or ITKas T cells isolated from IL17A-GFP/Foxp3-RFP reporter mice, under Th17
120 culture conditions. We found that the absence of ITK, or inhibition of ITKas results in the
121 inhibition of Th17 cell differentiation, along with the appearance of Foxp3⁺ Treg-like cells
122 (**Fig. 1a**). Similarly, stimulation of naïve WT IL17A-GFP/Foxp3-RFP reporter cells under
123 Th17 culture conditions in the presence of the covalent small molecule ITK inhibitor (23)
124 (CPI-818) resulted in dose dependent inhibition of Th17 cell differentiation, along with the
125 appearance of Foxp3⁺ Treg-like cells, with similar results observed when naïve ITKas T
126 IL17A-GFP/Foxp3-RFP reporter cells were used along with MBPP1 which we have
127 previously shown selectively inhibits the ITKas isoform (16) (**Fig. 1b,c**). This effect was
128 due to specific inhibition of ITK and not potential off-target effects since the covalent small
129 molecule ITK inhibitor (CPI-818) does not affect Th17 differentiation of naïve ITKas T
130 cells, nor does MBPP1 affect Th17 differentiation of naïve WT T cells (**Supplemental Fig.**
131 **1**). We refer to these Foxp3⁺ Treg-like cells generated under conditions of ITK inhibition
132 as *Switched Foxp3⁺ Treg-like cells*.

133

134 **ITK inhibition does not switch already differentiated Th17 cells to Foxp3⁺ Treg-like**
135 **cells.**

136 To determine whether inhibition of ITK can induce already differentiated Th17 cells to
137 become switched Foxp3⁺ Treg-like cells, we generated *in vitro* both the switched Foxp3⁺
138 Treg-like cells and Th17 cells, sorted them (by GFP⁺/RFP⁻ expression), and further
139 cultured the sorted cells under Th17 differentiation conditions in presence of the covalent
140 ITK inhibitor. While we there was no change in the switched Foxp3⁺ Treg-like cells,
141 inhibiting ITK led to a reduction in differentiated Th17 cells suggesting that ITK activity is
142 required for the maintenance of these cells. However, inhibiting ITK in these Th17 cells
143 did not lead to the development of Foxp3⁺ Treg-like cells under these conditions (**Fig. 1c**).
144 This data suggests that under Th17 differentiation conditions, inhibition of ITK switches
145 Th17 differentiation to Foxp3⁺ Treg-like cells, but is not able to do so once the cells have
146 already differentiated to the Th17 fate.

147

148 **Early events following TCR activation determines the ability of ITK to tune a switch**
149 **from Th17 to Foxp3⁺ Treg-like cells.**

150 To determine if early or late molecular events influence this switch to Foxp3⁺ Treg-like
151 cells upon ITK inhibition, naïve CD4⁺ T cells were activated under Th17 differentiation
152 conditions, followed by addition of the ITK inhibitor after 1, 2, 3, or 4 days post initiation
153 of the culture. We find that inhibition of ITK time dependently (after up to 2 days) blocks
154 Th17 differentiation (**Fig. 2a**). Analysis of the generation of switched Foxp3⁺ Treg-like
155 cells also showed a time dependent relationship between ITK inhibition, and the
156 generation of these cells, although optimal generation required early ITK inhibition, as ITK

157 inhibition at later time points beyond day 1 is less effective in inducing the switch, with
158 some Foxp3⁺ Treg-like cell generation upon ITK inhibition on day 2, and no significant
159 effect upon ITK inhibition between days 3 to 5 (**Fig. 2a**).

160 Next, we wanted to determine the time frame of ITK inhibition that results in this
161 switch response. Using a similar approach, naïve CD4⁺ T cells were activated under Th17
162 differentiation conditions, followed by removal of the ITK inhibitor after 1, 2, 3, or 4 days.
163 Removal of ITK inhibitor after 1 day led to an ~50% inhibition of Th17 cell differentiation,
164 although, with the greatest effect observed with 5 days of ITK inhibition (**Fig. 2b**).
165 Analogously, the generation of switched Foxp3⁺ Treg-like cells was observed when ITK
166 was inhibited for as little as 1 day (~40% of maximal generation), and close to maximum
167 after 3 days of ITK inhibition (**Fig. 2b**). Taken together this suggests that early events
168 following TCR activation determines the switch from Th17 to switched Foxp3⁺ Treg-like
169 cells upon ITK inhibition.

170

171 **Switched Foxp3⁺ Treg-like cells resemble true Foxp3⁺ Tregs and can suppress**
172 **naïve T cell proliferation *in vitro*.**

173 In addition to expression of Foxp3, Treg cells express a number of cell surface molecules
174 including CD25, CTLA4 and PD1 (1). To determine if the switched Foxp3⁺ Treg-like cells
175 generated upon ITK inhibition of naïve CD4⁺ T cells cultured under Th17 conditions,
176 resemble true Tregs, we compared the expression of these cell surface markers by flow
177 cytometry. We find that all Treg subsets (*in vitro* generated induced or iTregs, thymic
178 derived natural or nTregs and extra-thymic peripheral or pTregs) as well as the switched
179 Foxp3⁺ Treg-like cells express CD25, CTLA4 and PD1, with switched Foxp3⁺ Treg-like

180 cells closely resembling the iTregs in their level of expression of these markers (**Fig. 3a**).
181 Subsequently we investigated if the switched Foxp3⁺ Treg-like cells are able to suppress
182 proliferation of CFSE-labeled naïve CD4⁺ T cells when cocultured *in vitro*. In the absence
183 of coculture with Tregs, responding naïve T cells are able to undergo multiple rounds of
184 cell division when activated by anti-TCR antibodies (as determined by CFSE dye dilution).
185 However, when cocultured in presence of iTregs, this proliferation is suppressed (**Fig.**
186 **3b**). In presence of the switched Foxp3⁺ Treg-like cells, similar to the iTregs, this
187 proliferation of responding T cells is also suppressed (**Fig. 3b**). Therefore, the switched
188 Foxp3⁺ Treg-like cells, express surface markers and display suppressive function similar
189 to the iTregs.

190

191 **Switched Foxp3⁺ Treg-like cells have a transcriptome that resemble iTregs.**

192 We next investigated the transcriptomic profile of the switched Foxp3⁺ Treg-like cells,
193 comparing then to *in vitro* derived Th17 cells and iTregs, using RNA-Seq analysis.
194 Principal component analysis and heatmap analysis displaying expression of all
195 transcripts, indicated that the switched Foxp3⁺ Treg-like cells closely resemble iTregs
196 compared to Th17 cells (**Fig. 4a, b**). Using volcano plots to identify genes differentially
197 expressed, we find that Th17 cytokine Il17 and the critical Th17 transcription factor Rorc
198 were downregulated in switched Foxp3⁺ Treg-like cells compared to Th17 cells (**Fig. 4c**).
199 Furthermore, Foxp3, and Treg-related genes such as tgfr1 and tgfr2, as well as other
200 transcription factors smad3 and Ikzf2 were found to be upregulated in switched Foxp3⁺
201 Treg-like cells compared to Th17 cells (**Fig. 4c**). Notably, the switched Foxp3⁺ Treg-like

202 cells expressed lower levels of Treg related genes *foxp3*, *nrp1*, *tgfb1* and *tgfbr1*, although
203 higher levels of *tgfb3* (**Fig. 4c**).

204 To compare the transcriptomes of switched *Foxp3*⁺ Treg-like cells with other T cell
205 subsets, we compared the transcriptomes to transcriptomic data of Th1, Th2, pathogenic
206 pTh17 cells generated in presence of IL1, IL6 and IL23, non-pathogenic npTh17 cells
207 generated in presence of IL6 and TGF β and Tr1 cells (GSE158703)(24). We find that the
208 transcriptome of switched *Foxp3*⁺ Treg-like cells, Th17 and iTreg subsets we generated
209 cluster closer to the npTh17 and pTh17 subsets, and further from Th1 and Th2 subsets,
210 with Tr1 cells being furthest in the cluster comparison (**Fig. 4d**).

211

212 **Switched *Foxp3*⁺ Treg-like cells generated under ITK inhibition have a chromatin**
213 **profile distinct from both Th17 and iTreg cell subsets.**

214 We also analyzed the accessibility of the chromatin in switched *Foxp3*⁺ Tregs by ATAC-
215 Seq, comparing them to Th17 and iTreg cell subsets. Analysis by PCA and heatmaps
216 revealed that similar to the transcriptome, switched *Foxp3*⁺ Treg-like cells have chromatin
217 profiles that resemble iTreg compared to Th17 cells (**Fig. 5a,b**). Using volcano plots to
218 compare differentially open and closed chromatin regions, we find that the chromatin
219 region of genes associated with Th17 cells (e.g., *Rora*, *Stat3*, *Il17*, *Foxp1*) displayed
220 reduced accessibility in switched *Foxp3*⁺ Treg-like cells compared to Th17 cells (**Fig. 5c**).
221 By contrast, the chromatin regions of genes associated with iTreg cells (e.g., *Il10rb*,
222 *Tgfbr3*) displayed reduced accessibility in switched *Foxp3*⁺ Treg-like cells compared to
223 iTreg cells (**Fig. 5c**), while the Th17-related gene (e.g., *Rora*) was more open, perhaps
224 reflecting relatedness to Th17 cells (**Fig. 5c**). Individual chromatin region traces depict

225 these differences, where switched Foxp3⁺ Treg-like display reduced accessibility for Th17
226 genes (e.g., *Rorc*, *Il17*) but enhanced accessibility for genes associated with iTregs (e.g.,
227 Foxp3) (**Fig. 5d**).

228

229 **Enhanced calcium signaling prevents the ITK dependent switch response to**
230 **Foxp3⁺ Treg-like cells generated.**

231 Differentiation of naïve CD4⁺ T cells into Th17 cells requires both presence of cytokines
232 and TCR activation. An important component downstream of TCR activation is calcium
233 signaling, and we have previously shown that increased intracellular calcium is able to
234 rescue the development of Th17 cells in the absence of ITK (14). To determine whether
235 this pathway downstream of ITK is important for the ITK activity dependent switch
236 response, we used ionomycin to enhance cytosolic calcium levels during Th17
237 differentiation conditions in the presence of ITK inhibition. In the absence of ITK
238 expression (using naïve *Itk*^{-/-} CD4⁺ T cells), Th17 differentiation is prevented, and this is
239 rescued by ionomycin treatment, confirming the role of the calcium pathway in Th17
240 differentiation downstream of ITK (**Fig. 6a**). In addition, there is a switch to Foxp3⁺ Treg-
241 like cell generation, and notably, this switch is prevented by increasing intracellular
242 calcium, indicating a critical role for the calcium pathway in tuning this switch (**Fig. 6a**).
243 To examine if this is also observed with ITK inhibition, we enhanced cytosolic calcium
244 levels with ionomycin during Th17 differentiation conditions in the presence of ITK
245 inhibitor (CPI-818). Increasing intracellular calcium enhanced Th17 differentiation in the
246 absence of ITK inhibition, and importantly, rescued Th17 differentiation in the presence
247 of ITK inhibition (**Fig. 6b**). Together these results indicate that increases in intracellular

248 calcium signaling promotes generation of Th17 cells under the Th17 differentiation
249 condition, and suppresses the generation of switched Foxp3⁺ Treg-like cells in the
250 absence of ITK. Furthermore, while inhibiting ITK's activity led to increased differentiation
251 of iTregs as we have previously reported (17), when ITK is absent, or its ITK's activity is
252 inhibited (**Fig. 6c,d**). We also noted that inducing increases in intracellular calcium with
253 ionomycin led to suppression of induced Foxp3⁺ Treg cells differentiation, with some
254 observed differentiation of Th17 cells, when naïve CD4⁺ T cells were cultured under iTreg
255 conditions, suggesting that the negative regulation of Treg differentiation, and more
256 broadly, tuning of Th17/Treg differentiation by ITK also travels partly via calcium signaling.

257

258 **ITK dependent switch to Foxp3⁺ Treg-like cells involve deficits in molecules**
259 **involved in mitochondrial oxidative phosphorylation, and reduced expression of**
260 **BATF.**

261 Previous studies have shown that a select set of genes are associated with pathogenic
262 Th17 cells and non-pathogenic Th17 cells (25, 26). By comparing the expression levels
263 of these select genes, we find that the level of expression by switched Foxp3⁺ Treg-like
264 cells are distinct from Th17 cells and instead resemble iTregs (**Fig. 7a**). Several metabolic
265 changes are also known to be associated with Th17 cell development, and thus we
266 compared the expression level of select genes involved in these pathways during Th17
267 cell development (25, 26). For genes that are associated with tricarboxylic acid (TCA)
268 cycle activity and mitochondrial function, switched Foxp3⁺ Treg-like cells display
269 expression levels distinct from Th17 cells and instead resemble iTregs (**Fig. 7a**). In
270 contrast for genes that are associated with the hypoxia-induced factor (HIF) pathway and

271 glycolysis, switched Foxp3⁺ Treg-like cells resemble Th17 cells but not iTregs in the level
272 of expression of these markers (**Fig. 7a**). Therefore the switched Foxp3⁺ Treg-like cells
273 seem to have an intermediate metabolic phenotype, resembling in part both the Th17 and
274 iTreg metabolic phenotypes. Next we examined the cells for evidence of activation of
275 select molecules involved in the pathways of mitochondrial oxidative phosphorylation
276 (25), by estimating the phosphorylation status of S6K and mTOR, shown to be involved
277 in Th17 states. Using flow cytometric analysis of naïve CD4⁺ T cells cultured under Th17
278 differentiation condition either with or without ITK inhibitor (CPI-818), we find reduced
279 phosphorylated S6K and mTOR expressing CD4⁺ T cells when ITK's activity was inhibited
280 compared to control conditions, suggesting reduced mitochondrial oxidative
281 phosphorylation upon ITK inhibition (**Fig. 7b**).

282 The transcription factor BATF has been identified as a pioneer transcription factor
283 for Th17 differentiation involved in early events during Th17 differentiation (25). Our
284 results further indicated reduced overall BATF-expressing CD4⁺ T cells with ITK inhibition,
285 and that RORC expressing cells display reduced BATF expression under Th17 conditions
286 when ITK's activity was inhibited compared to control (**Fig. 7c**). Similarly we observed
287 reduced mRNA expression and reduced chromatin accessibility for BATF in the switched
288 Foxp3⁺ Treg-like cells compared to the Th17 and iTreg cells (**Fig. 7d,e**). Taken together
289 we demonstrate that ITK inhibition under Th17 differentiation conditions leads to deficits
290 in molecules which mediate mitochondrial oxidative phosphorylation and failure in
291 expression of BATF, which may be involved in the ITK dependent switch to Foxp3⁺ Treg-
292 like cells under Th17 differentiation conditions.

293

294 **DISCUSSION**

295 The ability of TCR signals to regulate the differentiation of Th17 and Treg cells has been
296 a focus of intense study, as a better understanding of this process may provide
297 approaches for differential regulation of these T effector subsets in specific immune
298 responses and autoimmune disease. We demonstrate here that the tyrosine kinase ITK
299 tunes a switch between Th17 cells and Foxp3⁺ Treg cells, and that is mediated by calcium
300 signaling, altered cellular metabolism, involving at least in part reduced mTORC signaling
301 with BATF functioning as one of the downstream components. Our findings provide
302 significant mechanistic understanding of how ITK regulates Th17 cells and Treg cells (14,
303 19, 27), and suggest that ITK signaling may control inflammation and anti-inflammation
304 during activation of naïve CD4⁺ T cells. Our work further shows that this ITK mediate
305 tuning of Th17 and Treg cells requires specific signals from ITK during the initial 24-48
306 hr. period of activation under Th17 conditions, indicating early molecular events regulate
307 the switch response.

308 In this study, along with expression of Foxp3, we found that switched Foxp3⁺ Treg
309 like cells, resemble iTregs both in terms of phenotypic profile based on the expression of
310 phenotypic Treg markers CD25, CTLA4 and PD1, as well as functionally suppressive
311 function *in vitro*. Foxp3 expression is critical for the development and function of Treg
312 cells, however more recently studies have shown expression of Foxp3 alone is not
313 sufficient for Treg mediated suppressive function (28, 29). Recent studies have reported
314 that based on the level of Foxp3 and CD45R expression within human lymphocytes, a
315 heterogenous Treg population could be established where each Treg subset varied in
316 both phenotype and function (28, 29). In addition to select phenotypic markers, several

317 studies have utilized comparison of the whole transcriptome and epigenetic landscape to
318 compare T cell subsets. Whereas the transcriptome allowed clear demarcations of
319 phenotypic profile of genes expressed in each T cell subset, investigation into chromatin
320 accessibility status provided a more nuanced view of these cellular states between the
321 different T cell subsets. Comparing the chromatin profile of Foxp3 loci by ChIP Seq for
322 example has revealed that selective activating histone modifications exist in iTregs and
323 not Th1 or Th2 cells, but surprisingly these activating marks were also present in Th17
324 cells, indicating some similarities in overall chromatin structure of iTregs and Th17 cells
325 (30). This previous study also suggested that the chromatin landscape of *in vitro* derived
326 T cells display considerable plasticity, because when studying the loci of the Th2
327 transcription factor Gata3, it was reported that in order to achieve full activation status
328 and associated histone modifications, two rounds of Th2 polarization were required
329 instead of a single Th2 polarization period (30). These observations may explain the
330 discrepancy we observed between transcriptomes and the chromatin accessibility profile.
331 Thus while the switched Foxp3⁺ Treg like cells appear to have attained the transcriptomic
332 profile of iTregs, but further rounds of polarization may be required to induce full transition
333 to iTreg chromatin accessibility profile.

334 We and others have reported that ITK functions to fine tune the TCR signal
335 strength, being a positive regulator for Th1, Th17 and Tr1 cells but a negative regulator
336 for Th2 and iTregs. Several studies reported that increasing the TCR signal strength
337 enhances differentiation of naïve CD4⁺ T cells into Th17 cells under Th17 polarization
338 conditions but inhibit differentiation of naïve CD4⁺ T cells into iTreg cells under iTreg
339 polarization conditions (14, 15, 31-34). Increased TCR signaling is associated with

340 enhanced calcium signaling (35, 36) and previously we and others have demonstrated
341 that increasing calcium dependent calmodulin and NFATc1 signaling restores the Th17
342 differentiation of naïve *Itk*^{-/-} CD4⁺ T cells polarized under Th17 conditions (14, 15, 34).
343 The findings of the present study showed that enhancing calcium increases using
344 ionomycin enhanced Th17 differentiation in naïve CD4⁺ T cells, but also rescued Th17
345 differentiation when ITK's activity was inhibited. Remarkably, this prevented the switch to
346 Foxp3⁺ Treg like cells when ITK's activity was inhibited under Th17 polarization condition.
347 Furthermore increased calcium not only prevented the switch to Foxp3⁺ Treg like cells
348 with ITK inhibition under Th17 polarization condition (IL6 and TGFβ), but also prevented
349 the increased iTreg differentiation with ITK inhibition under iTreg polarization condition
350 (IL2 and TGFβ), suggesting that the calcium regulated pathway is a key regulator of these
351 two fates.

352 Calcium signaling was previously reported to control mitochondrial metabolism,
353 such that deletion of stromal interaction molecule 1 (STIM1) in Th17 cells lead to reduced
354 expression of genes for molecules involved in mitochondrial oxidative phosphorylation
355 (26). Others have reported the role of enhanced mitochondrial oxidative phosphorylation
356 and glycolysis in Th17 cell differentiation (11, 12), which is consistent with our results of
357 increased expression of markers involved in mitochondrial function, oxidative
358 phosphorylation and glycolysis in the Th17 cells. Interestingly we find that the switched
359 Foxp3⁺ Treg like cells generated under ITK inhibition display reduced expression of these
360 markers for mitochondrial function, further emphasizing their altered fate from Th17 cells.

361 Altered cellular metabolism has been shown to alter T cell differentiation fate via
362 the regulation of the mTOR pathway (37, 38), where the inhibition of mTOR in naïve CD4⁺

363 T cells prevented the generation of IL17A producing Th17 cells and instead lead to
364 generation of Foxp3⁺ iTreg cells, under Th17 polarizing conditions (37). In addition
365 several studies have reported reduced mTOR signaling induces iTreg differentiation (14,
366 32, 33), whereas increased mTOR signaling induce Th17 differentiation (39, 40). Within
367 Th17 cells, the observed increase in mitochondrial function and oxidative phosphorylation
368 via enhanced mTOR signaling, was further shown to induce expression of the Th17
369 pioneer transcription factor BATF, allowing maintenance of differentiated Th17 cells (25).
370 In contrast the deletion of BATF in naïve CD4⁺ T cells, was reported to increase
371 expression of Foxp3 even under Th17 polarizing conditions (25, 41). Our results are
372 therefore consistent with these reports, and further demonstrate that with inhibition of ITK,
373 the switch in T cell fate from Th17 to Foxp3⁺ Treg like cells, under Th17 polarizing
374 conditions, similarly involve the mTOR pathway. These results suggest that with ITK
375 inhibition the reduced mitochondrial function, oxidative phosphorylation and glycolysis,
376 via reduced phosphorylation of mTOR and the mTOR substrate S6K, regulates the switch
377 to Foxp3⁺ Treg like cells. Reduced mTOR signaling via the reduced expression of Th17
378 transcription factor BATF, serves as a potential mechanism for the ITK dependent switch
379 from Th17 to Foxp3⁺ Treg like cells, under Th17 polarizing conditions.

380 In conclusion, our results suggest that under Th17 conditions, strong TCR
381 signaling in the presence of ITK activity that leads to increased intracellular calcium, naïve
382 CD4⁺ T cells, results in enhanced mitochondrial function and oxidative phosphorylation,
383 which can activate the mTOR pathway to induce Th17 transcription factor BATF. In the
384 absence of ITK activity, the reduction in expression of molecules involved in mitochondrial
385 function and oxidative phosphorylation, subsequent reduced activity of the mTOR

386 pathways and BATF expression could act as a potential mechanism for the switch in
387 naïve CD4⁺ T cell fate into generating Foxp3⁺ Treg like cells instead. The findings of this
388 study provide greater insight into how ITK controls the Th17/Treg dichotomy, and these
389 findings could have broader implications for immune disorders with an imbalance of
390 Th17/Treg.

391

392

393 **MATERIALS AND METHODS**

394 **Mice.** Male and female mice were on the C57BL/6 background aged between 6 to 8
395 weeks. Mice were housed in the specific pathogen free facilities and all experiments were
396 performed in accordance with the guidelines established by the Office of Research
397 Protection's Institutional Animal Care and Use Committee at Cornell University. WT, *Itk*^{-/-}
398 and ITKas IL17-GFP/Foxp3-RFP dual reporter strains were generated by crossing IL17-
399 GFP (B-IL17A-EGFP^{tm1}, Biocytogen, Worcester, MA) with Foxp3-RFP (C57BL/6-
400 *Foxp3*^{tm1Fiv/J}, Jackson Laboratory, Bar Harbor, ME) strain (24) as previously reported
401 (16). CD45.1 congenic (B6.SJL-*Ptprc*^a *Pepec*^b) and Rag1^{-/-} (B6.129S7-*Rag1*^{tm1Mom}) strains
402 were purchased from Jackson Laboratory.

403
404 **Flow Cytometry and antibodies.** The following antibodies for murine antigens were
405 used labelled as antigen (clone name; catalog number) at a dilution of 1:200 unless
406 indicated otherwise. Viability dye eF506 (65-0866-18) and antibody against CD16/32
407 (FcBlock) (93; 14016185) were purchased from Thermo Fisher Scientific (Waltham, MA).
408 Antibodies against PD1 (29F.1A12; 135224), mTOR (pSer2448) (MRRBY, 46971842),
409 CD25 (29F.1A12; 135224), CD4 (GK1.5; 100427), CD45.1 (A20; 110716), CD45.2 (104;
410 109819), CD3 ϵ (145-2C11; 100340) and CD28 (35.51; 102112) were purchased from
411 Biologend (San Diego, CA). Antibody for S6 (pS235/pS236) (N7-548; 561457) was from
412 BD (San Jose, CA). Flow cytometry data was generated using the Attune Nxt Flow
413 Cytometer (Thermo Fisher Scientific Waltham, MA) and FACS Aria II (BD, San Jose, CA),
414 which was analyzed using FlowJo (Tree Star, Ashland, OR).

415

416 **Th17 and iTreg differentiation *in vitro*.** Purified naïve CD4⁺ T cells were cocultured with
417 APCs for indicated period of days in RPMI media (10% FBS, 0.5% HEPES, 1 mM
418 Glutamine, 1 mM Sodium Pyruvate, 1 mM non-essential amino acids and 100 U/ml
419 Pen/Strep). Cells were treated with WT ITK inhibitor – CPI-818 (23) (Corvus
420 Pharmaceuticals, Burlingame CA), ITKas inhibitor – 3MB-PP1 (Cayman Chemicals, Ann
421 Arbor, MI) or DMSO (Sigma) control as indicated. For Th17 differentiation, naïve CD4⁺ T
422 cells were activated by anti-CD3 (2 µg/ml), anti-CD28 (1 µg/ml), in the presence of APCs
423 along with recombinant IL6 (406-ML-025, 10 ng/ml), recombinant TGFβ (240-B-002, 10
424 ng/ml), and recombinant human IL-2 protein (202-IL-010), all from R&D Systems
425 (Minneapolis, MN) as described (15). The iTregs were generated *in vitro* by activating
426 coculture of naïve CD4⁺ T cells and APCs with anti-CD3 (1 µg/ml), anti-CD28 (1 µg/ml),
427 recombinant IL2 (10 µg/ml) and recombinant TGFβ (10 µg/ml). Cells were stained with
428 cell surface markers and/or fixed and permeabilized with the Foxp3/Transcription Factor
429 Staining Kit (eBioscience) with staining for intracellular makers and fixable viability dye
430 eF506 for live/dead cell exclusion to analyze by flow cytometry. Where indicated, cells
431 were also stained with surface markers to sort purify (>95% purity) population of CD4⁺
432 IL17-GFP⁺ Th17 cells and CD4⁺ Foxp3-RFP⁺ Treg-like cells by BD FACS Aria II.

433
434 ***In vitro* suppression assay.** Foxp3⁺ Treg-like cells generated during WT ITK inhibition
435 (CPI-818) under Th17 conditions, and iTregs generated during activation under iTreg
436 conditions, were stained with cell surface markers and sort purified (>95% purity) to obtain
437 CD4⁺ Foxp3-RFP⁺ Treg-like cells and CD4⁺ Foxp3-RFP⁺ iTregs. Sort purified naïve
438 responding T cells were labelled with CFSE proliferation dye (Invitrogen) and cocultured

439 with Foxp3⁺ Treg-like cells or iTregs in presence of anti-CD3 (1 µg/ml), followed by
440 analysis using flow cytometry.

441

442 **RNA- and ATAC-Sequencing.** Total RNA was extracted from sort purified *in vitro*
443 generated Th17, Foxp3⁺ Treg-like cells and iTregs were by TRIzol reagent (Invitrogen).
444 The RNA library was generated using the NEB Ultra II Directional RNA Library Prep Kit
445 and quantified using Qubit Bioanalyzer. RNA sequencing was performed on an Illumina
446 NextSeq500 at 75bp reads and 20 million reads per sample (Transcriptional Regulation
447 and Expression Facility, Cornell University) as previously described (25). RNA
448 sequencing data was demultiplexed by BCL2FASTQ2 and FASTQC performed. RNA-
449 Seq data was mapped to the mm10 genome using STAR aligner and raw counts obtained
450 using HTSeq. Counts were normalized and differentially expressed genes identified using
451 DESeq2 (FDR<0.05). Gene set enrichment analysis (GSEA) was performed using
452 software developed by Broad Institute (26, 27) and heat map generated using R Studio
453 (Boston, MA) and Heatmapper (28). Data was compared to published RNA-Seq data
454 from GEO dataset GSE158703 with the indicated T helper cell subsets.

455 For ATAC-Seq, Foxp3⁺ Treg-like cells and iTregs generated *in vitro* were sort
456 purified and frozen in 10% DMSO in cell culture media. Nuclei was permeabilized (10 mM
457 Tris-HCl, pH 8.0, 10 mM NaCl, 2 mM Mg Acetate) and lysed (10 mM Tris-HCl, pH 8.0, 10
458 mM NaCl, 2 mM Mg Acetate, 6 mM CaCl₂, 0.2% Ipegal, 0.016% Tween20, 600 mM
459 Sucrose) as per instructions from Transcriptional Regulation and Expression Facility,
460 Cornell University. The lysed nuclei were provided to the Transcriptional Regulation and
461 Expression Facility, Cornell University for transposition reaction (25) by the

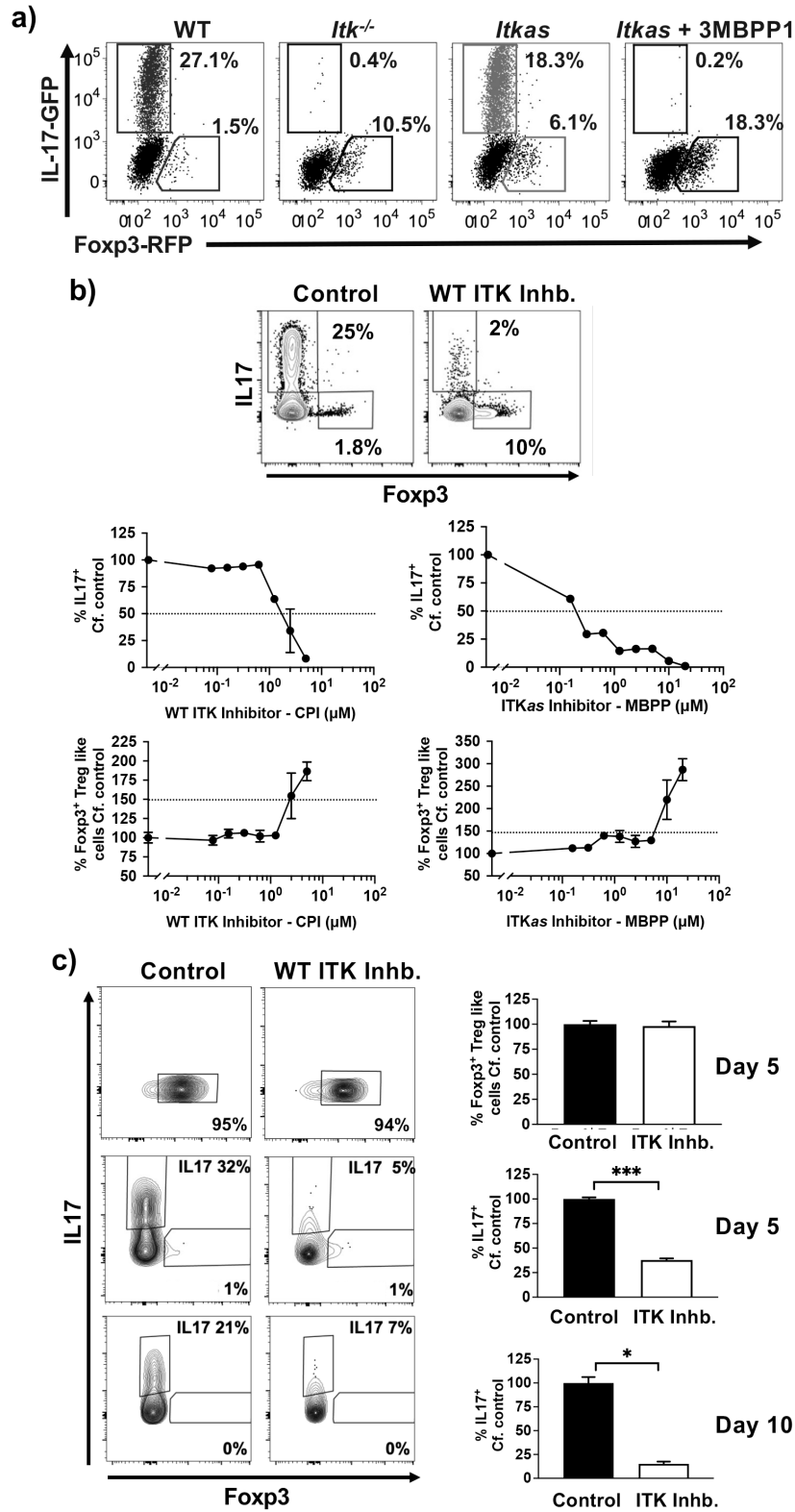
462 Transcriptional Regulation and Expression Facility, Cornell University. DNA library
463 generated was quantified using Qubit BioAnalyzer and sequencing performed on Illumina
464 NextSeq500 at 75bp reads and 20 million reads per sample followed by FASTQC analysis
465 for quality control. ATAC-Seq data was aligned to mm10 genome using Bowtie, ATAC-
466 Seq peaks were identified by MACS2 and promoter region associated peaks were
467 identified by bedtools as described in (29), by the Transcriptional Regulation and
468 Expression Facility, Cornell University. Analysis was performed in R Studio (Boston, MA)
469 and using the UCSC genome browser (University of California Santa Cruz).

470

471 **Statistical analysis.** Students T test and One way ANOVA was performed for
472 comparison between samples with $p < 0.05$ considered as statistically significant using
473 GraphPad Prism (San Diego, CA).

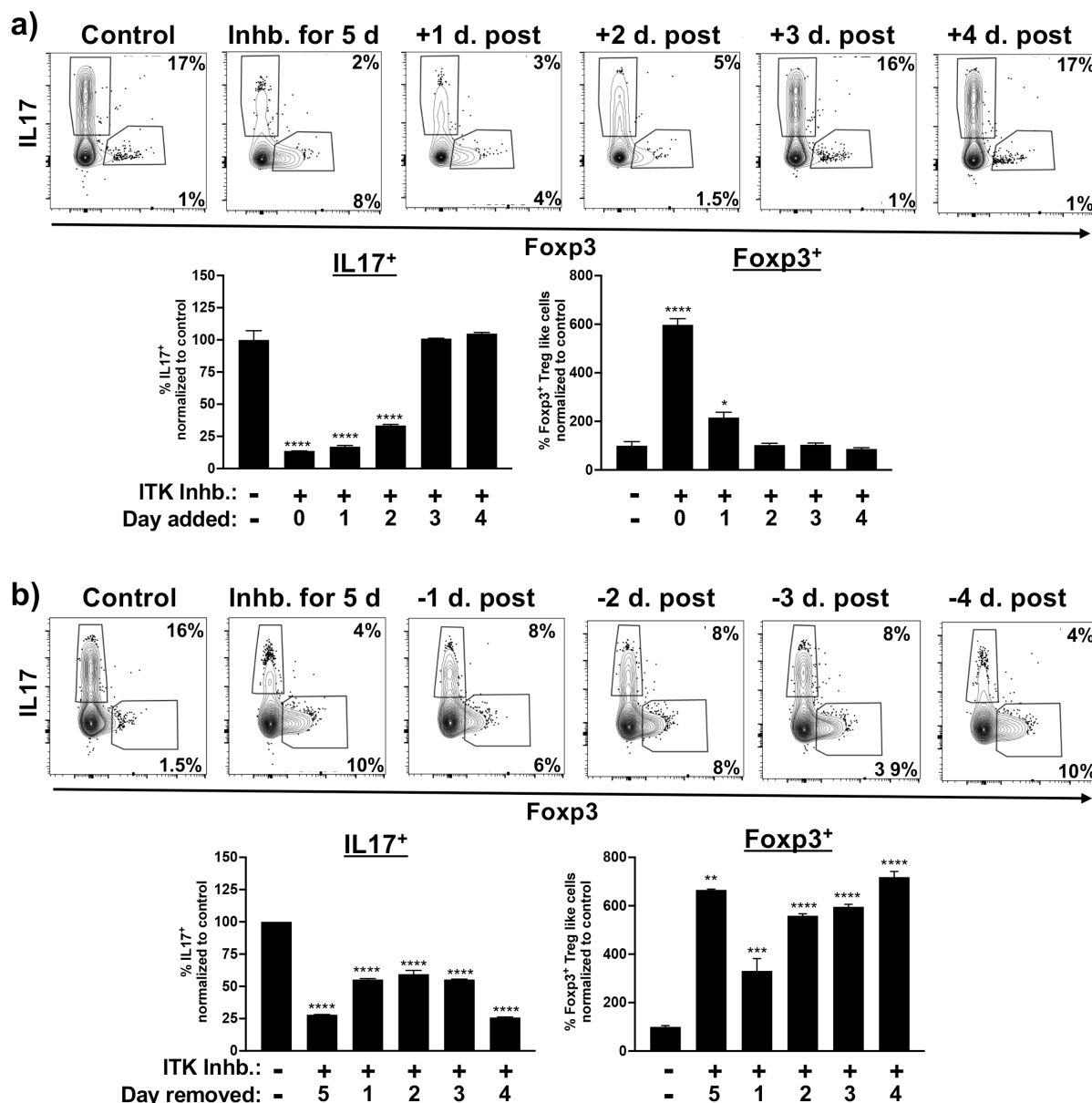
474

475 **Figure Legends:**



476

477 **Fig. 1. ITK controls a switch between Th17 differentiation and Foxp3⁺ Treg-like cells**
478 **when naïve CD4⁺ T cells are activated under Th17 differentiation conditions.**
479 (a) Naïve WT, *Itk*^{-/-} or ITKas IL17A-GFP/Foxp3-RFP CD4⁺ T cells were activated
480 under Th17 differentiation conditions (anti-CD3/28, IL6 and TGFβ) in presence of
481 ITKas inhibitor 2MBPP1 or DMSO control as indicated, followed by flow cytometric
482 analysis for percentage of GFP⁺/IL17⁺ cells and RFP⁺/Foxp3⁺ Treg-like cells. (b)
483 Top: Naïve WT IL17A-GFP/Foxp3-RFP CD4⁺ T cells were activated under Th17
484 differentiation conditions in presence of ITK inhibitor CPI-818 or DMSO control,
485 followed by quantification of percentage of GFP⁺/IL17⁺ cells and RFP⁺/Foxp3⁺
486 Treg-like cells. Bottom: Naïve WT (left) or ITKas (right) IL17A-GFP/Foxp3-RFP
487 CD4⁺ T cells were activated under Th17 differentiation conditions in presence of
488 ITK inhibitor CPI-818 (left) ITKas inhibitor 3MBPP1 (right) or DMSO control,
489 followed by quantification of percentage of GFP⁺/IL17⁺ cells and RFP⁺/Foxp3⁺
490 Treg-like cell. Mean ± SEM, one-way ANOVA was performed for statistical
491 significance where * p ≤ 0.05, ** p ≤ 0.005, *** p ≤ 0.001 and **** p ≤ 0.0001, 3
492 independent experiments. (c) Switched Foxp3⁺ Treg-like cells generated under
493 Th17 differentiation conditions in presence of ITK inhibitor, or *in vitro* generated
494 Th17 cells, were then further reactivated under Th17 differentiation conditions in
495 presence of ITK inhibitor CPI-818 or DMSO control for a duration of 5 days
496 (Switched Foxp3⁺ Treg-like cells and Th17 cells) or 10 days (Th17 cells). Mean ±
497 SEM, Student's T test was performed for statistical significance where * p ≤ 0.05,
498 ** p ≤ 0.005, 3 independent experiments.
499



500

501 **Fig. 2. Early ITK signals are required for ITK mediated switch between Th17 and**

502 **switched Fxp3⁺ Treg-like cells under Th17 differentiation conditions. (a)**

503 Naïve WT IL17A-GFP/Fxp3-RFP CD4⁺ T cells were activated under Th17

504 differentiation conditions followed by addition of ITK inhibitor CPI-818 after 1, 2, 3

505 or 4 days of culture, followed by analysis on day 5. (b) Naïve WT IL17A-

506 GFP/Fxp3-RFP CD4⁺ T cells were activated under Th17 differentiation

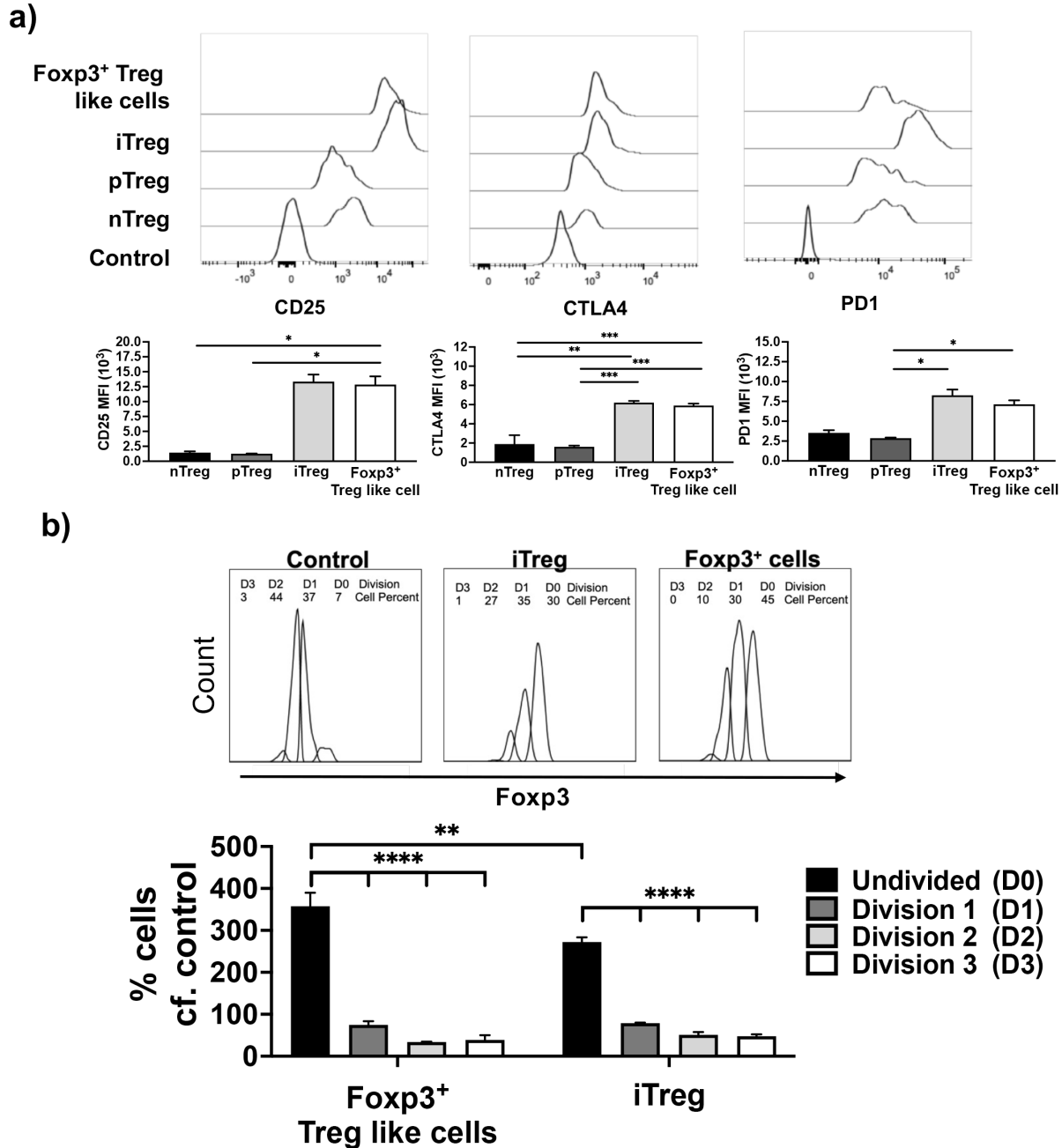
507 conditions in the presence of the ITK inhibitor CPI-818, followed by removal of inhibitor after

508 1, 2, 3 or 4 days of culture, with analysis on day 5. Mean ± SEM, one-way ANOVA

509 was performed for statistical significance where * p ≤ 0.05, ** p ≤ 0.005, *** p ≤

510 0.001 and **** p ≤ 0.0001, 2 independent experiments.

511



512

513 **Fig. 3. Switched Foxp3⁺ Treg-like cells generated under conditions of ITK inhibition**

514 **have a cell surface phenotype and suppressive function similar to iTregs. (a)**

515 Foxp3⁺ Treg-like cells that are generated from WT IL17A-GFP/Foxp3-RFP CD4⁺

516 T cells activated under Th17 differentiation conditions in presence of ITK inhibitor

517 CPI-818, and compared to iTregs, pTregs and nTregs for expression of select Treg

518 markers. Expression represented as mean fluorescence intensity. (b) CD45.2

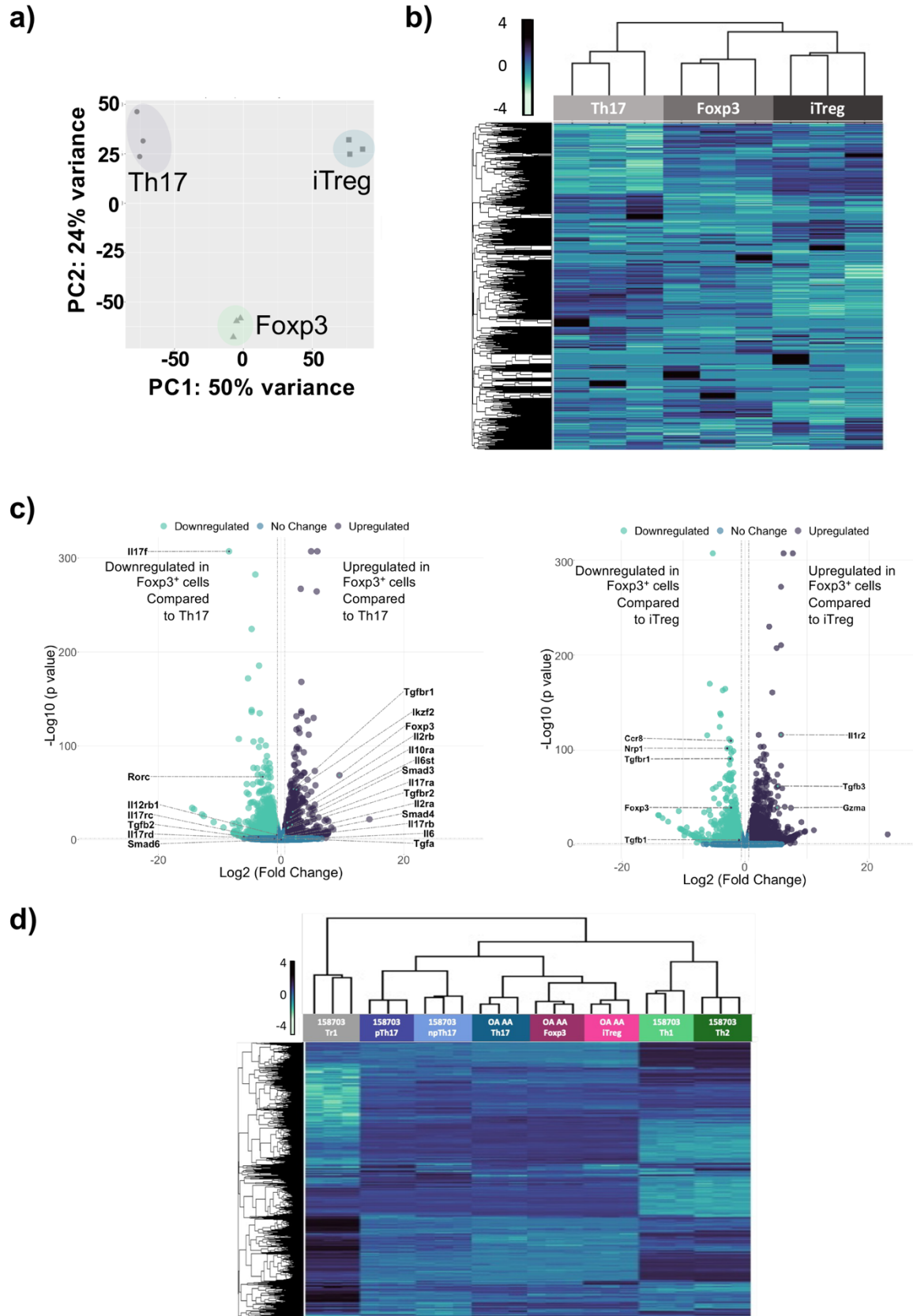
519 switched Foxp3⁺ Treg-like cells (generated in absence of ITK activity under Th17

520 conditions) and iTregs were sort purified, followed by co-culture with CFSE labelled

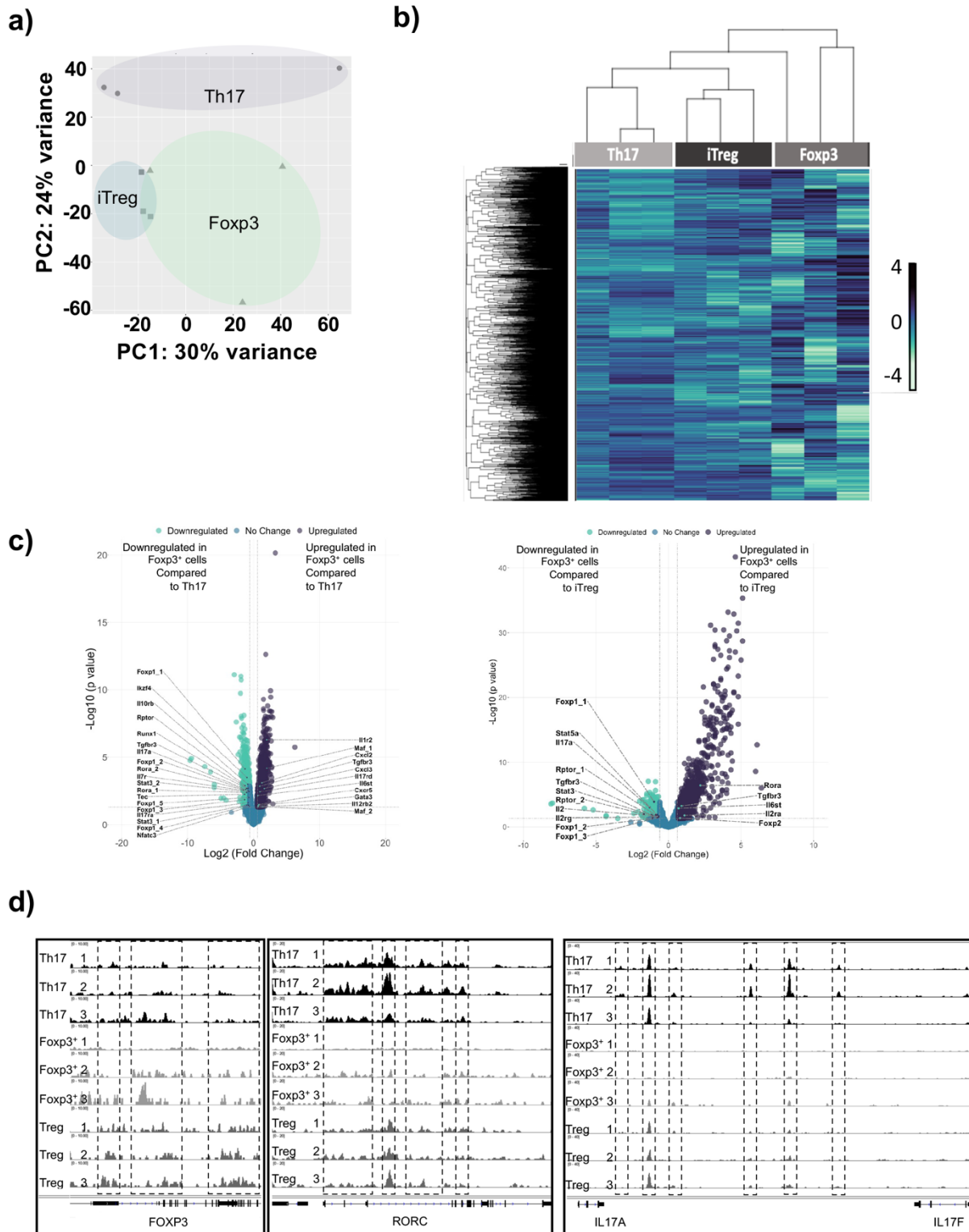
521 CD45.1 naïve CD4⁺ T cell responders. Representative CFSE plots (top).

522 Percentage suppression of proliferation of naïve CD4⁺ T cell responders by Foxp3⁺

523 Treg-like cells and iTregs was quantified (bottom). Mean \pm SEM for percentage of
524 cells undergoing division. One-way ANOVA performed for statistical significance
525 where * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.001$ and **** $p \leq 0.0001$, 3 independent
526 experiments.
527



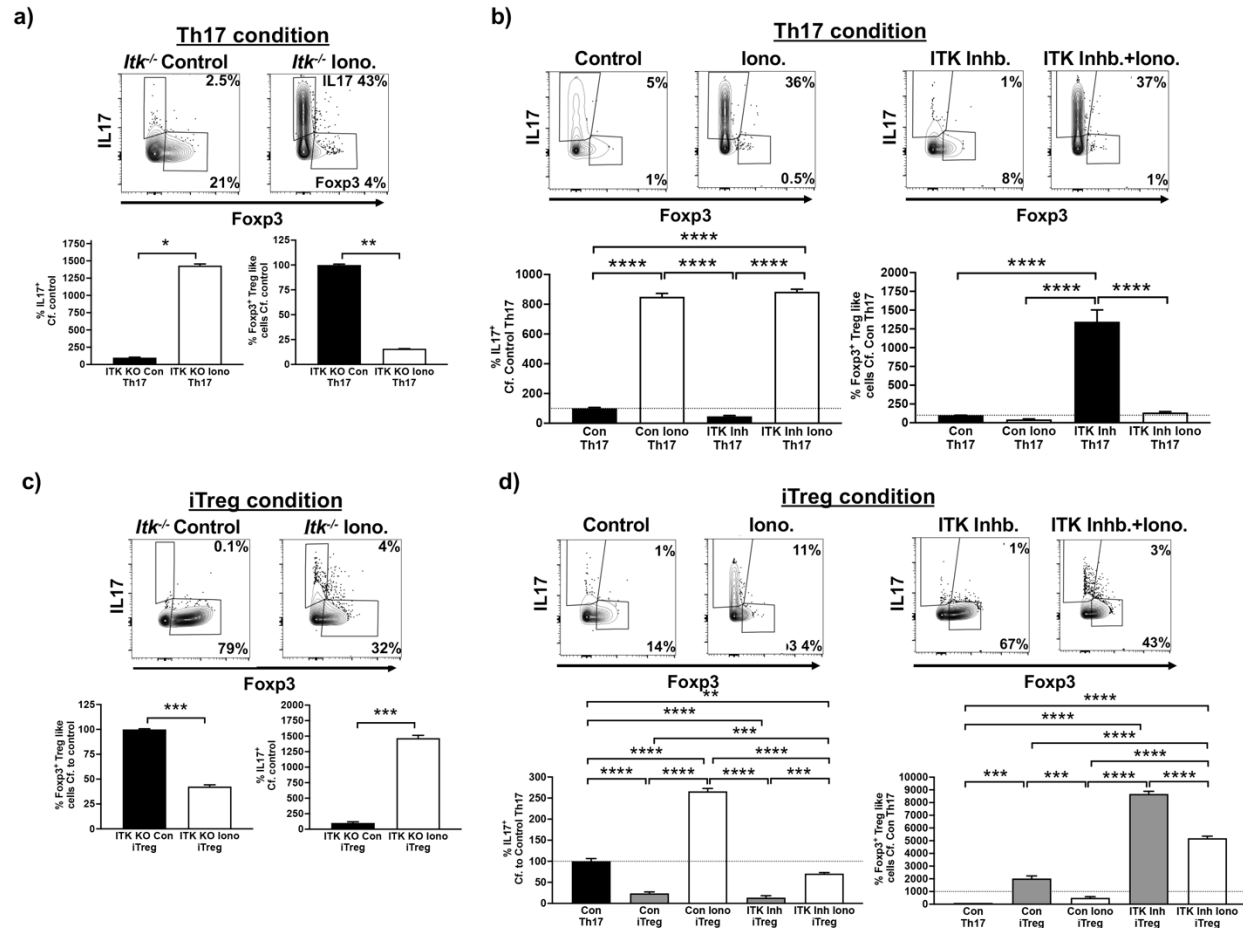
529 **Fig. 4. Switched Foxp3⁺ Treg-like cells generated under ITK inhibition have a**
530 **transcriptomic profile similar to iTregs.** Switched Foxp3⁺ Treg-like cells were
531 compared to *in vitro* generated Th17 and iTregs by RNA-Seq analysis. The
532 transcriptome was compared via (a) PCA analysis, (b) heatmap of global gene
533 expression, or (c) volcano plot of differentially expressed genes. N-3 for each
534 subset. (d) Switched Foxp3⁺ Treg-like cells were compared with GEOdata set from
535 GSE158703 by heatmap.
536



537

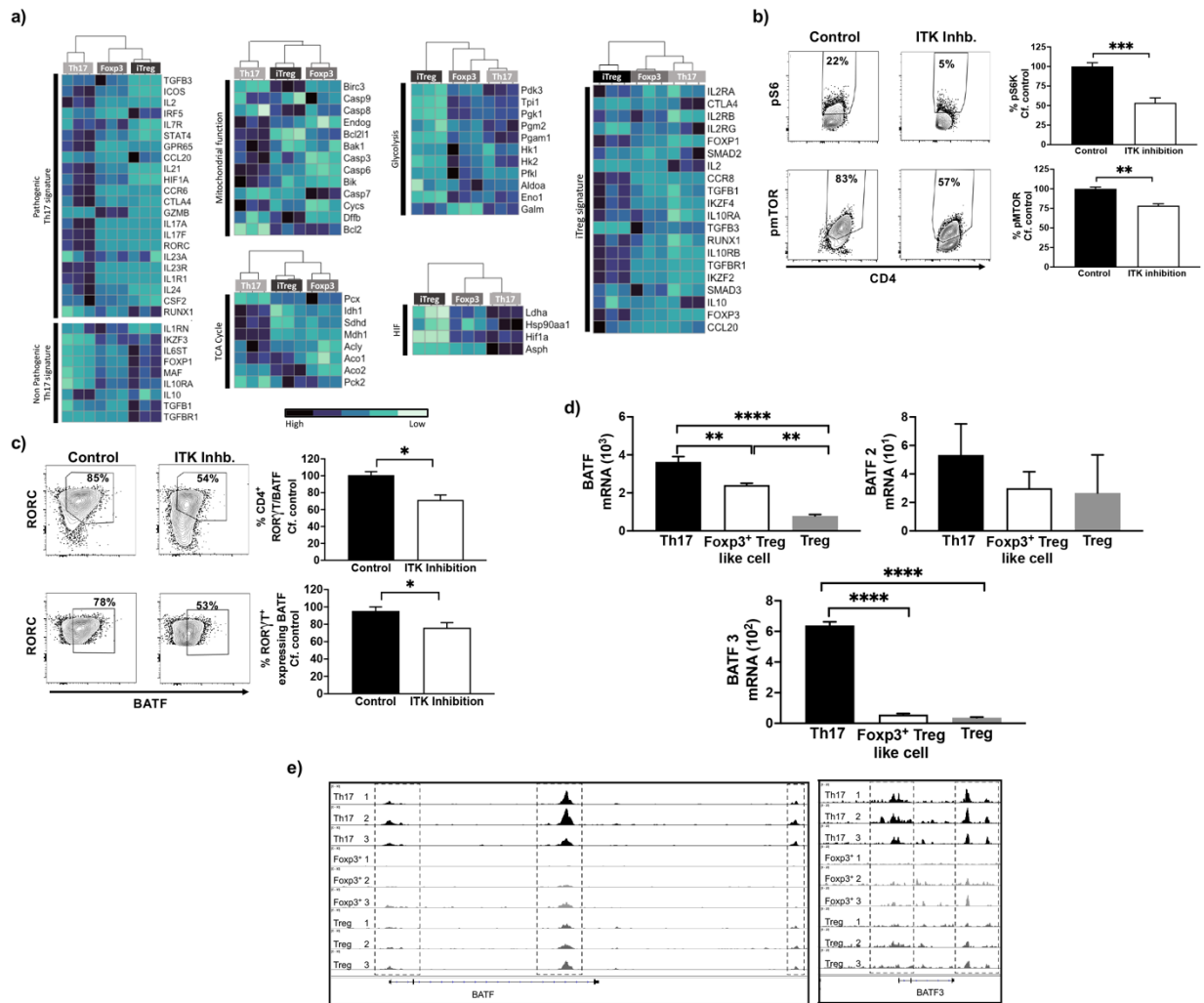
538 **Fig. 5. Switched Foxp3⁺ Treg-like cells generated under ITK inhibition have**
 539 **chromatin accessibility profiles distinct from Th17 and iTregs. Switched**
 540 **Foxp3⁺ Treg-like cells were compared with *in vitro* generated Th17 and iTregs by**
 541 **ATAC-Seq analysis. The chromatin accessibility profiles were compared via (a)**

542 PCA analysis, **(b)** heatmap of global differential peaks in chromatin accessibility,
543 and fold changes of global differential peaks in chromatin accessibility for switched
544 Foxp3 cells compared to **(c)** Th17 cells and iTregs. **(d)** Tracks indicate chromatin
545 areas chromatin accessibility for Foxp3, RORC, IL17A and IL17F in Foxp3⁺ Treg
546 like cells, compared to Th17 cells and iTreg cells.
547



548

549 **Fig. 6. Enhancing calcium signaling prevents the switch to Foxp3⁺ Treg-like cells**
 550 **during ITK inhibition.** (a) Naïve *Itk*^{-/-} IL17A-GFP/Foxp3-RFP CD4⁺ T cells were
 551 activated under Th17 differentiation conditions in presence of ionomycin or DMSO
 552 control, followed by analysis of percentage of GFP⁺/IL17⁺ cells and percentage of
 553 RFP⁺/Foxp3⁺ Treg-like cells. Representative flow plots (top), Quantified (bottom).
 554 (b) Naïve WT IL17A-GFP/Foxp3-RFP CD4⁺ T cells were activated under Th17
 555 differentiation conditions in presence of ionomycin or DMSO control, with or
 556 without ITK inhibitor CPI-818, followed by analysis of percentage of GFP⁺/IL17⁺
 557 cells and percentage of RFP⁺/Foxp3⁺ Treg-like cells. Representative flow plots
 558 (top), Quantified (bottom). (c) Naïve *Itk*^{-/-} IL17A-GFP/Foxp3-RFP CD4⁺ T cells
 559 were activated under iTreg differentiation conditions in presence of ionomycin or
 560 DMSO control, followed by analysis of percentage of GFP⁺/IL17⁺ cells and
 561 percentage of RFP⁺/Foxp3⁺ Treg-like cells. Representative flow plots (top),
 562 Quantified (bottom). (d) Naïve WT IL17A-GFP/Foxp3-RFP CD4⁺ T cells were
 563 activated under iTreg differentiation conditions in presence of ionomycin or DMSO
 564 control, with or without ITK inhibitor CPI-818, followed by analysis of percentage
 565 of GFP⁺/IL17⁺ cells and percentage of RFP⁺/Foxp3⁺ Treg-like cells.
 566 Representative flow plots (top), Quantified (bottom). Mean ± SEM, T test was
 567 performed for statistical significance where * p ≤ 0.05, ** p ≤ 0.005, *** p ≤ 0.001
 568 and **** p ≤ 0.0001, 3 independent experiments.



570

571

Fig. 7. Generation of switched Foxp3⁺ Treg like cells involve altered metabolic

pathways and BATF expression. (a) The transcriptomes of switched Foxp3⁺

Treg-like cells generated when naïve CD4⁺ T cells are activated under Th17

differentiation conditions in presence or absence of WT ITK inhibitor CPI-818, were

compared with signature genes that are associated with pathogenic/non-

pathogenic Th17 cells, and genes involved in the TCA cycle, mitochondrial

function, HIF1 α and glycolysis. (b) Flow cytometric analysis of phosphorylation of

Ribosomal S6 and mTOR in naïve CD4⁺ T cells are activated under Th17

differentiation conditions in presence or absence of WT ITK inhibitor CPI-818. (c)

Expression analysis by flow cytometry of BATF in RORC expressing naïve CD4⁺

T cells are activated under Th17 differentiation conditions in presence or absence

of WT ITK inhibitor CPI-818. (d) Analysis of mRNA expression and (e) chromatin

accessibility for BATF.

584

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722

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724 Investigation: OA, WH; Visualization: OA, WH, AA; Funding acquisition: AA, WH;
725 Project administration: AA; Supervision: AA; Writing – original draft: OA, AA; Writing –
726 review & editing: OA, WH, AA.

727

728 **Competing interests:** AA declares research funding from the 3M Company.

729

730 **Data and materials availability:** RNA-Sequencing and ATAC-Seq data will be
731 deposited in public databases. All other data are available in the main text or the
732 supplementary materials.