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## **CD5 signalosome coordinates antagonist TCR signals to control the generation of Treg cells induced by foreign antigens**

Gaëtan Blaize, Hélène Daniels-Treffandier, Meryem Aloulou, Nelly Rouquié, Cui Yang, Marlène Marcellin, Mylène Gador, Mehdi Benamar, Mariette Ducatez, Ki-Duk Song, et al.

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1           **CD5 signalosome coordinates antagonist TCR signals to control the**  
2           **generation of T reg cells induced by foreign antigens**

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26 **Abstract**

27

28 CD5 is characterized as an inhibitory co-receptor with important regulatory role during T cell  
29 development. The molecular mechanism by which CD5 operates has been puzzling and its  
30 function in mature T cells suggests promoting rather than repressing effects on immune  
31 responses. Here, we combined quantitative mass spectrometry and genetic studies to analyze  
32 the components and the activity of the CD5 signaling machinery in primary T cells. We found  
33 that TCR engagement induces the selective phosphorylation of CD5 tyrosine 429, which  
34 serves as a docking site for proteins with adaptor functions (c-Cbl, CIN85, CRKL),  
35 connecting CD5 to positive (PI3K) and negative (UBASH3A, SHIP1) regulators of TCR  
36 signaling. c-CBL acts as a coordinator in this complex enabling CD5 to synchronize positive  
37 and negative feedbacks on TCR signaling through the other components. Disruption of CD5  
38 signalosome in mutant mice reveals that it modulates TCR signal outputs to selectively  
39 repress the transactivation of *Foxp3* and limit the inopportune induction of peripherally-  
40 induced regulatory T cells during immune responses against foreign antigen. Our findings  
41 bring new insights into the paradigm of co-receptor signaling, suggesting that, in addition to  
42 providing dualistic enhancing or dampening inputs, co-receptors can engage concomitant  
43 stimulatory and inhibitory signaling events which act together to promote specific functional  
44 outcomes.

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46 **Keywords**

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48 T cells / Signaling / Co-receptors

49 **Significance statement**

50

51 T-cell co-receptors are often described as molecular switches that broadly regulate T-cell  
52 activation by enhancing or repressing TCR signaling according to the immunological context.  
53 However, many co-receptors act more selectively by instructing or restricting the generation  
54 of specific T-cell subsets. The molecular mechanisms by which these subtle regulations occur  
55 remain incompletely defined. In this study, we show that CD5 co-receptors engage a  
56 multimeric signaling complex which synchronize positive and negative feedback on TCR  
57 signaling to limit the induction of inopportune regulatory T cells during immune response.  
58 Our findings suggest that rather than exclusively acting as stimulators or inhibitors of TCR  
59 signaling, co-receptors may coordinate antagonist TCR signals which act together to promote  
60 specific T-cell responses.

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69 **Introduction**

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71 T cells have the ability to develop a wide variety of cellular responses following the  
72 stimulation of a single receptor, namely the T-cell antigen receptor (TCR). The recognition  
73 by TCRs of self or foreign peptides bound to the major histocompatibility complex (pMHC)  
74 triggers multiple signaling pathways, which lead to the activation of specific effector proteins  
75 involved in the transmission of distinct signaling responses. The relative intensity and the  
76 persistency by which signals are transmitted in each pathway play a critical role in specifying  
77 and driving specific T-cell responses. Because different pathways may have either synergistic  
78 or antagonist effects on these responses, their coordination in time and space (signaling  
79 patterns) is also critical to shape T-cell effector profiles and determine specific outcomes.

80

81 Signals transmitted by the TCR can be regulated by co-receptors that are engaged  
82 differentially based on their relative expression on the T-cell surface and on the availability of  
83 their cognate ligands in the extracellular environment. Initial work on co-receptor signaling  
84 led to the classification of these proteins into two main functional categories depending on  
85 their overall effect on T-cell activity. Stimulatory co-receptors, such as CD28, ICOS or OX40,  
86 which promote naïve T-cell activation and amplify effector T-cell responses, and inhibitory  
87 co-receptors, such as CTLA-4, PD-1 or BTLA which prevent the potential activation of T  
88 cells by self-antigens and contribute to terminate or tune down effector T-cell responses  
89 following antigen clearance. More recent investigations indicate that many co-receptors act  
90 more selectively on specific signaling pathways and contribute to shape the effector profile of  
91 T cells according to the immunological context (1-3). Although the mechanisms by which co-  
92 receptors positively or negatively regulate T-cell activity have been well documented (1, 4-7),

93 the molecular processes by which they convey signals to selectively modulate T-cell  
94 responses remain poorly understood.

95

96 CD5 is a type 1 transmembrane cell surface glycoprotein that is essentially expressed in T  
97 cells. Initial characterization of *Cd5*<sup>-/-</sup> mice indicated an inhibitory function for this receptor  
98 on TCR signaling (8). Later work showed that CD5 surface levels on thymocytes are  
99 correlated to the strength of TCR signaling that is dictated during positive selection by the  
100 affinity of the TCR for self-pMHC (9). The increased expression of CD5 in thymocytes that  
101 express TCRs with greater self-reactivity dampens TCR signals, possibly enabling some  
102 thymocytes that would otherwise be negatively selected to avoid activation-induced cell death  
103 and instead complete their maturation and be exported to peripheral lymphoid organs (10).  
104 Through this mechanism CD5 would enable the selection of T cells with higher self-  
105 reactivity, which are presumed to be more effective responders to foreign antigens (11).

106

107 Whereas the role of CD5 during thymic selection has been well characterized, its function in  
108 peripheral T cells is less clear. The expression level of CD5 remains correlated with the  
109 affinity of TCR for self-pMHC in peripheral T cells (11), suggesting that CD5 could be  
110 important for the maintenance of self-tolerance by dampening homeostatic TCR signals that  
111 could otherwise cause activation and autoimmunity. However, *Cd5*<sup>-/-</sup> mice do not exhibit signs  
112 of spontaneous autoimmune or inflammatory pathology and show, on the contrary, a reduced  
113 susceptibility to active experimental autoimmune encephalomyelitis (12) and inflammatory  
114 bowel disease (13). This suggests that in the absence of CD5 compensatory mechanisms  
115 might prevent the expansion and the full activation of T cells expressing TCRs with relatively  
116 high affinity to self-pMHC. Notably, previous studies showed that the numbers and

117 suppressive function of regulatory T cells (Treg) are increased in CD5 deficient mice (13, 14).  
118 However, more recent findings indicate that CD5 plays an instructive role in the generation of  
119 peripherally-induced Treg cells (iTreg) in response to tolerizing antigens (15), suggesting that  
120 CD5 could have different influences on this T-cell subset according to the immunological  
121 context.

122

123 Although several ligands have been reported for CD5 (16-18), it was shown that its  
124 extracellular domain is not required for negative regulation of TCR signaling in thymocytes  
125 (19), indicating that CD5 is engaged in a feedback loop that tunes down TCR signals  
126 following TCRs engagement. Accordingly, CD5 is constitutively associated with the TCR  
127 subunits at the cell surface (20) and contains several phospho-tyrosine-binding sites that are  
128 phosphorylated by SRC kinases following TCR engagement (21). Although many CD5-  
129 interacting partners have been reported, the relative importance of these interactions remains  
130 unclear because most of these binding partners were identified in independent studies or  
131 within distinct cellular models through approaches that do not always enable global  
132 comparisons of protein-protein interactions. Interestingly, whereas some of these proteins are  
133 well characterized inhibitors of TCR signaling (22, 23), others are known to be positive  
134 effectors (24-27), suggesting that CD5-mediated feedback on TCR signaling might be more  
135 complex than what was initially presumed.

136

137 In this study, we combined quantitative mass spectrometry and mouse genetics to analyze the  
138 composition, the mode of assembly and the molecular function of the CD5 transduction  
139 machinery in primary T cells. We found that CD5 coordinates the recruitment of a signaling  
140 complex composed of proteins with adapter functions (c-CBL, CIN85 and CRKL) that

141 connect CD5 to positive (PI3K) and negative (UBASH3A and SHIP1) regulators of TCR  
142 signaling. The recruitment of this complex is entirely dependent on the Y429 of CD5 which is  
143 predominantly phosphorylated following TCR engagement and serves as a docking site for c-  
144 CBL. Disruption of Y429 phosphorylation site in primary T cells shows that this signaling  
145 complex promotes on one hand AKT-mediated inhibition of FOXO1 and represses on the  
146 other hand ERK kinase activity to selectively dampen the transactivation of *Foxp3* gene  
147 expression. Analysis of antigen-specific Treg cells in CD5-Y429F mutant mice show that  
148 CD5 signaling selectively represses the generation of these cells presumably to promote the  
149 development of optimal immune responses.

150 **Results**

151

152 **Mass-spectrometry analysis of the CD5 interactome**

153

154 To investigate the molecular mechanism by which CD5 regulates TCR signaling, we  
155 performed an MS-based analysis of CD5-containing complexes in thymocytes. CD5 was  
156 immunoprecipitated from wild-type (WT) or *Cd5*<sup>-/-</sup> thymocytes that were treated with  
157 pervanadate for 1 minute to induce widespread activation of protein tyrosine kinases. CD5  
158 protein complexes were eluted, and the components of the different purified complexes were  
159 characterized by nanoflow liquid chromatography combined with tandem MS. To  
160 discriminate CD5-binding molecules from the background of contaminant proteins, a  
161 thorough quantitative comparison based on MS intensity values was performed for each  
162 identified protein between samples immunoprecipitated from WT versus *Cd5*<sup>-/-</sup> thymocytes.  
163 Candidate proteins were selected based on their significant enrichment in WT samples (fold  
164 change >2 and Student t-test p-value<0.001, n=8 replicate experiments, see Materials and  
165 Methods for details). On this basis, we identified 11 proteins as potential interacting partners  
166 of CD5 in thymocytes (Fig. 1A, *SI Appendix*, Fig. S1A and Dataset S1). Among these, six  
167 proteins were previously identified as regulators of TCR-mediated signaling (c-CBL,  
168 UBASH3A/STS-2, CIN85/SH3KBP1, SHIP1, CRKL and PI3K), two proteins are known  
169 components of the AP2 complex which is involved in clathrin-mediated internalization of  
170 CD5 (AP2a1, AAK1)(28) and three proteins have diverse reported functions not directly  
171 associated with TCR signaling (IGH, TRIM21, CYB5). The interactions of CD5 with c-CBL,  
172 UBASH3A, PI3K, CRKL and AP2 were reported previously in independent studies (22, 24,  
173 25, 28, 29) but not its interaction with SHIP1 and CIN85. Among these proteins, three are  
174 cytosolic adaptors (CRKL, CIN85 and AP2) and five are effector molecules with enzymatic

175 function (c-CBL, UBASH3A, SHIP1, AAK1 and PI3K). The association of these proteins  
176 with CD5 was still detected after 10 minutes of stimulation and no additional interactors were  
177 identified at this later time of stimulation (*SI Appendix*, Fig. S1B and Dataset S2).

178 To estimate the relative abundance of these signaling partners in the immunoprecipitated  
179 samples, we used the intensity-based absolute quantification (iBAQ) metric, which  
180 corresponds to the sum of all of the peptide intensities divided by the number of theoretically  
181 observable tryptic peptides of a protein. Analysis of these interactions (with normalized iBAQ  
182 intensities) shows that UBASH3A (IBAQ=604 x 10<sup>4</sup>) and c-CBL (IBAQ=560 x 10<sup>4</sup>) are more  
183 abundantly recruited to CD5 than CIN85 (IBAQ=147 x 10<sup>4</sup>), CRKL (IBAQ=143 x 10<sup>4</sup>),  
184 SHIP1 (IBAQ=55 x 10<sup>4</sup>) and PI3K (IBAQ=18 x 10<sup>4</sup>), suggesting an essential role for c-Cbl  
185 and UBASH3A in CD5-mediated regulation of T-cell activation (*SI Appendix*, Fig. S1A).  
186 Analysis by Western blot showed that CD5 interacts with c-CBL, UBASH3A, SHIP1, CIN85  
187 and PI3K both in thymocytes and in peripheral CD4<sup>+</sup> T cells, suggesting that CD5 engages  
188 similar signaling processes upon T cell development and primary T cell responses (Fig. 1B).  
189 Further analysis showed that c-CBL, UBASH3A, SHIP1, CRKL, CIN85 and PI3K interacted  
190 poorly with CD5 in resting cells but were recruited to CD5 upon TCR+CD4 cross-linking  
191 (Fig. 1C and Dataset S3), suggesting that CD5 contributes to TCR signaling through the  
192 activity of these proteins. Note that RAS-GAP (22), SHP-1 (23), CBL-b (30) and CK2 (27),  
193 which were previously reported to interact with CD5 in thymocytes or in T-cell lines, were  
194 either undetected (Ras-GAP), detected at the same level in immunoprecipitated samples from  
195 WT and *Cd5*<sup>-/-</sup> controls (SHP-1), or inconsistently detected across biological replicates  
196 without a statistically significant enrichment ratio in immunoprecipitated samples versus  
197 controls (CBL-b and CK2). Thus, these proteins were not selected in the list of major CD5-  
198 interacting proteins (Dataset S1).

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200

201 **Tyrosine 429 of CD5 is essential for assembly of the CD5 signalosome**

202

203 We next investigated the mechanism by which CD5 recruits these proteins following TCR  
204 stimulation. Previous studies performed on cell lines identified three potential tyrosine-  
205 phosphorylation sites on the intracytoplasmic domain of human CD5: tyrosine 429 (pY429),  
206 tyrosine 441 (pY441) and tyrosine 463 (pY463) (31, 32). To address which of these tyrosines  
207 is preferentially phosphorylated following TCR engagement in primary thymocytes, we  
208 analyzed the relative MS intensity of CD5 phosphorylated peptides encompassing these three  
209 modification sites compared to their respective unmodified forms. It must be noted that this  
210 percentage does not strictly measure the phosphorylation stoichiometry of each site (as  
211 phosphorylated and unphosphorylated forms may have different ionization efficiencies in the  
212 mass spectrometer). We nevertheless used this metric to qualitatively illustrate the  
213 phosphorylation occupancy before and after stimulation. Whereas the phosphorylation of  
214 these residues was barely detectable in unstimulated thymocytes, we found that CD5 was  
215 nearly exclusively phosphorylated on Y429 after TCR cross-linking with CD4 upon different  
216 times of stimulation (Fig. 2A, Dataset S3 and S4). All three tyrosine residues of CD5 were  
217 phosphorylated following pervanadate treatment, suggesting that Y441 and Y463 might be  
218 phosphorylated independently of TCR engagement upon additional co-stimulatory signals  
219 (Fig. 2A).

220

221 To determine the role of Y429 in the context of CD5 signaling, we expressed a wild-type  
222 (CD5tg<sup>Wt</sup>) or a mutated form of CD5, containing a tyrosine to phenylalanine substitution at  
223 position 429 (CD5tg<sup>Y429F</sup>), as transgenes under the control of the T-cell specific hCD2  
224 promoter/enhancer and crossed both transgenes into the *Cd5*<sup>-/-</sup> background (*Cd5*<sup>-/-</sup>; CD5tg<sup>Wt</sup>

225 or *Cd5*<sup>-/-</sup>; CD5tg<sup>Y429F</sup>, hereafter designated CD5tg<sup>Wt</sup> or CD5tg<sup>Y429F</sup>, respectively). We verified  
226 that the WT and Y429F CD5 transgenes were similarly expressed in thymocyte subsets and in  
227 peripheral CD4<sup>+</sup> and CD8<sup>+</sup> T cells (*SI Appendix*, Fig. S2A). The surface levels of CD5  
228 remained unchanged following stimulation with anti-CD3 antibodies and were comparable in  
229 CD5tg<sup>Wt</sup> and CD5tg<sup>Y429F</sup> thymocytes (Fig S2B). Moreover, the proportions and numbers of  
230 thymocytes and peripheral T cells in each subset were similar in CD5tg<sup>Wt</sup>, CD5tg<sup>Y429F</sup> and  
231 *Cd5*<sup>-/-</sup> mice (*SI Appendix*, Fig. S2C and S2D). Percentages of FOXP3<sup>+</sup> T cells in the thymus  
232 and the spleen (*SI Appendix*, Fig. S2E) and surface levels of activation/memory markers such  
233 as CD44, CD69 and PD-1 (*SI Appendix*, Fig. S2F and S2G) were also comparable between  
234 these three lines. To investigate the impact of this mutation on the formation of CD5 signaling  
235 complexes, we compared the interactome of the wild-type and the mutated form of CD5 in  
236 thymocytes stimulated with pervanadate. We verified that c-CBL, UBASH3A, PI3K, CRKL,  
237 SHIP1 and CIN85 interacts with WT CD5 in CD5tg<sup>Wt</sup> thymocytes similarly to what we  
238 observed in the C57BL/6 background (Fig. 2B and Dataset S5). IGH, TRIM21, CYB5 were  
239 not detected in this interactome suggesting that they represent low affinity or non-specific  
240 interactions. Notably, the mutation of tyrosine Y429 disrupted the association of c-CBL,  
241 UBASH3A, PI3K, CRKL, SHIP1 and CIN85 with CD5 following pervanadate treatment  
242 (Fig. 2B and Dataset S5) and TCR cross-linking (Fig. 2C), despite comparable  
243 phosphorylation of tyrosine Y463 of CD5<sup>Wt</sup> and CD5<sup>Y429F</sup> (Fig. 2B and Dataset S5).  
244 Quantitative analysis of the LFQ intensity metrics showed that the amount of each protein  
245 partner detected in the CD5tg<sup>Y429F</sup> interactome was drastically decreased compared to  
246 CD5tg<sup>Wt</sup>, and nearly comparable to those detected in *Cd5*<sup>-/-</sup> thymocytes (*SI Appendix*, Fig.  
247 S1C), suggesting that the mutation of Y429 almost entirely disrupted the assembly of the CD5  
248 signalosome. No additional proteins were detected in the interactome of CD5 when tyrosine  
249 429 was mutated (Fig. 2B).

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**c-CBL harbors a connective function that links CD5 to other regulators of TCR signaling**

We next investigated the mechanism by which CD5 interacting proteins are recruited by tyrosine phosphorylation of a single residue (Y429) on the cytoplasmic tail of CD5. Y429 is included in a D(N/D)XpY motif predicted to be a potential binding site for the phosphotyrosine binding (PTB) domain of c-CBL (33). In addition, UBASH3A, SHIP1, CRKL, PI3K and CIN85 were also previously characterized as direct or indirect c-CBL-interacting proteins, suggesting that c-CBL might be important to recruit these molecules to CD5 (34-36). To investigate this possibility, we immunoprecipitated CD5 in wild-type, *c-Cbl*<sup>-/-</sup> and *Cd5*<sup>-/-</sup> thymocytes and evaluated if UBASH3A, SHIP1, CRKL, PI3K and CIN85 were co-immunoprecipitated with CD5 by mass spectrometry (Fig. 2D and Dataset S3) and Western blot (Fig. 2E). The amount (Fig. 2D and 2E) and the phosphorylation level (Fig. 2E) of immunoprecipitated CD5 were slightly increased in *c-Cbl*<sup>-/-</sup> thymocytes compared to that in wild-type cells. Nevertheless, we found that the amounts of UBASH3A, SHIP1, CRKL, PI3K and CIN85 that co-immunoprecipitated with CD5 were strongly reduced in *c-Cbl*<sup>-/-</sup> thymocytes compared to those in wild-type thymocytes and were similar to those observed in *Cd5*<sup>-/-</sup> thymocytes, suggesting that c-CBL is required to recruit these molecules to CD5. In addition, we found that CD5 was not required for the phosphorylation of c-CBL (Fig S3A) or for its interaction with PI3K, SHIP1 and CIN85 (Fig S3B), suggesting that CD5 is important for the recruitment of these molecules at the membrane but not for their assembly with c-CBL.

275 **CD5 integrates both co-stimulatory and co-inhibitory signaling**

276

277 To elucidate the mechanism by which the CD5 regulates TCR signals, we next focused our  
278 study on c-CBL, which has well characterized inhibitory functions on TCR signaling (37). It  
279 has been proposed that c-CBL, an E3 ubiquitin-protein ligase, negatively regulates TCR  
280 signals by targeting TCRs and other components of the TCR signaling machinery to  
281 lysosomal or proteasomal degradation through ubiquitination. Consequently, TCR surface  
282 expression is increased in *c-Cbl*<sup>-/-</sup> CD4<sup>+</sup>CD8<sup>+</sup> thymocytes (referred to as double-positive (DP)  
283 thymocytes) compared to wild-type (*c-Cbl*<sup>+/+</sup>) DP thymocytes (Fig S3C). Contrasting with  
284 this observation, we found that CD5 deficiency did not affect TCR surface expression in DP  
285 thymocytes, suggesting that CD5 is not required for c-CBL-mediated TCR turnover.  
286 Supporting this conclusion, the degradation of the TCR ζ-chain, which is induced following  
287 TCR cross-linking, was reduced in *c-Cbl*<sup>-/-</sup> thymocytes but not in *Cd5*<sup>-/-</sup> thymocytes (*SI*  
288 *Appendix*, Fig. S3D and S3E). Expression of LCK, which was previously identified as a target  
289 of c-CBL in T cells (38), was slightly enhanced in *c-Cbl*<sup>-/-</sup> thymocytes but was also unaffected  
290 in *Cd5*<sup>-/-</sup> thymocytes (Fig S3F). Finally, we found that PI3K, SHIP1 and CIN85 are expressed  
291 similarly in WT, *c-Cbl*<sup>-/-</sup> and *Cd5*<sup>-/-</sup> thymocytes suggesting that c-CBL does not drive the  
292 degradation of the other CD5-interacting proteins (Fig S3F).

293 We next hypothesized that CD5 could use c-CBL, CIN85 and CRKL adapter functions to  
294 recruit positive (PI3K) and negative (UBASH3A, SHIP1) regulators of TCR signaling that  
295 would act more selectively to regulate specific signaling pathways. Recent studies show that  
296 CIN85 negatively regulates the phosphorylation of ZAP-70, SLP-76 and ERK by recruiting  
297 UBASH3A into TCR microclusters (39). CIN85 also interacts with SHIP1, which also exerts  
298 an inhibitory effect on ZAP-70 activity through the proteins Dok-1 and Dok-2 (40) that  
299 presumably act through CSK to inhibit SRC kinases activity (41, 42). This suggested that

300 SHIP1 and UBASH3A could act together to repress ZAP-70 activity. In addition, SHIP1  
301 negatively regulates calcium responses (43) and the phosphorylation of Tec kinases (44, 45)  
302 which play an essential role in PLC $\gamma$ 1 and ERK kinases activation. PI3K may interact directly  
303 with c-Cbl (46) or indirectly through CRKL (47). The co-recruitment of SHIP1 and PI3K by  
304 CD5 was surprising since these two proteins were reported to have antagonistic effect on  
305 AKT activity (40, 48). To analyze whether these effector proteins are involved in CD5  
306 signaling function, we stimulated thymocytes or peripheral CD4<sup>+</sup> T cells from CD5tg<sup>Wt</sup>,  
307 CD5tg<sup>Y429F</sup> and *Cd5*<sup>-/-</sup> mice with anti-CD3 and anti-CD4 antibodies and compared the  
308 intracellular increase of calcium and the phosphorylation of several of their downstream  
309 signaling targets. The calcium response was increased in CD5tg<sup>Y429F</sup> DP thymocytes  
310 compared to that in CD5tg<sup>Wt</sup> DP thymocytes (Fig. 3A). Accordingly, Western blot analysis  
311 showed that the phosphorylation of ERK and SLP-76 was increased in CD5tg<sup>Y429F</sup>  
312 thymocytes and peripheral CD4<sup>+</sup> T cells similarly to what was observed in *Cd5*<sup>-/-</sup> cells (Fig.  
313 3B and 3C). Although we could not detect significant ZAP-70 phosphorylation in CD5tg<sup>Wt</sup>  
314 thymocytes with the stimulation conditions used in these experiments, we found that TCR-  
315 stimulation induced phosphorylation of ZAP-70 was increased in both CD5tg<sup>Y429F</sup> and *Cd5*<sup>-/-</sup>  
316 CD4<sup>+</sup> T cells compared to that in CD5tg<sup>Wt</sup> CD4<sup>+</sup> T cells (Fig. 3C). By contrast, the  
317 phosphorylation of AKT was strikingly reduced in thymocytes and peripheral CD4<sup>+</sup> T cells  
318 from CD5tg<sup>Y429F</sup> and *Cd5*<sup>-/-</sup> mice compared to that in similar T-cell subsets from CD5tg<sup>Wt</sup>  
319 mice (Fig. 3B and 3C). In comparison, the phosphorylation of LCK and P38 were similar in  
320 thymocytes and CD4<sup>+</sup> T cells from the three different lines of mice (Fig. 3B and 3C). Finally,  
321 we found that the phosphorylation of CD5-interacting proteins such as SHIP1 and PI3K were  
322 not significantly impaired in CD5tg<sup>Y429F</sup> CD4<sup>+</sup> T cells despite a consistent decreased of Akt  
323 phosphorylation in those cells, suggesting that CD5 is important to relocate those proteins  
324 close to their molecular targets rather than directly controlling their activity (Fig. 3D).

325 Altogether these results suggest that CD5 acts through c-CBL-binding proteins to exert both  
326 enhancing and inhibitory effects on specific TCR generated signals.

327

328

329 **CD5-mediated signaling negatively regulates CD4<sup>+</sup> T-cell activation and restrains the**  
330 **generation of regulatory T cells induced by foreign antigens**

331

332 We next analyzed the functional consequences of these CD5-mediated signaling modulations  
333 on thymocyte and naïve CD4<sup>+</sup> T cell activation. We found that expression of Nur77, a  
334 quantitative sensor of TCR signal strength (49), was enhanced in CD5tg<sup>Y429F</sup> and *Cd5*<sup>-/-</sup> DP  
335 and CD4-SP thymocytes as compared to that in CD5tg<sup>Wt</sup> thymocytes following TCR  
336 crosslinking (Fig. 4A). TCR stimulation-induced surface expression of CD25 on CD4<sup>+</sup> T cells  
337 and the proportions of CD4<sup>+</sup> T cells expressing CD69 were also similarly augmented in  
338 CD5tg<sup>Y429F</sup> and *Cd5*<sup>-/-</sup> T cells as compared to those in CD5tg<sup>Wt</sup> thymocytes (Fig 4B and 4C).  
339 The up-regulation of CD25 surface expression was also increased in CD5tg<sup>Y429F</sup> CD4<sup>+</sup> T cells  
340 when cells were co-stimulated with anti-CD28 antibodies, indicating that CD28 signaling  
341 does not compensate for CD5 signaling deficiency (Fig. 4B). Finally, we found that  
342 CD5tg<sup>Y429F</sup> and *Cd5*<sup>-/-</sup> CD4<sup>+</sup> T cells proliferated more than CD5tg<sup>Wt</sup> CD4<sup>+</sup> T cells in response  
343 to anti-CD3 stimulation alone (Fig. 4D) or with anti-CD4 antibodies (Fig. 4E), suggesting that  
344 CD5 negatively regulates peripheral CD4<sup>+</sup> T-cell activation independently of CD4 co-  
345 stimulation.

346

347 The analysis of TCR signaling suggested that, rather than repressing broadly TCR signals,  
348 CD5 acts more selectively by promoting AKT and repressing ERK kinases and calcium  
349 response activities through a dedicated set of effector molecules. We thus suspected that CD5  
350 signaling could exert a more selective effect on T cell responses in addition to its role in  
351 controlling the threshold for T-cell activation. Among the many reported effects of AKT in  
352 CD4<sup>+</sup> T cells, it is well known that strong AKT-mediated signals reduce their ability to  
353 differentiate into FOXP3<sup>+</sup> regulatory T cells in presence of TGF- $\beta$  (50). One proposed  
354 mechanism to explain this effect is that AKT stimulates the cytoplasmic retention of FOXO1  
355 through the phosphorylation of two residues (Thr24 and Ser256) inhibiting the translocation  
356 of FOXO1 to the nucleus and the subsequent transactivation of *Foxp3* gene (51). The  
357 activation of ERK kinases was shown, in similar experimental settings, to have positive effect  
358 on *Foxp3* transactivation *in vitro* (52) suggesting that CD5 could coordinate these signaling  
359 pathways to selectively repress the induction of Treg cells.

360

361 To address this possibility, we first compared FOXP3 expression in CD5tg<sup>Wt</sup>, CD5tg<sup>Y429F</sup> and  
362 *Cd5*<sup>-/-</sup> CD4<sup>+</sup> T cells stimulated with different doses of anti-CD3 antibodies in presence of  
363 TGF- $\beta$ . We found that the percentages of CD5tg<sup>Y429F</sup> and *Cd5*<sup>-/-</sup> CD4<sup>+</sup> T cells expressing  
364 FOXP3 were enhanced, compared to that in CD5tg<sup>Wt</sup> CD4<sup>+</sup> T cells (Fig. 5A), upon weak but  
365 not high TCR stimulations, suggesting that CD5 may contribute to enhance the threshold at  
366 which the development of iTreg is engaged. By comparison, we found that CD5tg<sup>Wt</sup>,  
367 CD5tg<sup>Y429F</sup> and *Cd5*<sup>-/-</sup> CD4<sup>+</sup> T cells differentiated similarly into ROR $\gamma$ <sup>t</sup>IL-17<sup>+</sup> cells following  
368 TCR stimulation under Th17 polarizing conditions (Fig. 5B). Numbers of CD5tg<sup>Y429F</sup> and  
369 *Cd5*<sup>-/-</sup> FOXP3<sup>+</sup> cells were enhanced independently of the concentration of anti-CD3  
370 antibodies (Fig. 5A), suggesting that CD5 operates both by controlling the ability of CD4<sup>+</sup> T

371 cells to differentiate into iTreg and by repressing the proliferation/survival of conventional T  
372 cells prior or during their engagement into the Treg lineage. Accordingly, the percentages of  
373 cells expressing FOXP3 were higher in non-divided (CTV<sup>hi</sup>) CD5tg<sup>Y429F</sup> CD4<sup>+</sup> T cells  
374 compared to the same cell populations from CD5tg<sup>Wt</sup> CD4<sup>+</sup> T cells (*SI Appendix*, Fig. S4).  
375 Confirming the positive effect of CD5 on AKT activity, we found that the phosphorylation of  
376 FOXO1 on Thr24 and Ser256 was impaired in CD5tg<sup>Y429F</sup> and *Cd5*<sup>-/-</sup> CD4<sup>+</sup> T cells (Fig. 5C).  
377 The phosphorylation of ERK, which is also correlated with efficient generation of induced  
378 Treg cells (52), was enhanced in CD5tg<sup>Y429F</sup> and *Cd5*<sup>-/-</sup> CD4<sup>+</sup> T cells compared to that in  
379 CD5tg<sup>Wt</sup> CD4<sup>+</sup> T cells, showing further that CD5 delivers both co-stimulatory and co-  
380 inhibitory signals to modulate a specific T-cell response, namely the induction of Foxp3 and  
381 the generation of iTreg cells. Finally, the numbers of FOXP3<sup>+</sup> cells were similar in CD5tg<sup>Wt</sup>  
382 and CD5tg<sup>Y429F</sup> CD4<sup>+</sup> T cells when cells were incubated with high concentrations of FOXO1  
383 inhibitors in *in vitro* assays, suggesting that the increased activity of ERK observed in  
384 CD5tg<sup>Y429F</sup> CD4<sup>+</sup> T cells was not sufficient alone to enhance the generation of Treg cells  
385 (Fig. 5D). In comparison, the numbers of CD5tg<sup>Y429F</sup> CD4<sup>+</sup>FOXP3<sup>-</sup> T cells, reflecting T-cell  
386 expansion, remained increased compared to those in CD5tg<sup>Wt</sup> CD4<sup>+</sup>FOXP3<sup>-</sup> T cells when  
387 cells were treated with similar doses of FOXO1 inhibitors (Fig. 5D). Altogether these results  
388 suggested that CD5 signaling shape TCR signals to selectively repress FOXP3 expression.

389

390 The surface level of CD5 correlates with TCR signal intensity, which is dictated by the  
391 affinity of the TCR for self-ligands (9). To determine whether quantitative variations of CD5  
392 surface expression within normal physiological ranges influences the generation of induced  
393 FOXP3<sup>+</sup> cells, we next sorted naïve CD62L<sup>hi</sup> CD25<sup>-</sup> CD4<sup>+</sup> T cells expressing either high or  
394 low surface levels of CD5 and compared their ability to differentiate into FOXP3<sup>+</sup> cells in  
395 Treg-cell polarizing conditions. We found that CD5<sup>lo</sup>CD4<sup>+</sup> T cells differentiate more

396 efficiently into FOXP3<sup>+</sup> cells than CD5<sup>hi</sup>CD4<sup>+</sup> T cells under identical TCR stimulation  
397 conditions, suggesting that physiologically high levels of CD5 on naïve CD4<sup>+</sup> T cells might  
398 reduce the ability of these cells to differentiate into Treg cells upon antigenic recognition (Fig.  
399 6A). Contrasting with those results, previous studies suggested that CD5 promotes, rather  
400 than represses, the differentiation of peripherally-induced Treg cells when those cells are  
401 generated in a tolerogenic context such as the gut mucosa (15). Accordingly, we found that  
402 the percentages of FOXP3<sup>+</sup>CD4<sup>+</sup> T cells and of FOXP3<sup>+</sup>Neuropilin<sup>low</sup>CD4<sup>+</sup> T cells, which are  
403 essentially composed of iTreg cells (53), were decreased in the Peyer patches of CD5<sup>-/-</sup> and  
404 CD5tg<sup>Y429F</sup> mice as compared to those in CD5tg<sup>Wt</sup> mice (Fig. S5). We thus hypothesized that  
405 CD5 signaling might be influenced by the environment in which T cells are localized and  
406 that CD5 may operate differently in a non-tolerogenic environment when CD4<sup>+</sup> T cells  
407 encounter foreign antigens or pathogens. To examine this possibility, we first immunized  
408 CD5tg<sup>Wt</sup> and CD5tg<sup>Y429F</sup> mice with a peptide variant (EAWGALANKAVDKA, called 1W1K  
409 peptide hereafter) of the I-E alpha chain immunodominant peptide 52-68 in the presence IFA,  
410 which was shown to favor the polarization of iTreg cells (54). To follow antigen-induced  
411 FOXP3<sup>+</sup> T cells, we stained cells from the draining lymph nodes with 1W1K-pMHCII  
412 tetramer and analyzed FOXP3 expression in tetramer<sup>+</sup>CD4<sup>+</sup> T cells. We found that the  
413 percentages of CD44<sup>+</sup>tetramer<sup>+</sup>CD4<sup>+</sup> T cells were similar in CD5tg<sup>Wt</sup> and CD5tg<sup>Y429F</sup> mice,  
414 indicating that CD5 signaling does not significantly impact the clonal expansion of antigen-  
415 specific CD4<sup>+</sup> T cells in this experimental setting (Fig. 6B). We observed that the percentages  
416 and numbers of tetramer<sup>+</sup>FOXP3<sup>+</sup>CD4<sup>+</sup> T cells were increased in CD5tg<sup>Y429F</sup> mice compared  
417 to those in CD5tg<sup>Wt</sup> mice (Fig. 6C). In comparison, the percentages and numbers of  
418 tetramer<sup>+</sup>T-BET<sup>+</sup>CD4<sup>+</sup> T cells were similar in CD5tg<sup>Wt</sup> and CD5tg<sup>Y429F</sup> mice (Fig. 6D).  
419 Proportions and numbers of tetramer<sup>+</sup>FOXP3<sup>+</sup>Neuropilin<sup>low</sup>CD4<sup>+</sup> T cells were also higher in  
420 CD5tg<sup>Y429F</sup> mice compared to those in CD5tg<sup>Wt</sup> mice, suggesting that CD5 signaling restrains

421 the generation of iTreg cells following immunization with foreign antigens. To confirm this  
422 observation in a more pathophysiological model, we next analyzed whether CD5 signaling  
423 could repress the generation of Treg cells following mice infection with the respiratory virus  
424 influenza A, which was shown to drive important antigen-specific Treg-cell responses (55).  
425 Mice were infected intranasally and pathogen-specific Treg were analyzed with NP311-325-  
426 IA<sup>b</sup> tetramer in the draining lymph nodes 5 days following infection. We found that the  
427 percentages of virus-specific CD4<sup>+</sup> T cells were comparable in CD5tg<sup>Wt</sup> and CD5tg<sup>Y429F</sup> mice,  
428 indicating that CD5 signaling does not influence the overall expansion of antigen-specific  
429 CD4<sup>+</sup> T cell following infection (Fig. 6E). The proportions and numbers of virus-specific  
430 FOXP3<sup>+</sup>CD4<sup>+</sup> T cells and of FOXP3<sup>+</sup>Neuropilin<sup>low</sup>CD4<sup>+</sup> T cells were increased in CD5tg<sup>Y429F</sup>  
431 mice as compared to those in CD5tg<sup>Wt</sup>, showing that CD5 represses the development of those  
432 cells during pathogenic infection (Fig. 6F).

## 433 **Discussion**

434

435 In this study, we identified a multimeric signaling complex which acts through CD5 to limit  
436 the induction of regulatory T cells. This complex is composed of proteins with adaptor  
437 functions (c-CBL, CIN85, CRKL) that connect CD5 to distinct effector proteins (UBASH3A,  
438 SHIP1, PI3K) which acts cooperatively by repressing ERK and promoting AKT activity to  
439 inhibit the transactivation of *Foxp3*. This signalosome is recruited to CD5 Y429 following  
440 TCR engagement, suggesting that it is part of a feedback loop that is differentially engaged  
441 according to the strength of TCR signals that gradually regulates CD5 expression at the cell  
442 surface. It is generally admitted that stimulation of CD4<sup>+</sup> T cells with high dose of strong  
443 agonist exerts a repressive effect on the generation of iTreg cells, presumably because it  
444 promotes strong AKT-mediated signals that prevent the transactivation of *Foxp3* (50). A  
445 molecular model to explain this effect was that strong, but not weak, TCR signals repress the  
446 expression of the PtdIns(3,4,5)P3 phosphatase and tensin homologue (PTEN), which is an  
447 inhibitor of the AKT signaling pathway (56). Our study also suggests that CD4<sup>+</sup> T cells could  
448 finely tune AKT signaling by titrating the surface expression of CD5 to reduce the generation  
449 of iTreg cells upon TCRs stimulation with relatively high dose of high affinity ligands. It was  
450 shown that CD5<sup>hi</sup>CD4<sup>+</sup> T cells respond better than CD5<sup>lo</sup>CD4<sup>+</sup> T cells to diverse foreign  
451 antigens (11). This was explained by the enrichment, in the CD5<sup>hi</sup> population, of CD4<sup>+</sup> T cells  
452 with greater self-reactivity, which exhibit enhanced TCR signaling potential (57). An  
453 additional explanation inferred from our study is that CD5 could act by reducing the  
454 emergence of inopportune antigen-specific Treg cells that might occur during the recognition  
455 of foreign antigens and which may lead to the development of ineffective immune responses.  
456 This interpretation could also bring an explanation to the reduced development of active  
457 autoimmunity observed in CD5 deficient mice models (12).

458

459 The modes of interaction of c-CBL with UBASH3A, SHIP, CIN85, CRKL and PI3K have  
460 been extensively studied particularly in the context of EGF receptor (EGFR) signaling for  
461 which a similar molecular machinery as the one described in this study for CD5 has been  
462 described (36, 58, 59). PI3K can bind directly to c-CBL through a phospho-tyrosine binding-  
463 site located at the C-terminal end of c-Cbl (Y731) (60), or indirectly, through CRKL, which  
464 binds to two phospho-tyrosine binding sites located in the same region (Y700 and Y774) (61).  
465 CIN85 binds directly with SHIP1 and was shown to compete with UBASH3A for binding to  
466 c-CBL, suggesting that two distinct CD5 signaling subsets might co-exist in T cells (62).  
467 Previous studies have shown that CIN85/c-CBL complexes drive EGFR internalization and  
468 degradation in lysosomal compartments whereas UBASH3A/c-CBL complexes prevent these  
469 processes and sustain EGFR-mediated signals (36, 58). Thus, these distinct complexes could  
470 contribute to regulate the turnover of CD5 in addition to their regulatory function on TCR  
471 signaling. Although c-CBL was shown to mediate CD5 ubiquitylation resulting in its  
472 degradation in lysosomes following TCR/CD5 co-cross-linking (63), we found that the  
473 substitution of tyrosine 429 by phenylalanine does not significantly modify CD5 surface  
474 expression either before or after TCR engagement, suggesting that the direct engagement of  
475 CD5 by external ligands might be required for its degradation.

476

477 Several proteins previously identified as CD5-interacting proteins, such as Ras-Gap (22),  
478 SHP-1 (23), Vav1 (25) and ZAP70 (64), were not detected as CD5 interactors in our mass  
479 spectrometry analysis. Although initial studies suggested that SHP-1 interacts with CD5 in  
480 thymocytes (65) and Jurkat cells (23), several studies since then have failed to reproduce this  
481 interaction (22, 66). Moreover, both the phosphatase activity associated to CD5  
482 immunoprecipitates and the reduction in positive selection conferred by CD5 overexpression

483 were shown to be unaffected by SHP-1 deficiency, suggesting that CD5 operates  
484 independently of this phosphatase (67). CK2 (26) and Cbl-b (30), also previously described as  
485 CD5-binding proteins, were recruited to CD5 but not as robustly as other interactors,  
486 suggesting that CD5 could engage these proteins but in other molecular or cellular contexts  
487 than the one used in this study. It was shown that CD5 enhances Th17 responses when cross-  
488 linked to the TCR but not when the TCR is engaged alone (68, 69), suggesting that CD5  
489 could recruit distinct signaling effectors whether or not it is engaged by extracellular ligands.  
490 Accordingly, the deletion of a CK2 binding site on CD5 impairs the polarization of CD4<sup>+</sup> T  
491 cells into Th17 cells following stimulation with antigen-presenting cells but not following  
492 anti-CD3 antibodies (69).

493

494 We show that CD5 exerts a combined effect on TCR signaling, reducing ZAP-70 and ERK  
495 activity, presumably through the joint action of UBASH3A and SHIP1, which are known  
496 regulators of these signaling molecules (40, 44, 45, 70, 71), and enhancing AKT activity,  
497 likely through PI3K, which facilitates the retention of FOXO1 in the cytoplasm and prevents  
498 the consequent transactivation of *Foxp3* (51). This signaling complex does not detectably  
499 modulate the expression of ROR $\gamma$ t or T-BET in activated CD4<sup>+</sup> T cells *in vitro* and *in vivo*  
500 respectively, indicative of a selective effect of CD5 on the generation of iTreg cells.  
501 Remarkably, studies using SHIP1 and PI3K deficient murine models identified repressive  
502 functions for both proteins on the generation of induced Treg cells (72, 73). Whether  
503 UBASH3A is also directly involved in the control of this population has not yet been studied.  
504 However, a recent investigation performed in human T cells has shown that it represses NF $\kappa$ B  
505 signaling (74), a key pathway for the generation of induced Treg cells (75). Thus, CD5 could  
506 act as a scaffold that selectively engages specific signaling effectors involved in the

507 generation of peripheral Treg cells to selectively control the expansion of this population  
508 during immune responses.

509

510 Previous studies have shown that CD5 deficiency on the BALB/c but not on the C57BL/6  
511 genetic background leads to increase numbers of thymic Treg cells, suggesting that CD5 may  
512 also repress the generation of these cells according to the molecular context in which CD5  
513 operates in the thymus (13, 14). CD5 is highly expressed at the surface of thymic Treg cells,  
514 indicating that the ability of CD5 to negatively regulate FoxP3 expression in the thymus could  
515 also be overcome by additional signals (such as high TCR affinity for self-ligand) that impose  
516 FoxP3 expression despite the opposite force exerted by CD5. We thus speculate that CD5  
517 mediated inhibition may be sufficient to block Foxp3 expression and Treg lineage  
518 commitment in thymocytes that express TCRs that bind with intermediate affinity to self-  
519 ligands preventing their commitment to the Treg lineage. Also, co-receptors such as GITR,  
520 OX40 and TNFR2 are highly expressed by thymic Treg progenitors and were also shown to  
521 trigger co-stimulatory signals that induce thymic Treg differentiation (76). By contrast, these  
522 co-receptors are poorly expressed by naïve peripheral CD4 T cells suggesting that the  
523 negative effect of CD5 on FoxP3 expression may become predominant in this subset.

524

525 A recent study reported an effect of CD5 opposite to that shown in our study on the  
526 generation of peripherally-induced Treg cells in a mouse model in which tolerance to EAE is  
527 induced by direct delivery of encephalitogenic peptides to antigen-presenting dendritic cells in  
528 the absence of adjuvant (15). Those authors found that CD5 prevents the inhibition of Treg-  
529 cell induction potentially mediated by effector cell cytokines such as IL-6, IL-4 and IFN $\gamma$ .  
530 Although they do not provide a clear signaling mechanism by which CD5 operates here, they  
531 show that CD5 promotes the generation of Treg cells by inhibiting IL-6-mediated AKT

532 signaling leading to mTOR activation. One potential explanation for the apparent discrepancy  
533 between those results and our current findings is that CD5 might exert opposite effects on  
534 PI3K/ AKT signaling in tolerized and non-tolerized T cells. A recent proteomic study showed  
535 that CD5 interacts with both c-CBL and CBL-b in peripheral CD4<sup>+</sup> T cells (30), suggesting  
536 that distinct pools of CD5, connected either to a c-CBL or CBL-b signalosome, could be  
537 preferentially assembled under specific stimulation conditions. Whereas c-CBL has known  
538 positive effects on PI3K/AKT signaling (46), CBL-b blocks this pathway by inducing PI3K  
539 ubiquitylation (77) and by blocking Nedd4-mediated ubiquitylation of PTEN (78). Because  
540 CBL-b expression is up-regulated in CD4<sup>+</sup> T cells after tolerizing signals(79) and degraded in  
541 CD4<sup>+</sup> T cells that have received appropriate co-stimulatory signals (80), we speculate that  
542 CD5 signaling pools may vary in size or composition, tuning up or down the PI3K/AKT  
543 signaling axis according to the immunological context in which cells are stimulated.

544

545 Altogether the findings of our study suggest that CD5 engages a polymorphic signaling  
546 machinery that can transduce both stimulatory and inhibitory signals to selectively control  
547 specific T-cell responses depending on the immunological context. Our results also provide  
548 new insights into the paradigm of co-receptor signaling suggesting that, in addition to  
549 providing classical enhancing or dampening inputs, co-receptors coordinate TCR signals that  
550 may have antagonist effects to promote specific functional outcomes, such as the generation  
551 of iTreg cells.

552

553 **Materials and Methods**

554

555 Full details of materials and methods, including mice, antibodies, cell stimulation,  
556 immunoprecipitations, mass spectrometry analysis, calcium flux, Western blot, immunization  
557 and influenza virus infection, statistical analysis are provided in the SI Appendix.

558

559 **Data availability statement**

560 The data generated or analyzed during this study are included in the published article and its  
561 supplementary information appendix or dataset files. The mass spectrometry proteomics data  
562 have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository  
563 with the dataset identifier PXD017343. Raw data from figures 1A/S1A, S1B, 1C/2A/2F,  
564 2B/S1C can be respectively found in Datasets S1, S2, S3 and S5.

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579

580 The authors declare no competing interest.

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783

784 **Figures legends**

785

786 **Figure 1: Mass spectrometry analysis of CD5 interactome.** (A): Schematic of those  
787 proteins that preferentially interact with CD5 in thymocytes stimulated with pervanadate for 1  
788 minute. Layouts indicate the classification of these proteins according to their function (pink  
789 layout: TCR signaling; blue layout: AP2 complex; green layout: unknown function in T cell  
790 signaling). Data represent eight independent experiments with n=8 mice per group (n=8). (B)  
791 Thymocytes and peripheral CD4<sup>+</sup> T cells from *Cd5*<sup>+/+</sup> (+/+) or *Cd5*<sup>-/-</sup> (-/-) mice were  
792 stimulated with pervanadate for 5 minutes. Samples were then subjected to  
793 immunoprecipitation (IP) with antibodies specific for CD5 and then analyzed by Western  
794 blotting with antibodies specific for the indicated proteins. (C) Relative abundance of  
795 principal CD5 interacting partners after co-immunoprecipitation in thymocytes either non-  
796 stimulated (black bars) or stimulated with anti-CD3 and anti-CD4 antibodies (blue bars) or  
797 pervanadate (red bars). Protein abundances were estimated using the LFQ metric calculated  
798 by MaxQuant based on MS peptide signals intensities. Data are means ± SD of three mass  
799 spectrometry measurement and are representative of three independent experiments  
800 containing n=1 mouse per group.

801 **Figure 2: c-CBL is recruited to CD5 Y429 and is connecting CD5 with its signaling**  
802 **partners.** (A): MS analysis of CD5 phosphorylation sites. Thymocytes from WT mice were  
803 left unstimulated (Unstim.) or were stimulated with anti-CD3 + anti-CD4 antibodies or  
804 pervanadate (Perva.) for one minute. Samples were then subjected to immunoprecipitation  
805 (IP) with antibodies specific for CD5. MS intensity of peptide ions containing the three main  
806 phosphotyrosine residues of the protein were used to calculate for each site the percentage of  
807 MS signal deriving from modified phosphotyrosine forms. MS intensity values of  
808 phosphorylated ions were averaged for mass spectrometry replicates measurements of each

809 sample. Data are the means  $\pm$  SD and represent three independent experiments containing n=1  
810 mouse per group. (B) Volcano plots ( $-\log_{10}$ (Student t-test p-value) versus  $\log_2$ (fold change))  
811 showing abundances difference of proteins identified by MS analysis of samples obtained by  
812 immunoprecipitation of CD5 from cellular extracts of thymocytes stimulated with  
813 pervanadate. Left: comparison CD5tg<sup>Wt</sup> versus *Cd5*<sup>-/-</sup> thymocytes (n=6 independent samples);  
814 right: comparison CD5tg<sup>Wt</sup> versus *Cd5*<sup>-/-</sup> thymocytes (n=5 independent samples). Protein  
815 labeled in red show significant enrichment in CD5tg<sup>Wt</sup> samples compared to control *Cd5*<sup>-/-</sup>  
816 samples (fold change>2 and p<0.05). The same proteins plotted in red show no significant  
817 enrichment in CD5tg<sup>Y429F</sup> samples versus controls. Bar graph (insert) in the CD5tg<sup>Y429F</sup>  
818 volcano plot represent the MS intensity ( $\log_{10}$ ) of CD5 phosphorylated peptides containing  
819 the Y463 of CD5. (C) Thymocytes from CD5tg<sup>Wt</sup> or CD5tg<sup>Wt</sup> mice were stimulated with anti-  
820 CD3 + anti-CD4 for 1 minute. Samples were then subjected to immunoprecipitation (IP) with  
821 antibodies specific for CD5 and then analyzed by Western blotting with antibodies specific  
822 for the indicated proteins. (D)(E) CD5 was immunoprecipitated from cellular extracts of total  
823 thymocytes from the indicated genotypes stimulated with pervanadate. Samples were  
824 analyzed by mass spectrometry (D) or Western blot (E). Abundance of specific interacting  
825 partners was estimated based on MS signal intensity. Data are means  $\pm$  SD from at least two  
826 mass spectrometry measurements and are representative of two experiments containing n=1  
827 mouse per group. Western blots are representative of two independent experiments. WCL:  
828 whole cell lysate. Unpaired two-tailed Mann-Whitney *t* test. \*p<0.05; \*\*p<0.001.

829

830 **Figure 3: CD5 coordinates TCR signals in thymocytes and peripheral T cells.** (A)  
831 Analysis of calcium flux in DP thymocytes from CD5tg<sup>Wt</sup> and CD5tg<sup>Y429F</sup> mice stimulated  
832 with anti-CD3 antibodies at 5 or 10  $\mu$ g/ml (top and bottom graph respectively) in presence of  
833 anti-CD4 antibodies. Red arrows indicate maximum responses following stimulation with

834 ionomycine. Bar graphs represents the area under the curve and the maximum pic intensity of  
835 calcium flux normalized to the values obtain in DP thymocytes from CD5tg<sup>Wt</sup> mice. Data are  
836 means  $\pm$  SD and are representative of two independent experiments with n=1-2 mice per  
837 group. (B) Thymocytes from CD5tg<sup>Wt</sup>, CD5tg<sup>Y429F</sup> and *Cd5*<sup>-/-</sup> mice were stimulated with anti-  
838 CD3 + anti-CD4 antibodies for the indicated times. Total cytoplasmic extracts of the cells  
839 were then analyzed by Western blotting with antibodies against phosphorylated forms of  
840 SLP76, ERK, P38, LCK and AKT. Curves show the relative abundances of the indicated  
841 phosphorylated proteins as determined by calculating the ratios of the intensities of the bands  
842 corresponding to the phosphorylated proteins to those corresponding to glyceraldehyde-3-  
843 phosphate dehydrogenase (GAPDH), the loading control. The y axes represent means  $\pm$  SD of  
844 the relative values calculated after normalization to the highest value in the CD5tg<sup>Wt</sup> cells.  
845 Data are representative of three to five independent experiments each including one mouse of  
846 the indicated genotype. (C)(D) Peripheral CD4<sup>+</sup> T cells from CD5tg<sup>Wt</sup>, CD5tg<sup>Y429F</sup> and *Cd5*<sup>-/-</sup>  
847 mice were stimulated with anti-CD3 antibodies for the indicated times. Total cytoplasmic  
848 extracts of the cells were then analyzed by Western blotting with antibodies against  
849 phosphorylated forms of SLP76, ERK, P38, ZAP-70, PI3K, SHIP1 and AKT. Curves or  
850 values next to the blots show the relative abundances of the indicated phosphorylated proteins  
851 calculated as in (B). Data are representative of two to four independent experiments each  
852 including one mouse of the indicated genotype. Unpaired two-tailed *t* test. \**p*<0.05;  
853 \*\**p*<0.001; \*\*\**p*<0.0001.

854

855 **Figure 4: The CD5 signalosome negatively regulates the activation and the proliferation**  
856 **of CD4<sup>+</sup> T cells.** (A) Thymocytes from CD5tg<sup>Wt</sup>, CD5tg<sup>Y429F</sup> and *Cd5*<sup>-/-</sup> mice were stimulated  
857 or not with anti-CD3 and anti-CD28 antibodies for four hours. Nur77 protein abundance was  
858 then analyzed by flow cytometry in DP and CD4-SP cells after intracytoplasmic staining with

859 anti-Nur77 antibodies. Bar graphs represents the ratio of mean fluorescent intensities (MFI) of  
860 Nur77 in stimulated cells to unstimulated cells. Data are means  $\pm$  SD and are representative of  
861 two independent experiments with n=3 mice per group. (B) Peripheral CD4<sup>+</sup> T cells from  
862 CD5tg<sup>Wt</sup>, CD5tg<sup>Y429F</sup> and *Cd5*<sup>-/-</sup> mice were stimulated with the indicated doses of anti-CD3  
863 antibodies alone (curves) or with anti-CD28 antibodies (bar graphs) for 24 hours. Curves and  
864 bar graphs represents the ratio of mean fluorescent intensities (MFI) of CD25 in stimulated  
865 cells to unstimulated cells. Data are mean  $\pm$  SD and represent, for anti-CD3 alone, four  
866 independent experiments with n=12 mice per group or, for anti-CD3 + anti-CD28, two  
867 experiments with n=4 mice per group. (C) Peripheral CD4<sup>+</sup> T cells from CD5tg<sup>Wt</sup>, CD5tg<sup>Y429F</sup>  
868 and *Cd5*<sup>-/-</sup> mice were stimulated with the indicated doses of anti-CD3 antibodies for 24 hours.  
869 Curve graph represents the percentages of CD69<sup>+</sup> cells. Data are mean  $\pm$  SD and represent  
870 three independent experiments with n=4-8 mice per group. (D)(E) Peripheral CD4<sup>+</sup> T cells  
871 from CD5tg<sup>Wt</sup>, CD5tg<sup>Y429F</sup> and *Cd5*<sup>-/-</sup> mice previously stained with cell trace violet (CTV)  
872 were stimulated with anti-CD3 antibodies alone (D) or with anti-CD4 antibodies (E) for 72  
873 hours. Curve graphs represent the percentages (D) or the numbers (E) of CTV<sup>low</sup> CD4<sup>+</sup> T cells  
874 following stimulation with variable doses of anti-CD3 antibodies for 72 hours. Data are mean  
875  $\pm$  SD and represent three independent experiments with n=7-11 mice per group. Unpaired  
876 two-tailed Mann-Whitney *t* test. \*p<0.05; \*\*p<0.001; \*\*\*p<0.0001.

877

878 **Figure 5: CD5 signaling restrains the generation of induced Treg cells by promoting**  
879 **AKT-mediated inhibition of FOXO1 and repressing ERK activity.** (A) Naïve  
880 CD62L<sup>hi</sup>CD25<sup>lo</sup> CD4<sup>+</sup> T cells from CD5tg<sup>Wt</sup>, CD5tg<sup>Y429F</sup> and *Cd5*<sup>-/-</sup> mice were stimulated  
881 with anti-CD3 antibodies in presence of TFG $\beta$  for three days. Contour plots represent CD4  
882 versus FOXP3 staining profiles of CD4<sup>+</sup> T cells from the indicated genotypes. Curve graphs  
883 represent the percentages and numbers of FOXP3<sup>+</sup> CD4<sup>+</sup> T cells from mice of the indicated

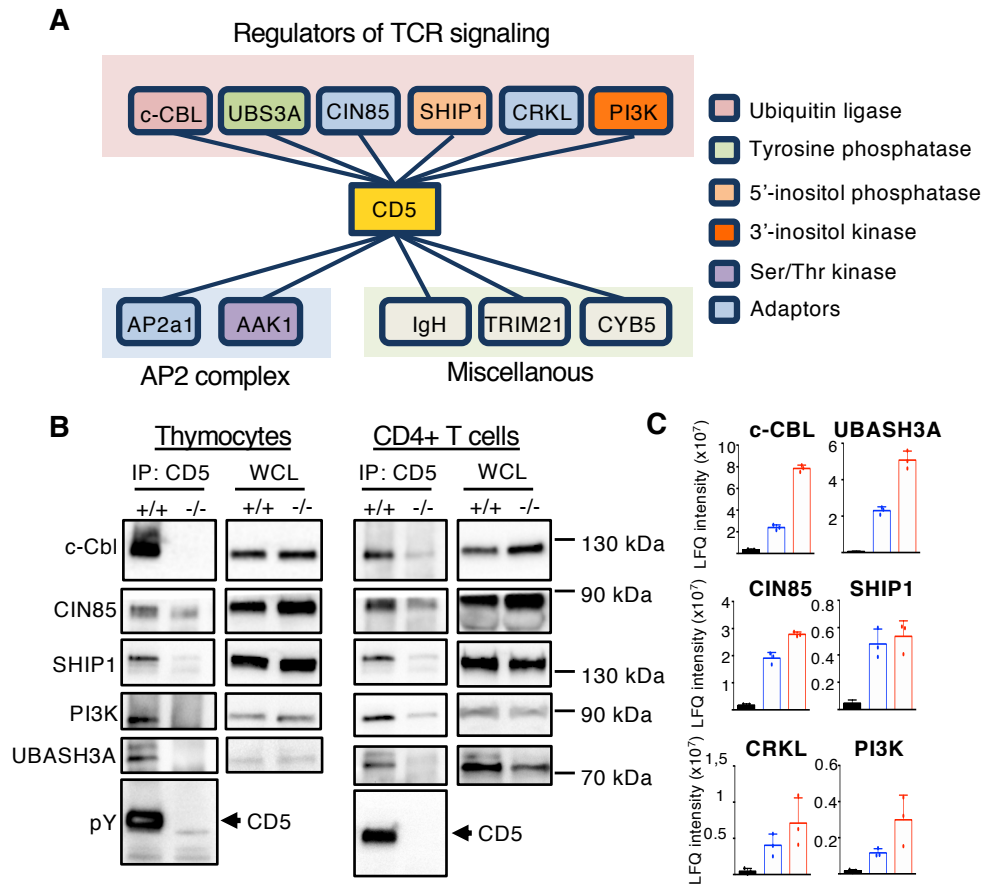
884 genotypes stimulated with the indicated doses of anti-CD3 antibodies in presence of TGF- $\beta$ .  
885 Data are means  $\pm$  SD and represent two independent experiments with n=6-9 mice per group.  
886 (B) Naïve CD62L<sup>hi</sup>CD25<sup>lo</sup> CD4<sup>+</sup> T cells from CD5tg<sup>Wt</sup>, CD5tg<sup>Y429F</sup> and *Cd5*<sup>-/-</sup> mice were  
887 stimulated with anti-CD3 antibodies in presence of TGF- $\beta$  and IL-6 for five days. Contour  
888 plots represent CD4 versus ROR $\gamma$ t staining profiles of CD4<sup>+</sup> T cells from the indicated  
889 genotypes. Bar graphs represent the percentages of ROR $\gamma$ t<sup>+</sup> and IL-17<sup>+</sup> CD4<sup>+</sup> T cells from  
890 mice of the indicated genotypes stimulated with the indicated doses of anti-CD3 antibodies in  
891 presence of TGF- $\beta$  and IL-6. Data are means  $\pm$  SD and represent two independent  
892 experiments with n=3 (0.5  $\mu$ g/ml anti-CD3) and n=6 (3  $\mu$ g/ml anti-CD3) mice per group. (C)  
893 Peripheral CD4<sup>+</sup> T cells from CD5tg<sup>Wt</sup>, CD5tg<sup>Y429F</sup> and *Cd5*<sup>-/-</sup> mice were stimulated or not  
894 (NS) with anti-CD3 antibodies for the indicated times. Total cytoplasmic extracts of the cells  
895 were then analyzed by Western blotting with antibodies against phosphorylated forms of  
896 FOXO1 and ERK. Western blots are representative of two independent experiments. (D)  
897 Naïve CD62L<sup>hi</sup>CD25<sup>lo</sup> CD4<sup>+</sup> T cells from CD5tg<sup>Wt</sup> and CD5tg<sup>Y429F</sup> mice were stimulated with  
898 anti-CD3 antibodies in presence of TGF $\beta$  with or without variable doses of FOXO1 inhibitors  
899 for three days. The expression FOXP3 was then analyzed by flow cytometry after  
900 intracytoplasmic staining of the cells with anti-FOXP3 antibodies. Bar graphs represent the  
901 numbers of FOXP3<sup>+</sup> (left) and FOXP3<sup>-</sup> CD4<sup>+</sup> T cells from mice of the indicated genotypes  
902 stimulated with anti-CD3 antibodies in presence of TGF- $\beta$  with or without the indicated doses  
903 of FOXO1 inhibitors. Data are means  $\pm$  SD and represent two experiments with n=6 mice per  
904 group. Unpaired two-tailed Mann-Whitney *t* test except for figures 5D where paired *t* test  
905 with Welch's correction was performed. \*p<0.05.

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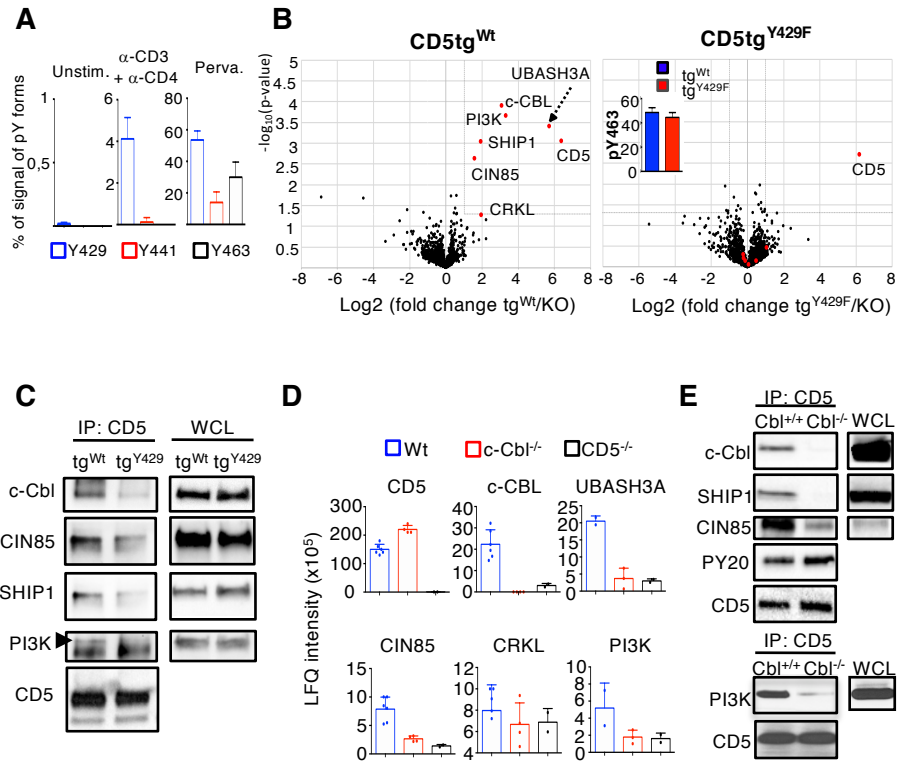
907 **Figure 6: CD5-mediated signaling restrain the generation of peripherally induced Treg**  
908 **cells following immunization with foreign antigens and viral infection.** (A) CD5<sup>lo</sup> and

909 CD5<sup>hi</sup> naïve CD62L<sup>hi</sup>CD25<sup>lo</sup> CD4<sup>+</sup> T cells from C57BL/6 mice were sorted prior to  
910 stimulation with anti-CD3 antibodies in presence of TFG- $\beta$  for three days. Histograms show  
911 the sorting strategy. Contour plots represent CD4 versus FOXP3 staining profiles of CD5<sup>lo</sup>  
912 and CD5<sup>hi</sup> CD4<sup>+</sup> T cells. Bar graphs represent the percentages of FOXP3<sup>+</sup> CD4<sup>+</sup> T cells  
913 following stimulation with the indicated doses of anti-CD3 antibodies in presence of TGF- $\beta$ .  
914 Data are means  $\pm$  SD and represent two independent experiments with n=7 mice per group.  
915 (B)(C)(D) CD5tg<sup>Wt</sup> and CD5tg<sup>Y429F</sup> mice were immunized with 1W1K peptide in presence of  
916 incomplete Freund's adjuvant (IFA). Draining lymph nodes were collected after seven days  
917 and analyzed by flow cytometry after staining of the cells with 1W1K-conjugated MHC  
918 tetramers. (E)(F) CD5tg<sup>Wt</sup> and CD5tg<sup>Y429F</sup> mice were intranasally infected with PR8 virus.  
919 Draining lymph nodes were collected after five days and analyzed by flow cytometry after  
920 staining of the cells with NP311-325-conjugated MHC tetramers. (B)(E) Contour plots  
921 represents CD44 versus tetramer staining profile on gated CD4<sup>+</sup> T cells. Bar graphs represent  
922 the percentages and absolute numbers of tetramer<sup>+</sup>CD44<sup>+</sup> T cells among CD4<sup>+</sup> T cells. (C)(F)  
923 Contour plots represents FoxP3 versus Neuropilin1 (Nrp1) staining profile on gated  
924 tetramer<sup>+</sup>CD44<sup>+</sup> CD4<sup>+</sup> T cells. Bar graphs represent the percentages and absolute numbers of  
925 either FoxP3<sup>+</sup> (solid gate) or FoxP3<sup>+</sup> neuropilin<sup>low</sup> (dashed gate) among tetramer<sup>+</sup>CD44<sup>+</sup>  
926 CD4<sup>+</sup> T cells. Data are representative of two independent experiments and are means  $\pm$  SD of  
927 at least ten mice for each genotype. Non-parametric t-test \*p<0.05; \*\*p<0.001.

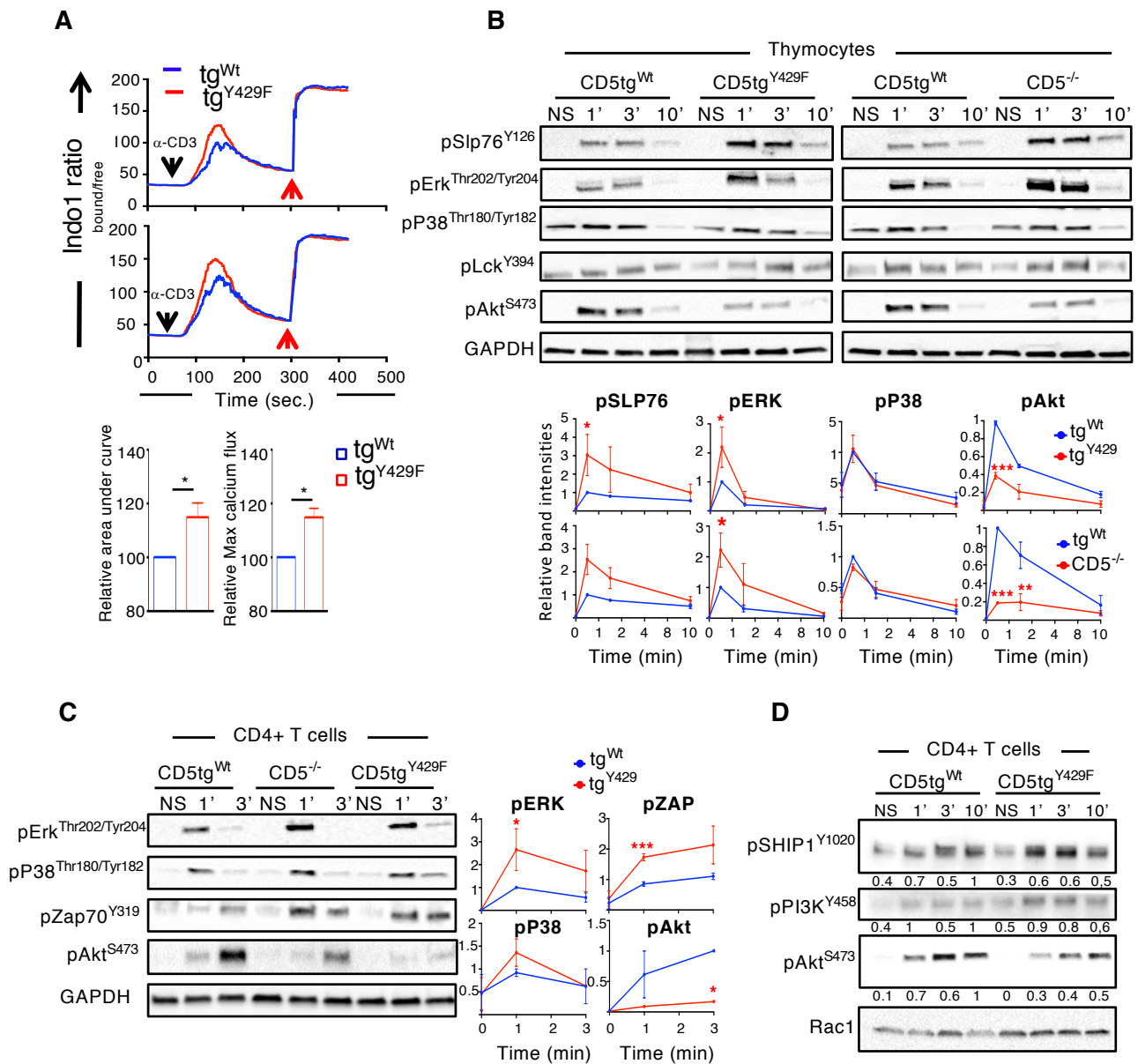
**Figure 1**



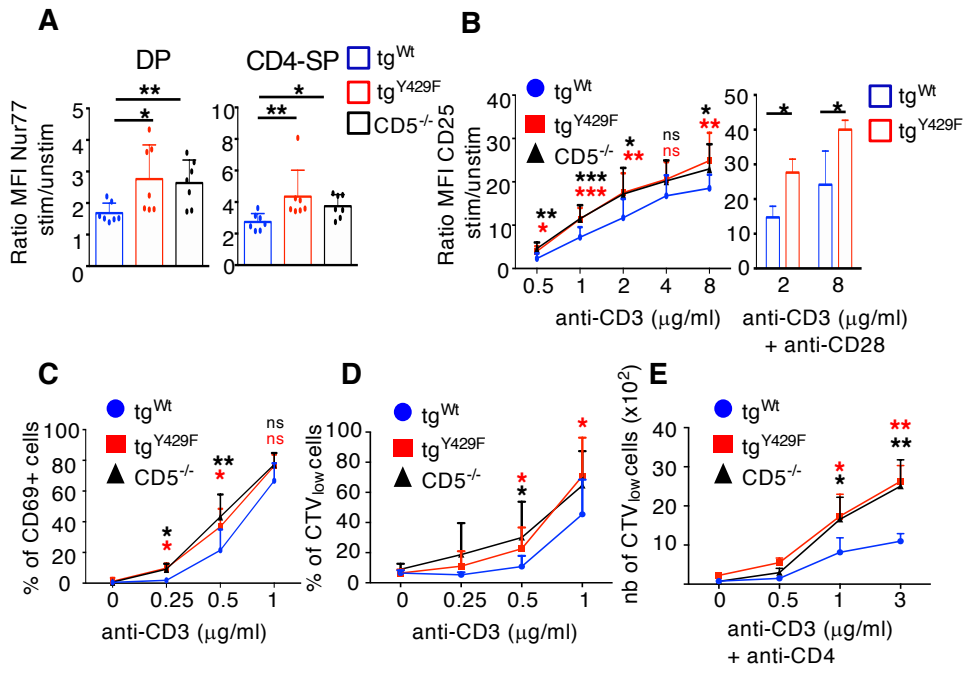
**Figure 2**



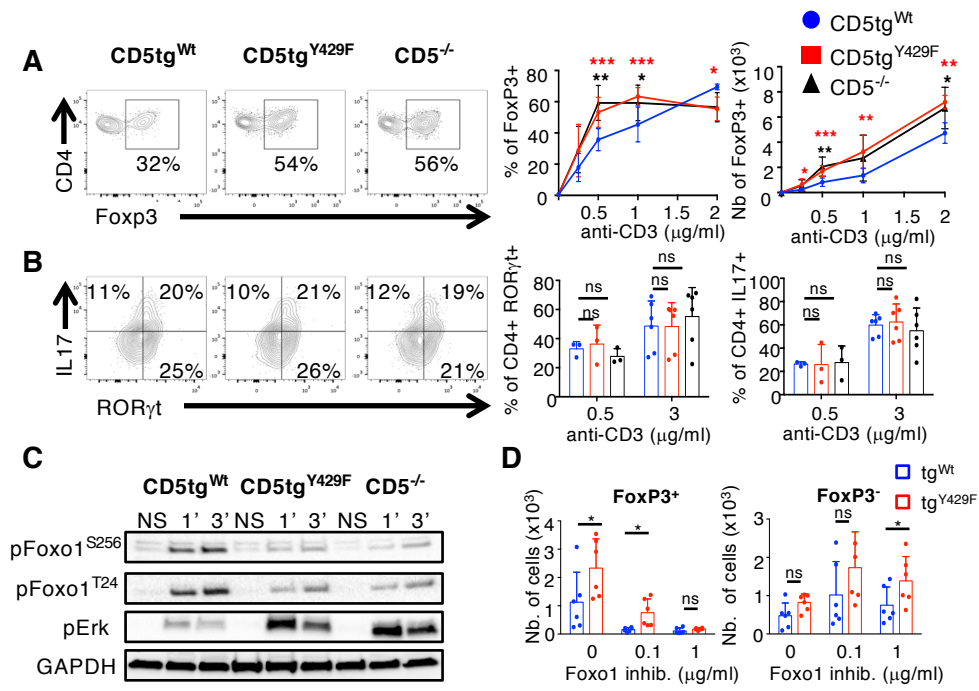
**Figure 3**



**Figure 4**



**Figure 5**



**Figure 6**

