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

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Abstract

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

Keywords

T cells / Signaling / Co-receptors

Significance statement

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

Introduction

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

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

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

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

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

Results

Mass-spectrometry analysis of the CD5 interactome

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

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

To estimate the relative abundance of these signaling partners in the immunoprecipitated samples, we used the intensity-based absolute quantification (iBAQ) metric, which corresponds to the sum of all of the peptide intensities divided by the number of theoretically observable tryptic peptides of a protein. Analysis of these interactions (with normalized iBAQ intensities) shows that UBASH3A (IBAQ=604 x 10⁴) and c-CBL (IBAQ=560 x 10⁴) are more abundantly recruited to CD5 than CIN85 (IBAQ=147 x 10⁴), CRKL (IBAQ=143 x 10⁴), SHIP1 (IBAQ=55 x 10⁴) and PI3K (IBAQ=18 x 10⁴), suggesting an essential role for c-Cbl and UBASH3A in CD5-mediated regulation of T-cell activation (*SI Appendix*, Fig. S1A). Analysis by Western blot showed that CD5 interacts with c-CBL, UBASH3A, SHIP1, CIN85 and PI3K both in thymocytes and in peripheral CD4⁺ T cells, suggesting that CD5 engages similar signaling processes upon T cell development and primary T cell responses (Fig. 1B). Further analysis showed that c-CBL, UBASH3A, SHIP1, CRKL, CIN85 and PI3K interacted poorly with CD5 in resting cells but were recruited to CD5 upon TCR+CD4 cross-linking (Fig. 1C and Dataset S3), suggesting that CD5 contributes to TCR signaling through the activity of these proteins. Note that RAS-GAP (22), SHP-1 (23), CBL-b (30) and CK2 (27), which were previously reported to interact with CD5 in thymocytes or in T-cell lines, were either undetected (Ras-GAP), detected at the same level in immunoprecipitated samples from WT and *Cd5*^{-/-} controls (SHP-1), or inconsistently detected across biological replicates without a statistically significant enrichment ratio in immunoprecipitated samples versus controls (CBL-b and CK2). Thus, these proteins were not selected in the list of major CD5-interacting proteins (Dataset S1).

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

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

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

225 or *Cd5*^{-/-}; CD5tg^{Y429F}, hereafter designated CD5tg^{Wt} or CD5tg^{Y429F}, respectively). We verified
226 that the WT and Y429F CD5 transgenes were similarly expressed in thymocyte subsets and in
227 peripheral CD4⁺ and CD8⁺ 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^{Wt} and CD5tg^{Y429F} thymocytes (Fig S2B). Moreover, the proportions and numbers of
230 thymocytes and peripheral T cells in each subset were similar in CD5tg^{Wt}, CD5tg^{Y429F} and
231 *Cd5*^{-/-} mice (*SI Appendix*, Fig. S2C and S2D). Percentages of FOXP3⁺ 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^{Wt} 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^{Wt} and CD5^{Y429F} (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^{Y429F} interactome was drastically decreased compared to
246 CD5tg^{Wt}, and nearly comparable to those detected in *Cd5*^{-/-} 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).

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*^{-/-} and *Cd5*^{-/-} 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*^{-/-} 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*^{-/-} thymocytes compared to those in wild-type thymocytes and were similar to those observed in *Cd5*^{-/-} 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.

CD5 integrates both co-stimulatory and co-inhibitory signaling

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

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

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

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

CD5-mediated signaling negatively regulates CD4⁺ T-cell activation and restrains the generation of regulatory T cells induced by foreign antigens

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

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

To address this possibility, we first compared FOXP3 expression in CD5tg^{Wt}, CD5tg^{Y429F} and *Cd5*^{-/-} CD4⁺ T cells stimulated with different doses of anti-CD3 antibodies in presence of TGF- β . We found that the percentages of CD5tg^{Y429F} and *Cd5*^{-/-} CD4⁺ T cells expressing FOXP3 were enhanced, compared to that in CD5tg^{Wt} CD4⁺ T cells (Fig. 5A), upon weak but not high TCR stimulations, suggesting that CD5 may contribute to enhance the threshold at which the development of iTreg is engaged. By comparison, we found that CD5tg^{Wt}, CD5tg^{Y429F} and *Cd5*^{-/-} CD4⁺ T cells differentiated similarly into ROR γ ⁺IL-17⁺ cells following TCR stimulation under Th17 polarizing conditions (Fig. 5B). Numbers of CD5tg^{Y429F} and *Cd5*^{-/-} FOXP3⁺ cells were enhanced independently of the concentration of anti-CD3 antibodies (Fig. 5A), suggesting that CD5 operates both by controlling the ability of CD4⁺ T

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

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

efficiently into FOXP3⁺ cells than CD5^{hi}CD4⁺ T cells under identical TCR stimulation conditions, suggesting that physiologically high levels of CD5 on naïve CD4⁺ T cells might reduce the ability of these cells to differentiate into Treg cells upon antigenic recognition (Fig. 6A). Contrasting with those results, previous studies suggested that CD5 promotes, rather than represses, the differentiation of peripherally-induced Treg cells when those cells are generated in a tolerogenic context such as the gut mucosa (15). Accordingly, we found that the percentages of FOXP3⁺CD4⁺ T cells and of FOXP3⁺Neuropilin^{low}CD4⁺ T cells, which are essentially composed of iTreg cells (53), were decreased in the Peyer patches of CD5^{-/-} and CD5tg^{Y429F} mice as compared to those in CD5tg^{Wt} mice (Fig. S5). We thus hypothesized that CD5 signalosome might be influenced by the environment in which T cells are localized and that CD5 may operate differently in a non-tolerogenic environment when CD4⁺ T cells encounter foreign antigens or pathogens. To examine this possibility, we first immunized CD5tg^{Wt} and CD5tg^{Y429F} mice with a peptide variant (EAWGALANKAVDKA, called 1W1K peptide hereafter) of the I-E alpha chain immunodominant peptide 52-68 in the presence IFA, which was shown to favor the polarization of iTreg cells (54). To follow antigen-induced FOXP3⁺ T cells, we stained cells from the draining lymph nodes with 1W1K-pMHCII tetramer and analyzed FOXP3 expression in tetramer⁺CD4⁺ T cells. We found that the percentages of CD44⁺tetramer⁺CD4⁺ T cells were similar in CD5tg^{Wt} and CD5tg^{Y429F} mice, indicating that CD5 signaling does not significantly impact the clonal expansion of antigen-specific CD4⁺ T cells in this experimental setting (Fig. 6B). We observed that the percentages and numbers of tetramer⁺FOXP3⁺CD4⁺ T cells were increased in CD5tg^{Y429F} mice compared to those in CD5tg^{Wt} mice (Fig. 6C). In comparison, the percentages and numbers of tetramer⁺T-BET⁺CD4⁺ T cells were similar in CD5tg^{Wt} and CD5tg^{Y429F} mice (Fig. 6D). Proportions and numbers of tetramer⁺FOXP3⁺Neuropilin^{low}CD4⁺ T cells were also higher in CD5tg^{Y429F} mice compared to those in CD5tg^{Wt} 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^b tetramer in the draining lymph nodes 5 days following infection. We found that the
427 percentages of virus-specific CD4⁺ T cells were comparable in CD5tg^{Wt} and CD5tg^{Y429F} mice,
428 indicating that CD5 signaling does not influence the overall expansion of antigen-specific
429 CD4⁺ T cell following infection (Fig. 6E). The proportions and numbers of virus-specific
430 FOXP3⁺CD4⁺ T cells and of FOXP3⁺Neuropilin^{low}CD4⁺ T cells were increased in CD5tg^{Y429F}
431 mice as compared to those in CD5tg^{Wt}, showing that CD5 represses the development of those
432 cells during pathogenic infection (Fig. 6F).

Discussion

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

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

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

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

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

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

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

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

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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The authors declare no competing interest.

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Figures legends

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

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

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

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

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

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

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

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

genotypes stimulated with the indicated doses of anti-CD3 antibodies in presence of TGF- β . Data are means \pm SD and represent two independent experiments with n=6-9 mice per group. (B) Naïve CD62L^{hi}CD25^{lo} CD4⁺ T cells from CD5tg^{Wt}, CD5tg^{Y429F} and *Cd5*^{-/-} mice were stimulated with anti-CD3 antibodies in presence of TGF- β and IL-6 for five days. Contour plots represent CD4 versus ROR γ t staining profiles of CD4⁺ T cells from the indicated genotypes. Bar graphs represent the percentages of ROR γ t⁺ and IL-17⁺ CD4⁺ T cells from mice of the indicated genotypes stimulated with the indicated doses of anti-CD3 antibodies in presence of TGF- β and IL-6. Data are means \pm SD and represent two independent experiments with n=3 (0.5 μ g/ml anti-CD3) and n=6 (3 μ g/ml anti-CD3) mice per group. (C) Peripheral CD4⁺ T cells from CD5tg^{Wt}, CD5tg^{Y429F} and *Cd5*^{-/-} mice were stimulated or not (NS) with anti-CD3 antibodies for the indicated times. Total cytoplasmic extracts of the cells were then analyzed by Western blotting with antibodies against phosphorylated forms of FOXO1 and ERK. Western blots are representative of two independent experiments. (D) Naïve CD62L^{hi}CD25^{lo} CD4⁺ T cells from CD5tg^{Wt} and CD5tg^{Y429F} mice were stimulated with anti-CD3 antibodies in presence of TGF β with or without variable doses of FOXO1 inhibitors for three days. The expression FOXP3 was then analyzed by flow cytometry after intracytoplasmic staining of the cells with anti-FOXP3 antibodies. Bar graphs represent the numbers of FOXP3⁺ (left) and FOXP3⁻ CD4⁺ T cells from mice of the indicated genotypes stimulated with anti-CD3 antibodies in presence of TGF- β with or without the indicated doses of FOXO1 inhibitors. Data are means \pm SD and represent two experiments with n=6 mice per group. Unpaired two-tailed Mann-Whitney *t* test except for figures 5D where paired *t* test with Welch's correction was performed. *p<0.05.

Figure 6: CD5-mediated signaling restrain the generation of peripherally induced Treg cells following immunization with foreign antigens and viral infection. (A) CD5^{lo} and

909 CD5^{hi} naïve CD62L^{hi}CD25^{lo} CD4⁺ T cells from C57BL/6 mice were sorted prior to
 910 stimulation with anti-CD3 antibodies in presence of TFG- β for three days. Histograms show
 911 the sorting strategy. Contour plots represent CD4 versus FOXP3 staining profiles of CD5^{lo}
 912 and CD5^{hi} CD4⁺ T cells. Bar graphs represent the percentages of FOXP3⁺ CD4⁺ T cells
 913 following stimulation with the indicated doses of anti-CD3 antibodies in presence of TGF- β .
 914 Data are means \pm SD and represent two independent experiments with n=7 mice per group.
 915 (B)(C)(D) CD5tg^{Wt} and CD5tg^{Y429F} 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^{Wt} and CD5tg^{Y429F} 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⁺ T cells. Bar graphs represent
 922 the percentages and absolute numbers of tetramer⁺CD44⁺ T cells among CD4⁺ T cells. (C)(F)
 923 Contour plots represents FoxP3 versus Neuropilin1 (Nrp1) staining profile on gated
 924 tetramer⁺CD44⁺ CD4⁺ T cells. Bar graphs represent the percentages and absolute numbers of
 925 either FoxP3⁺ (solid gate) or FoxP3⁺ neuropilin^{low} (dashed gate) among tetramer⁺CD44⁺
 926 CD4⁺ 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

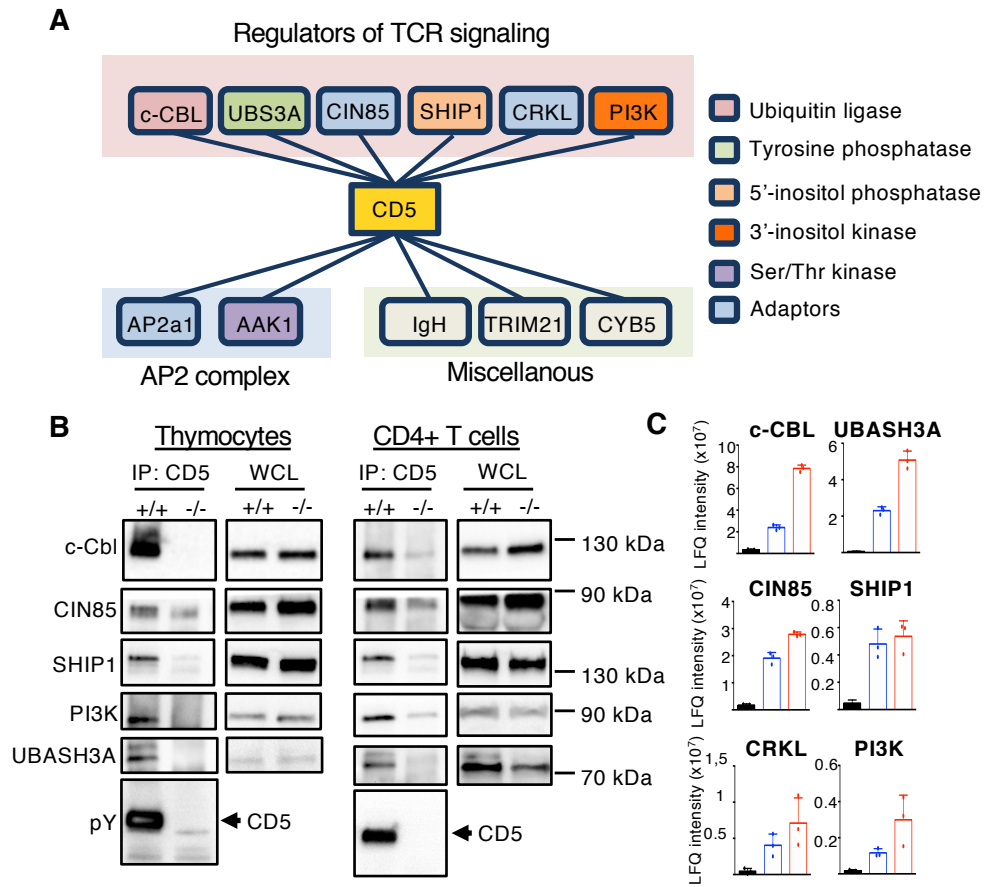


Figure 2

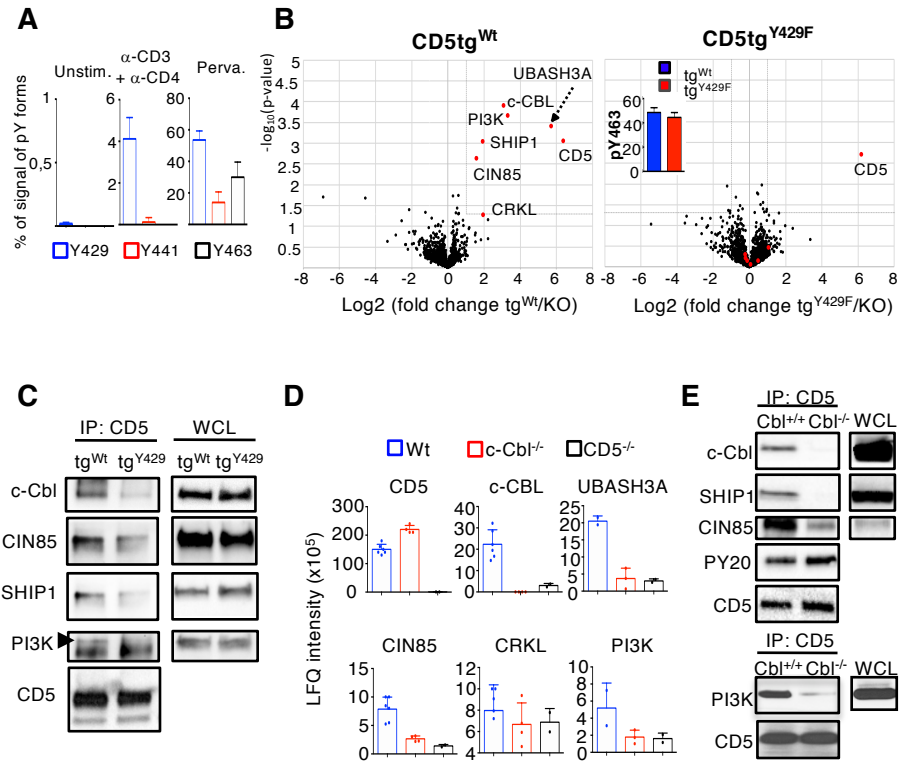


Figure 3

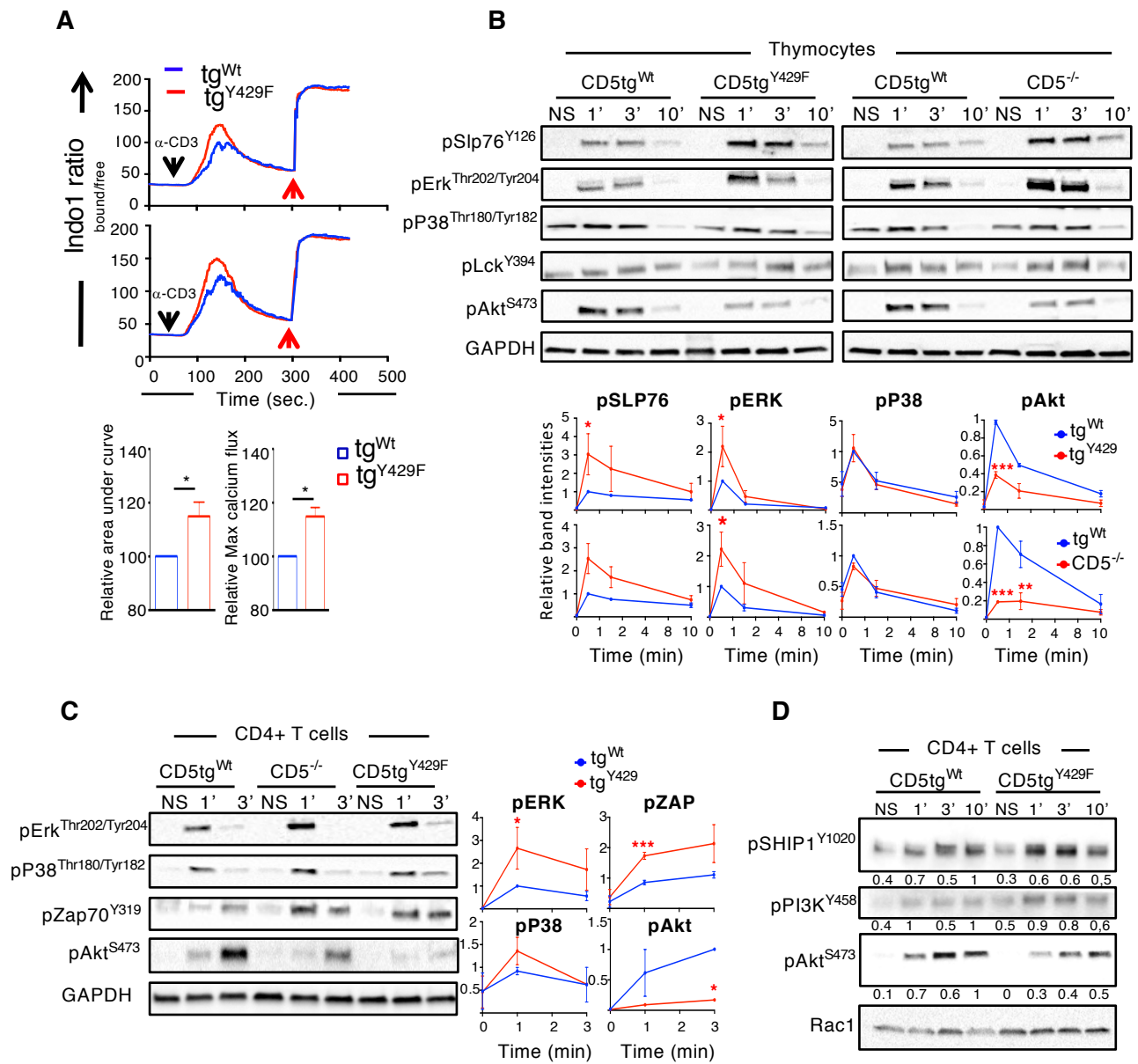


Figure 4

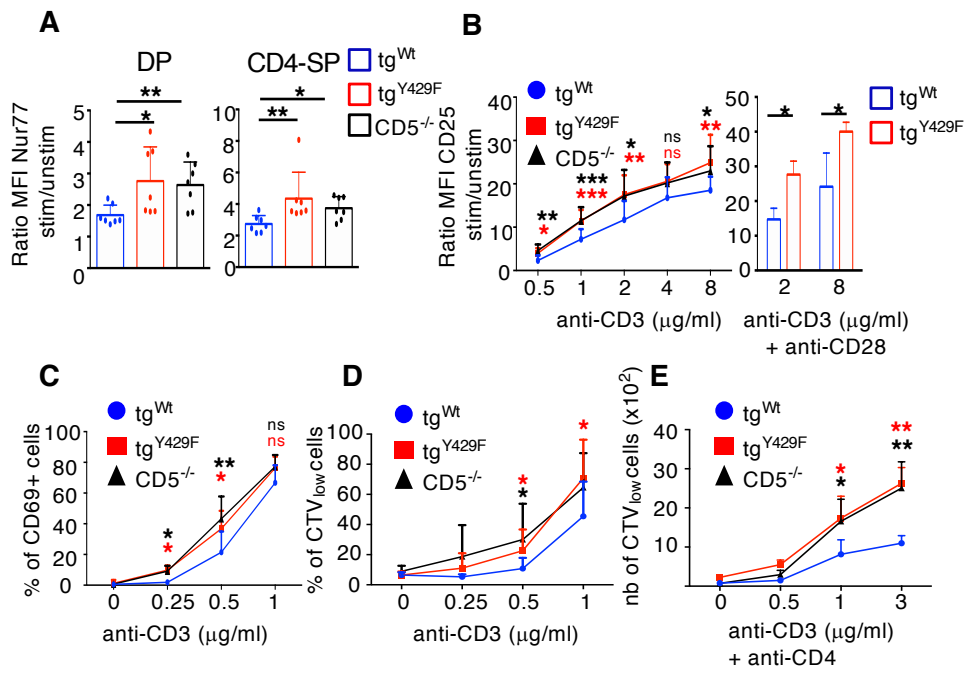


Figure 5

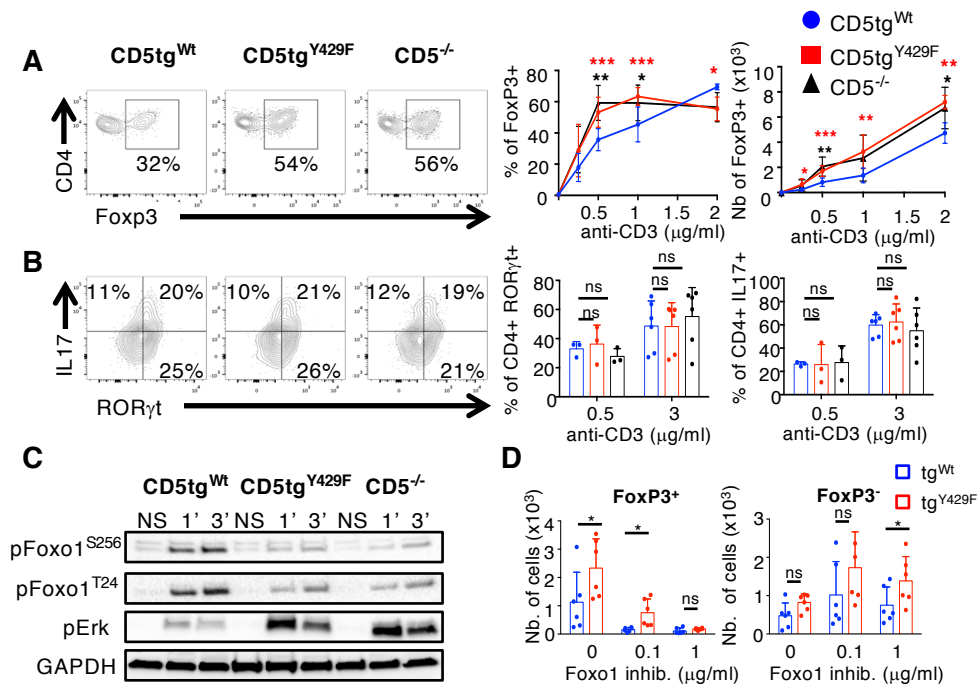


Figure 6

