p85β phosphoinositide 3-kinase regulates CD28 co-receptor function

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Abstract

CD28 is a receptor expressed on T cells that regulates their differentiation following antigen-stimulation to long-term-survival memory T cells. CD28 enhances T cell-receptor signals and reduces expression of CBL ubiquitin ligases, which negatively control T cell activation. In the absence of CD28 ligation during the primary stimulation, CBL levels remain high and T cells fail to mount an efficient secondary response. CD28 associates with p85α, one of the regulatory subunits of phosphoinositide-3-kinase (PI3K), but the relevance of this interaction is debated. We examined here the contribution of the other ubiquitous PI3K regulatory subunit, p85β, in CD28 function. We describe that p85β bound to CD28 and to CBL with greater affinity than p85α. Moreover, deletion of p85β impaired CD28-induced intracellular events, including c-CBL and CBL-b downregulation as well as PI3K pathway activation. This resulted in defective differentiation of activated T cells, which failed to exhibit an efficient secondary immune response. Considering that p85β-deficient T cells fail in recall responses and that p85β binds to and regulates CD28 signals, the presented observations suggest the involvement of p85β in CD28-mediated activation and differentiation of antigen-stimulated T cells.

Keywords: Cbl / CD28 / p85 PI3K / T cells activation / T cell co-stimulation
Introduction

T cell activation involves cooperation of signals triggered by the T cell receptor (TCR) and co-stimulatory molecules such as CD28\(^1\). TCR engagement induces a cascade of early signals including activation of Src family tyrosine kinases (TyrK) pp56\(^{ck}\) and pp59\(^{fyn}\), which in turn phosphorylate the TCR\(\zeta\) chain and activate the Syk family TyrK ZAP-70. ZAP-70 phosphorylates and associates numerous signaling molecules like LAT and SLP-76, which propagate the activation signal\(^2,3\). These cascades elicit expression of genes required for activation of hematopoietic cells, and for T cell expansion, effector function, and differentiation\(^4\).

CD28 binding contributes to T cell activation by stabilizing the immunological synapse, by enhancing the magnitude and duration of TCR-induced signaling cascades\(^5\) and by inducing long-term survival\(^6\). CD28 is therefore necessary for optimal activation of T cells, which become anergic (non-responsive) in the absence of CD28-derived signals\(^7,8\). Although CD28 co-receptor function was initially described for CD4+ T cells\(^9\), it is also required for optimal primary CD8+ T cell responses to most pathogens as well as for their recall responses\(^10-13\). CD28 can evade the anergy program by inducing downregulation of CBL-b (Casitas B-lineage lymphoma b)\(^14-17\). CBL-b ubiquitinates the p85\(\alpha\) regulatory subunit of PI3K; this reduces p85\(\alpha\) binding to CD28 and TCR\(\zeta\), resulting in diminished PI3K activation\(^18,19\). CBL-b downregulation, thus, is required for optimal PI3K pathway activation, and in turn for Rac-induced actin polymerization and immunological synapse stability\(^20\).

c-CBL is another CBL family member that limits T lymphocyte activation\(^21\). c-CBL exerts this action by downregulating ZAP-70 TyrK, although c-CBL enhances PLC\(\gamma\) activation\(^21\). c-CBL-deficient mice show increased positive selection of T cells in thymus as well as peripheral lymphoid hyperplasia\(^22-24\). Whereas changes in c-CBL expression following T cell activation have not been reported, this process causes an early reduction in CBL-b levels, an
event required for optimal T cell activation. CBL-b levels recover at later time points contributing to trigger TCR trafficking to the lysosomal compartment.

Class I PI3K enzymes are formed by a p110 catalytic subunit and a regulatory subunit; the p110 subunit catalyzes formation of phosphatidylinositol (3,4)P$_2$ and phosphatidylinositol (3,4,5)P$_3$ following receptor stimulation. PI3K are classified as TyrK-controlled class IA enzymes (p110$\alpha$, p110$\beta$ and p110$\delta$) that associate p85-like regulatory subunits, and G protein-regulated class IB PI3K, p110$\gamma$. In the case of class IA PI3K, three genes ($pik3r1$, $pik3r2$, and $pik3r3$) and their alternative splice forms give raise to five regulatory subunits. Whereas p85$\alpha$ and p85$\beta$ expression is ubiquitous, p55$\gamma$, p55$\alpha$, and p50$\alpha$ expression is restricted to certain tissues. PI3K binds to the TCR and to CD28 through p85, thereby contributing to trigger T cell activation, survival and division, nonetheless, the contribution of PI3K to CD28-mediated signaling remains incompletely understood.

We previously described that p85$\beta$-deficient T cells exhibit a moderately enhanced primary immune response. In the course of these studies, we noticed that secondary responses in these mice were defective. Considering that CD28 binds to p85 subunits, and that CD28 regulates recall T cell responses, we examined whether p85$\beta$ contributes to mediate CD28 signals. We describe that p85$\beta$ associates with CD28, controls CD28-mediated signals and is required for efficient secondary immune responses.
Materials and methods

Mice and cDNA

p85β^−/− mice, reported previously^33, were maintained in heterozygosis. F5TCR (Vβ11Vα4) Tg (transgenic) mice^41 were provided by D. Kioussis (MRC, London, UK). The CNB ethics committee approved all animal studies. F5TCR Tg mice were crossed with p85β^−/− mice; offspring were analyzed by PCR and flow cytometry to confirm the TCR transgene and correct MHC expression. Mice were bred and maintained in specific pathogen-free conditions in our animal facility; the CNB ethics committee approved all studies.

Recombinant- Haemaglutinin (rHA)p85α and rHAp85β were donated by L. Williams (Chiron Corp., Emeryville, CA) and J. Janssen (Heidelberg Univ., Germany); we cloned both into the pEF-BOS vector. Mutant forms of human p85β where cloned by PCR using primers encoding HA peptide including and the appropriate coding sequence encompassing the SH3 domain (pEF-BOS HA-SH3β) or SH2-SH2β region (pEF-BOS HA-SH2-SH2β). pSG5 p65β^{PI3K}, similar to the previously described p65α^{PI3K},^42, was prepared by introducing a stop codon at residue 567 by point mutation.

Antibodies and Reagents

Antibodies used for T cell activation were hamster anti-mouse CD3ε (145-2C11) and CD28 (37.51), mouse anti-human CD3ε (UCHT1) and CD28 (CD28.2), mouse anti-Armenian and -Syrian hamster IgG1 (G94-56), mouse anti-Armenian hamster IgG (G192-1) (all from BD Pharmingen), and rabbit anti-mouse IgG (Fcγ fragment-specific; Jackson Immunoresearch, West Grove, PA). For immunoprecipitation, protein A Sepharose beads and the following antibodies were used: anti-mouse CD28 (37.51, BD Biosciences, and M-20, Santa Cruz Biotechnology), -human CD28 (152-2E10, Biosource, Caramillo, CA), -PI3K pan-p85 (Upstate Biotechnology), -SH3 PI3K p85α (Upsate), -cCBL (BD Bioscience), -CBLb (Santa Cruz Biotechnology) and -HA (Covalance). We prepared an anti-p85β Ab using His-tagged
full-length murine- p85β, the Ab recognizes murine and human p85β and not α (in preparation). For Western blot, we used the appropriate antibodies and developed with ECL (enhanced chemiluminescence; Amersham Bioscience, Bucks, UK). We measured activity of PKC (protein kinase C) using anti-phospho PKC (γThr514), PKB (protein kinase B; also termed Akt) using anti-phospho-Akt (Ser473), phospho-Akt (Thr308), and that of p44/42 MAPK (mitogen-activated protein kinase) using anti-phospho-p44/42MAPK (Thr202/Tyr204), and of p38 MAPK using anti-phospho-p38MAPK (Thr180/Tyr182), all from Cell Signaling Technology (Beverly, MA).

Anti-PKCα (C-20, Santa Cruz Biotechnology), -Akt1/PKBα (Upstate Biotechnology), -p42/44 MAPK and -p38 MAPK (Cell Signaling) antibodies were used to control loading. We analyzed the profile of tyrosine-phosphorylated proteins using anti-PTyr antibody (4G10, Upstate Biotechnology). We also used anti-CBL-b (G-1, C-20, Santa Cruz Biotechnology), -c-CBL (clone 17) and -mouse-Nedd4 (clone 15; both from BD Transduction Laboratories), -human Nedd4 (Upstate Biotechnology), -βactin (Sigma), -HA (Covalence) and pan-p85 antibody (Upstate Biotechnology). Ly294002 were from Calbiochem.

Cell lines and transfections

Jurkat T cells were maintained in complete DMEM (Dulbecco’s modified Eagle medium; BioWhittaker, Walkersville, MD) with 10% FBS (fetal bovine serum; Sigma), 2 mM glutamine, 50 μM 2-mercaptoethanol, and 100 U/ml penicillin/streptomycin. Cells (1.5 x 10⁷) in 400 μl complete medium were transfected by electroporation of 25 μg DNA using a Gene Pulser (270V, 950 μF; Bio-Rad, Hercules, CA). Cells were immediately transferred to 10 ml complete medium containing 50 μg/mL Z-VAD (benzyloxy carbonyl-Val-Ala-Asp(OMe)-fluoromethylketone) and assayed after 36 h, when FACS or Western blot analyses showed maximum expression of the transfected protein.
Spleen and lymph node cell suspensions were T cell-enriched by depletion of B cells using mouse pan B (B220) Dynabeads (Dynal, Oslo, Norway) followed by 2 h incubation on plastic plates at 37°C to deplete adherent cells, or using mouse T cell negative isolation kits (Invitrogen, Carlsbad, CA); T cells were more than 95% CD3+ by FACS analysis (Epics XL-MCL, Beckman Coulter). For CD8+ cells, T cell-enriched suspensions were depleted of CD4+ cells using Dynabeads. Primary T and B cells were maintained in RPMI 1640 (BioWhittaker). Antigen-presenting cells (APC) were prepared by depleting T cells from spleen suspensions with mouse pan-T (Thy1.2) Dynabeads.

**Two-dimensional gel electrophoresis**

Before activation, purified mouse or Jurkat T cells were incubated (2 h) in DMEM with 0.1% BSA (Fraction V low endotoxin; Sigma), after which cells were washed and resuspended in serum-free medium. For activation, 5-10 x 10^6 cells were incubated with soluble anti-CD3+anti-CD28 Ab and crosslinked with secondary antibody as for in vivo T cell activation. Cells were lysed in CHAPS buffer (5% CHAPS, 20 mM Tris-HCl pH 8, 1 mM MgCl₂, 1 mM CaCl₂, 10% glycerol, 137 mM NaCl, 5 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM EDTA, 1 μg/ml each aprotinin and leupeptin, and 2 nM okadaic acid) (4°C, 90 min). Extracts were immunoprecipitated with anti-p85 (1 μg/ml) or CD28 (1 μg/ml) (4°C, 16 h). Two-dimensional electrophoresis was performed following standard procedures.

**Statistical analyses**

Statistical analyses were performed using StatView 512+ (Calabasas, CA). Gel bands were quantitated with ImageJ software.

**Online supplemental material**

T cell activation protocols, Coupling of peptides to Actigel resin, in vitro transcription translation, PI3K and PKB kinase assays, in vitro binding of proteins to peptide columns, immunoprecipitation and Western blot assays, TCR downregulation analyses, flow cytometry
assays, quantitative RT-PCR and proliferation assays are available in Supplementary Materials and Methods.
Results

CD28 preferentially associates with p85β

CD28 binds directly to the p85 regulatory subunit of PI3K. T cells express both of the ubiquitous p85 isoforms: α and β, however, the relative contribution of these subunits to CD28-mediated signals has not been elucidated. We first examined the relative abundance of p85α and β in Jurkat cells and wild type (WT) murine T cells. Since p85α and β have to be recognized by distinct Ab, for comparison rather than Western Blot (WB), we performed quantitative RT-PCR (QRT-PCR) as p85 mRNA levels do correlate with p85 protein levels. Whereas the ratio of p85α: p85β was close to one in Jurkat cells, in murine T cells p85β levels were approximately ~15% those of p85α (Fig. 1A).

We then examined p85-CD28 complex formation in Jurkat T cells expressing similar levels of recombinant HA-tagged p85α or p85β (rHAp85α or rHAp85β), which were approximately double to that of endogenous proteins (Fig. 1B). We immunoprecipitated CD28, and examined CD28-p85 association by WB detection with anti-HA antibody. Despite similar abundance of rHAp85α and -β, CD28 bound a larger amount of p85β than α (Fig. 1B).

We also examined endogenous CD28-p85 complex formation in mouse T cells. As mentioned above, p85β mRNA levels were roughly one sixth those of p85α; a moderate increase in p85α mRNA levels was found in ~two thirds of p85β−/− mice (Fig. 1C, WB of a p85β−/+ representative mouse with enhanced p85α levels). We analyzed total WT T cell extracts as well as CD28 immunoprecipitates obtained from CD3+CD28-activated T cells in two-dimensional gel electrophoresis. Whereas in WT cells the p85β spot (the most acidic spot) represented ~15% of the total p85, in CD28 immunoprecipitates p85β proportion was double, showing that relatively to its abundance p85β exhibits a greater affinity for CD28 (Fig. 1D). Similarly, in Jurkat cells, the human p85β right most spot (the more basic spot) was ~50% of the total signal in whole extracts, and ~80% in CD28 immunoprecipitates (Fig. 1D).
We also compared the amount of endogenous p85β bound to CD3 or CD28 receptors following T cell activation by CD3, CD28, or both. CD3 activation did not induce a significant association of p85β to either CD3 or CD28 (Fig. 1E). In contrast, CD28 activation induced the binding of p85β to CD28 but triggered as well p85β binding to CD3 (Fig. 1E); suggesting that CD28 stimulation is required to bring p85β into complex with CD3. Finally, following CD3+CD28 stimulation, a larger amount of p85β was found in CD28 immunoprecipitates, suggesting a greater affinity of p85β for CD28 than for CD3 (Fig. 1E). A similar analysis performed with p85α showed that CD3 stimulation triggered association of p85α to CD3, whereas CD28 activation favored p85α association to CD28; a similar amount of p85α was found in CD3 and CD28 receptors following CD3+CD28 stimulation (Fig. 1E). Therefore, whereas p85α associates similarly to both CD3 and CD28, p85β association to CD28 is greater than to CD3, moreover, CD28 activation is required for association of p85β to CD28 and to CD3. The analysis of CD28-bound p85 using a pan-p85 Ab resembled the pattern of p85β supporting that CD28 binds greater levels of p85β than p85α (Fig. 1E).

To map p85-binding regions in the CD28 cytoplasmic tail, and to quantify more accurately the amount of p85α and p85β bound to CD28, we analyzed direct binding of purified in vitro transcribed and translated p85 subunits to CD28 peptide-columns. We selected four CD28 peptides including the YMNM motif (M1), the C-terminal YRS region (M2), and the Tyr-phosphorylated forms of these peptides (M1p, M2p) (Fig. 1F), regions involved in CD28 binding to the p85 subunit40.

Translated murine and human 35S-Met labeled p85β and α (h-p85α fused to GFP) were resolved in SDS-PAGE gels to measure p85 levels (Fig. 1F), equal amounts of purified p85α and β were loaded onto CD28 peptide-columns, and we examined the binding of each isoform to the columns. As negative controls we used columns with no-peptide, as positive controls we used PDGF receptor (R) peptide columns42. p85 association to CD28 peptides was
analyzed by SDS-PAGE and autoradiography. Both p85β and α bound slightly better to phosphorylated-CD28 peptides, in addition, p85β bound slightly better to M1 peptides and markedly better to M2 and M2p CD28 peptides, compared to p85α (Fig. 1F). We also compared p85 binding to CD28 peptides and to PDGFR peptide. p85α binding to PDGFR was within the range of p85α and β binding to M1p peptide; binding to M2p was one third to one half (human and mouse p85β, respectively) that observed using PDGFR peptide (Fig. 1F, $n = 3$). Although in these assays both p85α and β bound better to M1p than to M2p columns, the scenario in vivo might be distinct; deletion of the last 10 residues of CD28 (in M2) reduces p85 binding and PI3K activity by 90%\textsuperscript{40}. The greater affinity of p85β for the M2p columns explains the greater binding of p85β to CD28 in vivo.

**Lower PKB activation in p85β-deficient cells**

To determine whether the p85 regulatory subunits that bind to CD28 upon T cell activation exhibit p110-associated PI3K activity, we transfected Jurkat cells with rHAp85α or β in combination with p110δ. Cells were activated with CD3+CD28 and we measured CD28-associated PI3K activity in vitro. CD28 exhibited a similar associated-PI3K activity in both cases, suggesting that CD28 bound p85α and β are in complex with p110 (Fig. 2A). A faster PI3K activity association was observed in some cases in p85β cells, but the difference ($n = 3$) was not statistically significant. CD28 immunoprecipitates from CD3+CD28-activated murine WT or p85β−/− T cells also contained a similar associated PI3K activity (Fig. 2B), suggesting that p110-recruitment to CD28 is observed in the presence or absence of p85β.

PI3K assay might under estimate quantitative differences in vivo; we thus examined the activation of the PI3K effectors PKB and p70S6K upon stimulation of WT and p85β−/− T cells via CD3, CD28 or both. p-p70S6K and p-PKB cell content was ∼ one third lower in p85β−/− T cells upon CD3 activation, in contrast, CD28 activation resulted in p-p70S6K and p-PKB levels that were ∼60% lower in p85β−/− T cells than control cells (Fig. 2C), showing that a
greater signaling defect is observed upon stimulation of CD28. CD3+CD28-activation also yielded significant lower levels of p-p70S6K and p-PKB in p85$^{β/-}$ cells (Fig. 2C); we confirmed the lower PKB activity in p85$^{β/-}$ T cells in kinase assays (Fig. 2D).

**SH2 domains, and to lesser extent SH3 domains, are involved in p85 binding to CD28**

The Tyr170 region (M1 peptide) is Tyr phosphorylated following T cell activation; the C-terminal M2 region contains a phosphorylatable Tyr and a Pro-rich motif. Since pTyr residues bind to SH2 domains and Pro-rich regions bind to SH3 domains, we tested the binding of p85$^β$ to these regions. Previous studies showed that the CD28 Tyr170 region binds to p85$^α$ C-SH2 domain *in vitro*, whereas the phosphorylated M2 region, containing Pro residues, binds both to the C-SH2 and the SH3 p85$^α$ domains$^{40}$. We examined in extracts of CD3+CD28 activated Jurkat cells, the binding of CD28 to p85$^β$ SH2-SH2 region, or to the SH3 region, or to a previously described p65$^{PI3K}$ mutant encompassing the SH3, Bcr and the N-SH2 domains$^{42}$, *in vivo*. Cells were transfected with the different HA-tagged p85$^β$ constructs, 36h latter the cells were activated and the extracts immunoprecipitated with CD28; association to p85$^β$ constructs was determined in WB using HA Ab. When binding was normalized considering the expression levels, the binding of wild type p85$^β$ was lower than that observed with p85 fragments, suggesting that p85 folding partially masks the interaction regions (Fig. 2E). In the mutants, the greatest binding was observed with the SH2-SH2 region, whereas binding to p65$^{PI3K}$ mutant, which lacks the C-SH2 domain, was lower, suggesting that the C-SH2 domain (or else both SH2 domains) is required for binding to CD28 (Fig. 2E). Moreover, the SH3 domain also bound CD28, showing that although to lesser extent SH3-binding to Pro residues also contributes to CD28- p85$^β$ association (Fig. 2E). Thus, p85$^β$ C-SH2 and SH3 domains participate in p85$^β$ binding to CD28.

**c-CBL and CBL-b preferentially associates with p85$^β$**
CD28 binds to p85<sup>36-40</sup>, which in turn associates with CBL-b and c-CBL<sup>18, 44</sup>. Therefore, p85 might link CD28 and CBL. Since c-CBL associates better to p85β than to p85α<sup>in vitro</sup><sup>44</sup>; we tested whether p85β might also associate better than p85α to CBL proteins<sup>in vivo</sup>. We measured CBL association to p85α and β in Jurkat T cells transfected with rHAp85α or β. Although rHAp85α and β were expressed to a similar extent, CBL-b and c-CBL immunoprecipitates contained more rHAp85β than α (Fig. 3A). p85β thus not only binds CD28 preferentially, but also shows greater affinity for CBL-b and c-CBL, potentially acting as a bridge between CD28 and the CBL. CBL contain Tyr phosphorylated residues, and a Pro rich domain<sup>24</sup> (Fig. 3B, bottom) that might bind SH2 and SH3 regions, respectively. To determine the p85 region involved in association with CBL-b and c-CBL, cells were transfected with the different HA-tagged p85β constructs (as above), we immunoprecipitated the CBL and determined association to different p85β constructs in WB using HA Ab. Expression levels were as in Fig. 2D. As in p85β/CD28 association, p85β fragments showed greater binding than full-length p85 to both CBL (Fig. 3B). In addition, whereas CBL-b bound preferentially to the SH3 domain and to the p65<sub>PI3K</sub> mutant (which includes the SH3 domain), c-CBL bound preferentially to SH2-SH2 and to p65<sub>PI3K</sub> (which includes the NSH2 domain), showing that the SH3 and NSH2 domains are likely the main regions of p85β binding to CBL-b and c-CBL, respectively.

**Defective CBL regulation in p85β-deficient T cells**

CBL limits T cell activation, indeed, defects in CBL-b downregulation yield T cells anergic<sup>45-47</sup>. For this reason, one of the critical actions of CD28 is to induce CBL-b downregulation following T cell activation<sup>15-20</sup>. Considering that p85β binds preferentially to CD28 and the CBL, we examined whether p85β deletion impaired CBL downregulation. T cells from p85β<sup>+/+</sup> and p85β<sup>−/−</sup> mice were activated<sup>in vitro</sup> with anti-CD3 or with anti-CD3+anti-CD28 antibodies; in control cells CD3 activation reduced CBL-b levels by 2 h and
reduction was more pronounced following CD3 + CD28 activation (Fig. 4A). In contrast, CD3-induced CBL-b reduction was significantly less pronounced in p85β−/− T cells and was unaffected by the presence of CD28 (Fig. 4A). Similarly, whereas in controls CD3 induced a moderate c-CBL downregulation at ~2 h upon stimulation that was enhanced in the presence of CD28Ab, neither CD3 nor CD3+CD28 reduced c-CBL levels in p85β−/− T cells (Fig. 4A). Although we detect CBL downregulation defects upon CD3 stimulation this might also reflect CD28 signaling defects since CD28−/− mice show CD3-induced CBL downregulation defects16, implying that activation via TcR also involves CD28.

CBL downregulation was also observed in vivo (Fig. 4B). p85β+/− and p85β−/− F5TCRTg mice were injected with by antigenic peptide. Antigenic peptide-loaded APC activate T cells through TcR and CD285. Primary antigen stimulation in vivo induced a pronounced, transient reduction in CBL-b and c-CBL levels in control mice T cells, p85β−/− T cells, however, showed a significantly lower CBL-b and c-CBL downregulation (Fig. 4B).

Considering that PI3K/PKB pathway activation was lower in p85β−/− T cells we examined whether PI3K activity is required to trigger CBL downregulation. Jurkat T cells were activated in the presence or absence of the PI3K inhibitors worthmanin (not shown) or Ly294002 (Fig. 4C). CD3 stimulation induced and CD3+CD28 enhanced CBL downregulation in Jurkat cells; however, PI3K inhibition did not block but moderately enhanced CBL downregulation, more clearly that of CBL-b (Fig. 4C). Since p85β deletion impairs CBL downregulation despite reduction of PI3K/PKB pathway activity, these observation suggest that p85β controls CBL downregulation in a PI3K-activity independent manner.

c-CBL and CBL-b regulate TCR downregulation at late time points25, 27, 48. Considering that p85β links CD28 and the CBL, it was possible that p85β-deficient T cells exhibited defects in activation-induced TCR downregulation. Indeed, whereas CD3 crosslinking induced a similar TCR downregulation in WT and p85β−/−purified T cells, CD3+CD28
triggered TCR downregulation was significantly lower in p85β−/− T cells than in WT cells (Fig. 5A), confirming the signaling defects of CD28 in p85β−/− T cells.

**Nedd4 is not responsible for the CBL downregulation defects of p85β−/− mice**

The CBL regulation defects of p85β-deficient T cells might result from defects in Nedd4 ubiquitin ligase, which targets c-CBL and CBL-b for degradation. Anti-CD3 stimulation triggered Nedd4 expression at 72 h in both p85β+/− and p85β−/− cells, moreover, CD3+CD28 activation accelerated Nedd4 expression in control cells but not in p85β−/− cells (Fig. 5B). These observations support the CD28 signaling defects in p85β−/− T cells, since whereas CD3 induced Nedd4 expression similarly in control and p85β−/− T cells, CD28 failed to enhance Nedd4 expression in p85β−/− cells. In addition, these data shows that defective Nedd4 expression is not the cause of CBL downregulation defects since CBL levels decrease at 2-to-6 h and Nedd4 increases at ~36h following T cell activation.

**Increased expression of p85β enhances CBL and TCR downregulation**

Since p85β deletion impaired CBL and TCR downmodulation, we analyzed whether p85β overexpression might enhance these processes. We expressed rHAp85α or -p85β in Jurkat cells. Whereas in Jurkat cells transfected with empty vector, CD3+CD28 stimulation triggered a reduction in CBL-b and c-CBL levels at 4-to-6 h (Fig. 5C), both rHAp85α and β expression resulted in a greater and more sustained CBL downregulation, more markedly in the case of p85β. Moreover, in agreement with the idea that p85 links CD28 to CBL, increased p85α expression and even more markedly that of p85β, enhanced TCR downregulation compared to controls (Fig. 5D). These observations confirm the role of p85 as a molecule regulating TCR-CD28 induced CBL downregulation with a greater action of p85β, which might cooperate with p85α for this function.

**CD28 defective signaling in primary activation of p85β−/− T cells**
APC-antigenic T cell stimulation activates T cells in the context of CD28 co-stimulation, which stabilizes the immunological synapse and prolongs TCR-induced signaling cascades. Since CD28 binds preferentially to p85β, we analyzed the effect of p85β deletion on the signaling cascades triggered by APC-induced T cell activation in vivo using phospho-antibodies to analyze TyrK, PKC, PKB, p38MAPK, and p44/42MAPK activation.

In agreement with the lower PKB activation in CD3+CD28 stimulated p85β−/− T cells, primary antigenic stimulation in p85β−/− F5TCRTg T cells induced a reduced pPKB content compared to controls (Fig. 5E). In addition, whereas at 15-30 min post-stimulation, p44/42MAPK and p38MAPK activation occurred similarly in p85β−/− and p85β+/− F5TCRTg cells, activation at 60 min was lower in p85β−/− T cells (Fig. 5E). As CD28 prologues signals induced by the TCR, these observations concur with the idea that CD28 signaling is defective in p85β−/− cells. A similar defect was detected in TyrK stimulation (not shown). PKC activation was greater in p85β−/− F5TCRTg T cells than in controls (Fig. 5E), in agreement with the higher stability of c-CBL in these cells, as c-CBL enhances PLCγ/PKC activation.

Thus, a higher PKC activation, a less sustained MAPK activation and lower pPKB content were observed in primary-stimulated p85β−/− T cells.

Reduced secondary immune response in p85β-deficient mice

CD28-deficient T cells undergo an incomplete primary activation resulting in a defective differentiation of activated T cells, which become unresponsive on a secondary Ag challenge. As p85β binds to CD28 and regulates CD28-downstream signaling, we examined whether p85β deficiency impaired secondary immune responses. We first compared the primary and secondary immune responses in p85β−/− and p85β+/− mice using as antigen Candida albicans, which triggers mainly a CD4+ cell response. The primary response to Candida albicans was slightly higher in p85β−/− mice than in controls; in contrast, p85β−/− mice showed a lower CD4+ spleen and lymph node secondary immune response (Fig. 6A).
We also examined primary and secondary antigen-specific immune responses. p85β+/− F5TCRTg and p85β+/− F5TCRTg mice were immunized once with the NP366-374 influenza peptide (primary) or challenged at day 0, and treated again 10 days later (secondary). The primary response was moderately enhanced in p85β+/− F5TCRTg mice but the secondary response was significantly lower than in controls (Fig. 6B). IL-2 partially restores anergic cells proliferation7-9, IL-2 addition enhanced T cell proliferation in p85β+/− mice secondary response (Fig. 6C), suggesting that primary activated p85β+/− T cells behave as anergic cells.Confirming a defective secondary response in p85β+/− mice, the expression of the activation (CD25, CD69) and memory markers (CD44high, CD62Llow) was significantly lower upon secondary challenge in p85β+/− mice (Fig. 6D). These data shows that p85β deficiency results in reduced CD4+ and CD8+ T cell secondary immune responses.

**CD28-mediated defects upon secondary p85β+/− T cell stimulation**

We examined CBL downregulation and intracellular pathways in secondary responses. We immunized p85β+/− and p85β+/− F5TCRTg mice with the NP366-374 peptide and 11 days later, mice were reinjected with the peptide; we examined CBL levels in vivo, in extracts of cells collected at different times post-injection. Basal levels of CBL-b and c-CBL were moderately higher in p85β+/− T cells compared to controls, as in anergic cells46; in addition, restimulation triggered a large CBL downregulation in controls, and a minor reduction in p85β+/− T cells (Fig. 7A). For intracellular signals analysis, mice were immunized as above and 11 days later, purified T cells were re-stimulated in vitro. PKB activation was reduced in p85β+/− T cells compared to controls (Fig. 7B), since CBL-b ubiquitinates p85α and reduces PI3K activation18,19 this concurs with the higher CBL-b levels in p85β+/− cells. TyrK activation and p44/42MAPK activation were lower and delayed in p85β+/− T cells, as in anergic cells52 whereas the basal activity of PKC was higher in p85β+/− T cells (Fig. 7B), in agreement with the larger c-CBL levels in p85β+/− T cells, since c-CBL enhances PLCγ/PKC activity21-24,27.
Discussion

The presented observations show that p85β associates with CD28 and regulates CD28-signalling delivery. p85β binds to CD28, CBL-b and c-CBL more efficiently than p85α. This function of p85β explains the reduced transmission of signals by CD28 in p85β−/− T cells, including a lower PKB activation, as well as a defective downregulation of CBL-b and c-CBL. Since PI3K/PKB activity is required for long-term survival 30 and CBL downregulation by CD28 is required for activated T cells to escape the anergy program 15, CBL and PKB defects likely contribute to the impaired secondary immune response in p85β−/− mice.

Effective activation of naïve T cells requires a signal dependent on TCR engagement by the peptide-MHC and a secondary signal provided by interaction between the antigen-presenting cell (APC) and additional receptors in the T cell. CD28 provides a potent co-stimulatory signal following engagement of its B7 ligands on the APC. CD28 contributes to enhancing and prolonging the signals induced by the TCR, synapse stability and long-time survival 5-15, these events regulate differentiation of primary stimulated T cells, as activation of CD28−/− T cells induces anergy 7-9. p85, the regulatory subunit of PI3K, is one of the signaling molecules that bind to CD28 36-40. We show that the p85β isoform exhibits preferential binding to CD28 receptor compared to p85α, as examined in Jurkat cells expressing similar levels of rp85α or β or in transformed and normal T cells analyzing endogenous p85α and β subunits. Greater p85β binding to CD28 was confirmed in vitro using purified p85 and CD28 peptides.

p85β binding to CD28 contributed to CD28-mediated early signals as determined examining antigenic stimulation of normal murine T cells, occurring in the context of CD28 stimulation 5-15, which resulted in lower PKB activation and less sustained MAPK activation in p85β−/− T cells. The CD28 signaling defects in p85β−/− T cells were confirmed examining activation of the PI3K effectors PKB and p70S6K following CD3 or CD28 crosslinking, which showed greater signaling defects upon CD28 activation that following CD3 activation.
T cell activation induces phosphorylation of CD28 on Tyr170 (on the M1 peptide, Fig. 1). This phosphorylated site was initially considered the main residue responsible for p85 binding\textsuperscript{37}. However, reconstitution of CD28-deficient mice with the transgenic Y170F-CD28 mutant rescued the T cell anergy phenotype of CD28\textsuperscript{-/-} mice\textsuperscript{53}, arguing against the relevance of this residue or of PI3K for anergy prevention. Nevertheless, whereas CD28-deletion reduces PI3K activity following T cells antigenic stimulation, the Y170F-CD28 mutant did not reduce CD28-mediated PI3K activation\textsuperscript{54}, showing that PI3K binds to CD28 on different site/s. There is a second, non-canonical CD28 binding site for p85 at the C-terminus of CD28\textsuperscript{40}. This motif is in the last 10 residues on CD28 (included in peptide M2, Fig. 1). Although \textit{in vitro} the C-terminal CD28 region bound a lower amount of p85 regulatory subunit than the Tyr170-containing peptide, \textit{in vivo} the scenario might differ, as deletion of the C-terminal motif induced a 90\% reduction on PI3K activation and a defective CD28 co-stimulatory function\textsuperscript{40}, suggesting that binding of PI3K to this motif is critical for CD28 function. We showed that this region exhibits a high selectivity for p85\(\beta\) binding, pointing at binding of p85\(\beta\) to CD28 as a critical event for CD28- co stimulation.

p85\(\beta\) binding to CD28 also modulated CBL recruitment and downregulation. We show that rHAp85\(\beta\) bound better than p85\(\alpha\) to CBL-b and c-CBL. p85\(\beta\) contribution to CD28-induced CBL downregulation was revealed examining antigen or CD3+CD28 induced CBL downregulation, which was markedly impaired in p85\(\beta\)\textsuperscript{-/-} T cells. We show that p85\(\beta\) regulated not only CBL-b downregulation, but also that of c-CBL, which was not previously reported. A less marked defective downregulation was also observed upon CD3 stimulation, however, as CD3-induced CBL-b downregulation also involves CD28\textsuperscript{16}, this defect might be secondary to the CD28-signalling defect. Alternatively, p85\(\beta\) might also affect the signaling properties of TCR\(\zeta\),Lck, LAT, and TRIM, which also associate p85 subunits\textsuperscript{2,55,56}. Nonetheless, comparison of the signaling defects of CD3 or CD28 stimulated p85\(\beta\)\textsuperscript{-/-} T cells
(Fig. 2C) support a greater contribution of p85β to CD28-induced signal transduction. The requirement of p85β for CD28-induced CBL downregulation was not consequence of the reduced PI3K/PKB activity in p85β−/− T cells, since PI3K activity is not required for CBL downregulation.

We investigated the p85β regions responsible for association to CD28, c-CBL and CBL-b. The principal regions involved in binding to CD28 were the SH2 domains with a lower contribution of the SH3 domain. Since p65β, a mutant including the BcR, SH3 and N-SH2 domains, did not bind CD28 efficiently, either the two SH2 are required for p85β binding to CD28 or the p85β C-SH2 domain (which is not present in p65β) is critical for this interaction. Studies in vitro using p85α domains support the contribution of C-SH2 and SH3 regions for the binding of CD28 to p8540. As the SH3 regions are less conserved between p85α and β (~50% homology) than the SH2-SH2 domains (~82%), the greater p85β association might result from the contribution of the SH3 domain.

In the case of the CBL, both c-CBL and CBL-b bound to p85β N-SH2 and SH3 regions, nonetheless, the relative contribution of these domains was distinct. Whereas c-CBL exhibited a greater binding to N-SH2 region CBL-b bound preferentially to the SH3. Therefore, one might envision a quadruple complex involving p85β C-SH2 domain associating to CD28, the N-SH2 domain binding c-CBL, and the SH3 region bringing CBL-b into complex. More likely, as association of CD28 and CBL to p85β were not exclusively dependent on single domains, it is possible that several p85β molecules associated to the T cell synapse (through Lck, TCRζ, LAT, TRIM, etc) establish a more complex network of interactions in which different p85β associate with CD28, c-CBL or CBL-b. We found that isolated domains bound better to CD28, c-CBL and CBL-b than the full-length p85, supporting the idea that the binding of p85/p110 to receptor/s or its activation induced conformational changes facilitating subsequent interactions with p85.
p85α−/p85β−/ double deficient mice developed of a rare-autoimmune disorder similar to Sjögren’s syndrome. T cells from these mice exhibit a dramatic reduction in PI3K activation since p85 molecules protect p110 from degradation. Considering that PI3K activity is required for regulatory T cells differentiation, it is possible that defective development of this population causes the Sjögren’s syndrome in p85α−/p85β−/ mouse.

Together, we conclude that the p85β regulatory subunit of class IA PI3K binds to CD28 more efficiently than to CD3, and with greater affinity than p85α thereby regulating CD28-mediated downstream signaling. In the absence of the p85β, primary T cell activation induces a flawed PI3K pathway activation, and an impaired c-CBL and CBL-b downregulation, an effect that is independent of p85β-associated PI3K activity. These defects result in an impaired differentiation of activated T cells, which are then unable to develop an efficient secondary immune response. Considering the contribution of PI3K/PKB for long term T cell survival as well as the need of CBL downregulation for CD28-mediated differentiation of activated T cells to functionally competent memory T cells, we propose that impaired PKB activation and CBL downregulation in p85β−/− cells contributes to cause the defective secondary responses in these mice, implying that p85β plays an important role in CD28-mediated T cell activation and differentiation.
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Authorship

Contribution: IA, DFB and IC performed experiments, IA, DFB and ACC analyzed the results and made figures, DAF contributed vital reagents, participated in discussion of the results and reviewed the paper, CH analyzed animal phenotypes, DFB and ACC designed the research and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

IA and DFB contributed equally to the experiments done in this study.

DFB and ACC are co-senior authors on this manuscript.
References


Legends to the figures

Figure 1. CD28 preferentially associates to p85β. (A) Ratio of p85α/p85β molecules per ng of total mRNA determined by QRT-PCR. (B) Jurkat cells transfected with control, rHAp85α or β vectors (36 h), were activated via CD3+CD28 (15 min). WB examined protein expression in total extracts, extracts were also immunoprecipitated with anti-CD28 Ab and associated p85 was analyzed in WB using anti-HA Ab (long and short exposure). (C) Number of p85α or β molecules per ng of total mRNA (from p85β−/− and p85β+/+ T cells) by QRT-PCR; each dot represents a mouse. WB analysis of p85 in extracts of a representative mouse (vertical line indicate a repositioned gel lane). (D) T cells from p85β−/− and p85β+/+ mice and Jurkat cells were activated as in (A). Total extracts or CD28 immunoprecipitates were resolved in two-D electrophoresis and analyzed in WB using anti-pan-p85 antibody. β indicates the p85β spot (absent in p85β−/− mice). The isoelectric point (pl) of mp85β was 5.74, and 5.96, 5.9 and 5.8 those of mp85α and its phosphorylated forms, the pl for hp85β was 6.03, and 5.84, 5.77 and 5.71 those of hp85α and its phosphorylated forms. Graphs show the mean ± SD (n = 3) of the β spot signal relative to the combined signal for all spots (100%). (E) Jurkat cells were activated as in (B), WB show the amount of p85 present in CD3 or CD28 immunoprecipitates. Graphs show the mean signal ± SD (AU, arbitrary units) of p85 bound to CD3 or CD28. (F) cDNA encoding human (h) or mouse rp85α or β were transcribed/translated in vitro (rhp85α was fused to GFP) in the presence of 35S-Met. Purified proteins were examined by SDS-PAGE and autoradiography (top, left). Sequence of the hCD28 peptides; the dots represent the non-conserved aminoacids in mCD28 (H>P and P>A). In the bottom, p85 bound to the different CD28-peptide columns analyzed by SDS-PAGE and autoradiography. PDGFR (P) peptide was used as a positive control; column with no peptide and mp85α was used as the negative control. The histograms show the absolute binding of
each rp85 form to CD28 motifs (mean signal ± SD in AU, arbitrary units) on the right, rp85 relative binding to M peptides and to P column ($n = 3$). * $t$-test, $P < 0.05$.

**Figure 2. Lower PKB activation in p85β deficient cells.** (A, B) Jurkat cells were transfected with control, rHAp85α or β in combination with p110δ vectors and incubated for 36 h (A). T cells were purified from WT and p85β+/− and mice (B). Cells were activated with CD3+CD28 antibodies (7 or 15 min) and collected. PI3K activity was assayed in CD28 immunoprecipitates *in vitro*. WB examined recombinant protein expression levels in cell extracts. The histogram represents the mean ± SD ($n = 3$) of the PIP3 signal in arbitrary units (A.U.) (C, D) T cells were purified from p85β+/− and p85β+/+ spleen and lymph node cell suspensions, then activated by CD3, CD28 or CD3 + CD28 crosslinking (indicated). In (C), total cell lysates were examined in WB using anti-pSer473-PKB, pThr308-PKB, pThr389-p70S6K or -PKB antibodies. Graphs show the percentage of p-PKB or p-p70S6K signal compared to maximal (with CD3 + CD28 at 5 min). In (D), PKB was immunoprecipitated from cell extracts and assayed *in vitro* using Histone H2B as substrate. Graphs were as in (A). (E) Jurkat cells were transfected with cDNA encoding the indicated p85β mutant forms, and 36h later cells were activated as in (A) and total extracts or CD28 immunoprecipitates resolved by SDS-PAGE and examined by WB using anti-HA Ab. Vertical lines have been inserted to indicate a repositioned gel lane. The graph shows the mean signal ± SD (AU, arbitrary units) of CD28 bound rp85β forms normalized for their expression levels. Scheme showing the potential interaction domains of p85 and CD28 and after activation *$P < 0.05$.

**Figure 3. CBL-b and c-CBL associated preferentially to p85β.** (A) Jurkat T cells were transfected with empty vector, or with vectors encoding rHAp85α and β. At 36 h post-transfection, cells were collected and activated with CD3+CD28 for different times. Total extracts were immunoprecipitated with CBL-b or c-CBL antibodies and p85 binding was analyzed in WB using anti-HA antibody. Total p85 was used as a loading control.
Histograms show relative signal ± SD ($n = 3$) of rHAp85α or rHAp85β binding to CBL-b and c-CBL. AU, arbitrary units. (B) Extracts from the assay shown in figure 2D were immunoprecipitated using anti-c-CBL or CBL-b Ab, resolved by SDS-PAGE and examined by WB using anti-HA Ab. The graphs are as in figure 2D. Scheme showing the potential interaction domains of p85β to CD28 and CBL after activation. * $P < 0.05$.

**Figure 4. Defective CBL and TCR downregulation in p85β-deficient cells.** (A) Purified peripheral T cells from p85β$^{+/−}$ and p85β$^{-/-}$ mice were stimulated with CD3+CD28 Ab for the indicated times. CBL-b and c-CBL levels were analyzed in WB. Actin was used as loading control. (B) For primary responses, purified peripheral CD8$^+$ T cells from p85β$^{-/-}$ and p85β$^{+/−}$/F5TCRTg mice were stimulated *in vitro* with peptide-pulsed APC for the times indicated. Extracts were examined in WB using the indicated antibodies. (C) Jurkat T cells were activated with CD3 or CD3+CD28 for the indicated times in the presence or absence of 10μM Ly294002. We analyzed CBL-b and c-CBL levels in cells extracts by WB. Graphs (A-C) show CBL-b and c-CBL signal intensity in arbitrary units, mean ± SD ($n = 5$). *P<0.05.

**Figure 5. p85β overexpression enhances CBL-b and TCR downregulation.** (A) TCR down modulation was measured by flow cytometry in purified peripheral CD8$^+$ T cells from p85β$^{+/−}$ and p85β$^{+/−}$/F5TCRTg mice stimulated *in vitro* with CD3 or CD3+CD28 for the times indicated. TCR downregulation was estimated as the reduction in the mean fluorescence index of TCRβ expression on stimulated CD8$^+$ vs. non-stimulated CD8$^+$ T cells (mean ± SD, $n = 3$). (B) Cells were activated as in (A) and Nedd4 levels were examined in WB. The graph quantitates the mean ± SD ($n = 3$) of Nedd4 signal relative to maximal levels (100%). (C,D) Jurkat T cells were transiently transfected with empty vector or rHAp85α or β. Cells were activated as above 36 h post-transfection and then collected. We analyzed CBL-b and c-CBL levels in cell extracts by WB (C) or TCR down modulation as in (A); MFI of TCRβ expression on stimulated CD4$^+$ vs. gated non stimulated CD4$^+$ T cells (mean ± SD, $n = 4$) (D).
Graphs (C) show CBL signal intensity in arbitrary units (AU); mean ± SD (n = 3). (E) Purified peripheral CD8+ T cells from p85β−/− and p85β+/− F5TCRTg mice were stimulated in vitro with peptide-pulsed APC. Cell extracts were examined in WB (indicated). The graphs represent the mean ± SD (n = 3) of the signal for each of the proteins in three different assays, compared to the maximal signal (100%) in WT cells at 15 min (pPKC), 30 min (pPKB) or 1 h (p-p38, p-p44/42) and normalized in comparison with loading controls (n = 3). P values are shown for the time points of maximal signal in WT cells. * P <0.05.

**Figure 6. Reduced secondary responses in p85β-deficient mice.** (A) p85β−/− and p85β+/− mice received an i.p. injection of heat-inactivated *Candida albicans* to induce a primary response; 21 days later, some mice received an identical injection to induce the secondary response. Splenocytes or lymph nodes (LN) were isolated and the cells analyzed by flow cytometry using appropriate antibodies. The figure shows CD4+ cell numbers at different times post-injection (mean ± SD; n = 3). P values were calculated at day 5. (B) The NP366-374 influenza peptide was injected into p85β−/− and p85β+/− F5TCRTg mice for a primary response; injection was repeated after 11d for the secondary response. The figure shows CD8+/Vβ11+ spleen cell numbers at different times post-injection (mean ± SD, n = 3). Arrows indicate peptide injection. (C) For primary in vitro proliferation assays, peripheral T cells from p85β−/− and p85β+/− F5TCRTg mice were activated with peptide-pulsed APC at different peptide doses, alone or in the presence of IL-2 (10 U/ml). For secondary IR, mice received a peptide injection and were sacrificed after 10 d, when T cells were purified from spleen and lymph nodes. T cells were then activated in vitro. [3H]thymidine incorporation was measured at 48 h (mean ± SD, n = 3). The arrows indicate the compared data for P value calculation (2ng/ml peptide). (D) p85β−/− and p85β+/− F5TCRTg mice received NP366-374 peptide at day 0 (arrow) to induce a primary IR; injection was repeated at day 11 for the secondary IR. Mice were sacrificed at different times after peptide injection. T cell activation/memory
markers were analyzed in the CD8+/Vβ11+ population by flow cytometry. P values were calculated at 6h following secondary IR. *P<0.05.

**Figure 7. CBL downregulation and pPKB defects upon secondary stimulation of p85β−/− T cells.** (A-B) We measured CBL expression levels in antigen-experienced T cells; p85β−/− and p85β+/−F5TCRTg mice were injected with the NP366-374 peptide; 11 days later both mice groups were reinjected with the NP366-374 peptide, and CBL-b or c-CBL were analyzed in WB at different times post-injection (A). Alternatively, 11 days following first priming, mice were sacrificed and purified T cells were stimulated *in vitro* with peptide-pulsed APC for the times indicated. Cell extracts were examined in WB using antibodies as indicated (B). Figures are representative of at least three experiments with similar results. Graphs in (A) show the mean ± SD (n = 3) of CBL-b or c-CBL signal. Graphs in (B) show the mean percentage ± SD (n = 3) of the signal for each of the proteins, compared to the maximal signal in control cells (100%) and normalized in comparison with loading controls (n = 3). P values are shown for the times of maximal signal. * P < 0.05.
Figure 1

A

B

C

D

E

F
Figure 2
Figure 4

A.

- **Primary Response**
  - WT T cells vs. p85β⁻/⁻ T cells
  - Signal (A.U.) for CD3 and CD3+CD28
  - Time (h) from 0 to 24

B.

- **B:** Primary Response
  - p85β⁻/⁻ TCR vs. p85β⁻/⁻ TCR
  - Time (h) from 0 to 6
  - Signal (A.U.) for CBL-b and c-CBL

C.

- **C:** CD3 and CD3 + CD28
  - Time (h) from 0 to 24
  - Signal (A.U.) for CBL-b and c-CBL
  - ± + Ly 10μM
  - n = 3 for CBL-b, n = 5 for c-CBL
Figure 5

(A) % TCR Downregulation over time in WT, CD3+CD28, β−/− CD3, and β−/− CD3+CD28 cells. (B) Western blots showing Nedd4 and Actin expression in WT mice and rHAp85α and rHAp85β transduced Jurkat cells. (C) Bar graphs comparing CBL-b and c-CBL protein levels in Jurkat, rHAp85α, and rHAp85β transduced Jurkat cells. (D) % TCR Downregulation in p85β−/− and p85β+ transduced Jurkat cells. (E) % P-PKB and % P-p38 in p85β−/− and p85β+ transduced Jurkat cells.
Figure 6

A

B

C

D

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Figure 7

A

Secondary Response

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Time (h)

CBL-b

[p85β+/−] [p85β−/−]

Signal (A.U.)

Actin

c-CBL

n = 3

(*)

B

Secondary Response

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<th>p85β+/−</th>
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Time (min)

PKB

P-PKB

P-p38

p38

P-p44/42

p44/42

P-PKC

PKC

P-Tyr

n = 3

(*)
p85β phosphoinositide 3-kinase regulates CD28 co-receptor function

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