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## **ICOS ligation recruits the p50 $\alpha$ PI3K regulatory subunit to the immunological synapse<sup>1</sup>.**

**Camille Fos**<sup>\*, †, ‡</sup>, **Audrey Salles**<sup>\*, †, ‡</sup>, **Valérie Lang**<sup>§, ¶</sup>, **Florent Carrette**<sup>§, ¶</sup>, **Stéphane Audebert**<sup>\*, †, ‡, 2</sup>, **Sonia Pastor**<sup>\*, †, ‡</sup>, **Marguerite Ghiotto**<sup>\*, †, ‡</sup>, **Daniel Olive**<sup>\*, †, ‡</sup>, **Georges Bismuth**<sup>§, ¶</sup> and **Jacques A. Nunès**<sup>\*, †, ‡, 3</sup>.

\* Institut National de la Santé et de la Recherche Médicale, Unité **891**, Centre de Recherche en Cancérologie de Marseille, F-13009 Marseille, France; <sup>†</sup>Institut Paoli-Calmettes, Marseille, F-13009, France; <sup>‡</sup> Univ Méditerranée, F-13007, Marseille, France ; <sup>§</sup> Institut Cochin, Centre National de la Recherche Scientifique (UMR 8104), Université Paris Descartes, Paris, France. Equipe labellisée par la Ligue Nationale contre le Cancer. <sup>¶</sup> Institut National de la Santé et de la Recherche Médicale, Unité 567, Paris, France

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<sup>2</sup>Mass Spectrometry and Proteomics Platform, Institut de Cancérologie et d'Immunologie de Marseille (IFR137) CRCM, UMR **891** Inserm-Institut Paoli-Calmettes, 13009 Marseille, France.

<sup>3</sup>Address correspondence and reprint requests to Dr. Jacques A. Nunès, Centre de Recherche en Cancérologie de Marseille, 27 Bd Lei Roure, 13009 Marseille, France. [nunes@marseille.inserm.fr](mailto:nunes@marseille.inserm.fr)

<sup>4</sup>Abbreviations used in this paper: SH2, Src homology 2 domain ; IS, immunological synapse; PtdInsP<sub>2</sub>, phosphatidylinositol-4,5-bisphosphate; PtdInsP<sub>3</sub>, phosphatidylinositol-3,4,5-trisphosphate; WT, wild type; WB, western blot.

## **Abstract**

Inducible costimulator (ICOS) ligation in concert with TcR stimulation results in strong phosphoinositide 3-kinase (PI3K) activation in T lymphocytes. The ICOS cytoplasmic tail contains an YMF<sub>M</sub> motif that binds the p85 $\alpha$  subunit of class IA PI3K, similar to the YMN<sub>M</sub> motif of CD28, suggesting a redundant function of the two receptors in PI3K signaling. However, ICOS costimulation shows greater PI3K activity than CD28 in T cells. We show in this report that ICOS expression in activated T cells triggers the participation of p50 $\alpha$ , one of the regulatory subunits of class IA PI3Ks. Using different T-APC cell conjugate systems, we report that p50 $\alpha$  accumulates at the immunological synapse in activated but not in resting T cells. Our results demonstrate that ICOS membrane expression is involved in this process and that p50 $\alpha$  plasma membrane accumulation requires a functional YMF<sub>M</sub> SH2-binding motif in ICOS. We also show that ICOS triggering with its ligand, ICOSL, induces the recruitment of p50 $\alpha$  at the synapse of T cell/APC conjugates. In association with the p110 catalytic subunit, p50 $\alpha$  is known to carry a stronger lipid kinase activity compared to p85 $\alpha$ . Accordingly, we observed that ICOS engagement results in a stronger activation of PI3K. Together, these findings provide evidence that p50 $\alpha$  is likely a determining factor in ICOS mediated PI3K activity in T cells. These results also suggest that a differential recruitment and activity of class IA PI3K subunits represents a novel mechanism in the control of PI3K signaling by costimulatory molecules.

## Introduction

T lymphocyte activation is based on multiple and complex interactions between T cells and antigen presenting cells (APC). It is now commonly accepted that optimal T cell activation requires several independent signals (1). The first one, which determines the specificity of the immune response, is delivered through the antigen receptor interaction with the MHC-antigen complex on the APC surface. An additional costimulatory signal is crucial for complete T cell activation which leads to cytokine production and cell proliferation. On T cells, the best characterized costimulatory molecule is CD28, but several other costimulatory molecules like the ICOS inducible costimulator have also been described (2, 3). CD28 and ICOS (also known as CD278) deliver positive signals during T cell activation. They bind respectively to B7 molecules (B7.1/CD80 and B7.2/CD86) and ICOS ligand (ICOSL/CD275) expressed in various tissues, including APCs (4, 5). These two costimulatory receptors have unique and overlapping functions that synergize to regulate CD4<sup>+</sup> T cell differentiation (6). CD28 is expressed in resting and activated T cells, while ICOS expression is induced upon T cell activation. ICOS binds to a specific ligand, ICOSL, that is expressed on both lymphoid and non-lymphoid cells (4, 5). Recent studies indicated that the ICOS-ICOSL pathway plays an important role in Th2 responses in asthma and allergies (7, 8), Th1-Th2 responses during bacterial infections (9, 10), tumor cell rejection and during the pathologic process of chronic graft rejection *in vivo* (11, 12). ICOS mediated costimulation leads predominantly to the production of effector cytokines such as IL-4 and IL-10 and to a lesser extent in the production of IL-2, IFN- $\gamma$  and TNF- $\alpha$  (4, 13, 14).

In humans, signaling pathways induced by the costimulatory molecule ICOS remain poorly understood. ICOS bears a unique YXXM signaling motif in its intracellular tail that binds the p85 $\alpha$  regulatory subunit of phosphoinositide 3-kinase (PI3K) (7, 15). This is in contrast to CD28 that has several SH2<sup>4</sup> and SH3 binding motifs for proteins such as Grb2, GRID, Lck, Itk and Tec (16). ICOS signaling has been shown to essentially involve the PI3K pathway since so far, p85 $\alpha$  is the only identified signaling molecule that interacts with ICOS (17). Although ICOS seems to be more limited in generating intracellular signals, ICOS stimulation in combination with TcR shows a much stronger capability to activate PI3K than CD28 (18, 19). Class IA PI3K is composed of a p110 catalytic subunit (either the  $\alpha$ ,  $\beta$  or  $\delta$  isoforms) and a regulatory subunit which can be p85 $\alpha$ , p55 $\alpha$  or p50 $\alpha$  (encoded by the *pik3r1* gene), p85 $\beta$  (encoded by the *pik3r2* gene) or p55 $\gamma$  (encoded by the *pik3r3* gene) (20).

p85 $\alpha$ , p55 $\alpha$  and p50 $\alpha$  regulatory subunits share a common C-terminal region consisting of two SH2 domains flanking the p110 catalytic binding site and have a unique N-terminal region of 304, 34 and 6 amino acids respectively (21).

Although it has been showed that p50 $\alpha$  associated PI3K activity in response to insulin stimulation is greater than p85 $\alpha$  or p55 $\alpha$  (21-23), little is known about their relative function and contribution during T cell activation. We therefore made the hypothesis that the difference in PI3K activity between ICOS and CD28 costimulation could be linked to a differential recruitment of the regulatory isoforms.

In this study, we show that the p50 $\alpha$  PI3K regulatory subunit is recruited by costimulatory receptors only on activated T cells. Despite similar expression levels before and after cell activation, we also report that p50 $\alpha$  accumulates at the immunological synapse (IS) in activated but not in resting T cells. We show that ICOS may have a specific role in recruiting p50 $\alpha$  at the membrane, as ICOS triggering induces p50 $\alpha$  localization to the plasma membrane through its YMFM intracellular motif. **Upon ICOS ligation, p50 $\alpha$  associated PI3K activity is stronger than that associated with p85 $\alpha$ .** Finally ICOS but not CD28 ligation, induces a strong phosphorylation of Akt in activated T cells. Collectively, our results suggest that ICOS makes use of the p50 $\alpha$  regulatory subunit to sustain PI3K activity in activated T cells.

## Materials and Methods

### Cells

Human CD4<sup>+</sup> T cells and monocytes were isolated from healthy blood donors by Ficoll density gradient centrifugation followed by negative and positive depletion respectively on magnetic beads (CD4<sup>+</sup> T Cell Isolation Kit II human, CD14 microbeads, Miltenyi). To induce ICOS expression, CD4<sup>+</sup> T cells were activated with CD3-CD28 coated beads (Dynabeads® CD3/CD28, Invitrogen) according to manufacturer's instructions. Beads were removed after 24 hrs of stimulation and cells were harvested for an additional 24 hrs. Peripheral blood monocytes were derived into dendritic cells (monocyte-derived DCs) in RPMI 10% FCS, 20 ng/ml IL-4, 100 ng/ml GM-CSF (Abcys) for 5 days before a 24 hrs maturation period in the presence of 2.5 µg/ml of LPS (Sigma-Aldrich). The Jurkat cell line JA16 and the JICOS.1 clone stably expressing the ICOS receptor were cultured in RPMI 10% FCS. To make the JICOS.1 cell line, human ICOS cDNA was cloned into a βDNA4 vector (24) between the NotI-SpeI restriction sites. The Raji B cell line was cultured in RPMI 10% FCS. L cells stably expressing the ICOS-L protein (L-LICOS cells) (25) or the B7.1 protein (L-B7.1 cells) (26) were cultured in DMEM 10% FCS. L-LICOS cells are a kind gift from R.A. Kroczeck and H.W. Mages (RKI, Berlin, Germany).

### ICOS, CTLA-4 and PD-1 Fc fusion proteins

Extracellular domains of ICOS, CTLA-4 or PD1 were amplified by PCR from human activated T cell cDNAs, and cloned in frame with the Fc fragment of the human IgG1 sequence using the Cos Fc link vector (SmithKline Beecham Pharmaceuticals, King of Prussia, PA). Fc fusion proteins were produced in COS cells harvested in CHO-S-SFM II medium (Invitrogen). Culture supernatants were collected 7 days after transfection, filtered and loaded on a 5-ml Affigel protein A column according to the manufacturer's protocol (Bio-Rad). After washing, the proteins were eluted with a 0.1mol/L citrate buffer, pH 3.5, concentrated, and dialyzed against phosphate-buffer saline (PBS). Purification steps were monitored by ELISA using a sandwich revelation system with coated antibody anti-human IgG-UNLB and human IgG-AP (Southern Biotechnology Associates) and revealed by pNPP substrate (Sigma). Purity and quality of the human Ig fusion proteins were controlled by gel electrophoresis and by cell surface staining on L-LICOS cells, L-B7.1 cells or PD-L1 transfected COS cells.

## T cell stimulation/Immunoprecipitation/Immunoblotting

Prior to immunoprecipitation or in order to measure the phosphorylation status of Akt, cells were stimulated with anti-CD3 antibody (clone 289) and anti-CD28 (clone CD28.2 [(27)]) or anti-ICOS antibody (# C398.4A, BioLegends) (10 µg/ml each), or with L-LICOS, L-B7.1 or L-LTK<sup>-</sup> cells for 15 min at a 1:2.5 ratio. Following stimulation, 1% NP40 cell lysates were subjected to immunoprecipitation with 5 µg of CD28.2 or ICOS antibodies and blotted with a polyclonal rabbit antiserum that recognizes all regulatory alpha subunits (anti-pan p85α, # 06.195, Millipore). GFP-p50α and GFP-p85α constructs were transfected in Jurkat cells. Cell lysates were immunoprecipitated with anti-GFP antibodies (clones 7.1 and 13.1, Roche Diagnostic) and immunoblot analyses were performed with rabbit GFP polyclonal antibody (#ab6556, Abcam) and Myc Tag antibody (# ab9106, Abcam). In order to disrupt receptor-ligand interactions, L cells were pre-incubated with 10 µg/ml anti-LICOS (# 136726, R&D Systems) or anti-CD80 (clone 2D10.4, (26)) antibodies prior to stimulation. Cells were lysed and subjected to Western blot analyses with anti-phospho Akt, anti-Akt, **anti-phospho-GSK-3α/β (Ser<sup>21/9</sup>)** and **GSK-3α** antibodies (#4060, #9272, #9331, #9338 respectively, Cell Signaling Technology).

## Constructs

ICOS constructs consist of a Myc Tag epitope inserted between the signal peptide and the extracellular part of murine CD28 followed by the transmembrane and intracellular tail of human ICOS (see diagram Fig. 3B). In the ICOS Y180F construct, the tyrosine residue at the position 180 was mutated to phenylalanine. Both constructs were cloned into a βDNA4 vector. The GFP-p85α and GFP-p50α constructs were cloned according to the same procedures (28). Briefly, human p85α and p50α were cloned into pCR2.1-TOPO (Invitrogen Life Technologies) and then subcloned into the pEGFP-C1 fusion vector (BD Clontech) as an *EcoRI* restriction enzyme fragment, resulting in expression of a N-terminal GFP fusion protein. The Myc-p110δ construct is a kind gift from B. Vanhaesebroeck (Institute of Cancer, London, UK) and has been cloned according to previously published data (29).

## Cell transfections

Freshly prepared human unstimulated (U-023 program) or CD3+CD28 stimulated (U-024 program) primary CD4<sup>+</sup> T cells were transfected with the Nucleofector technology (AMAXA biosystems) according to the manufacturer's instructions with 5 µg of the indicated construct.

Jurkat cell lines were transfected using the Biorad apparatus Gene Pulser Xcell (250V, 25ms, 4mm) with 20 µg of the indicated construct for 24 hrs.

#### Determination of PI3K activity

$10^7$  activated CD4<sup>+</sup> T cells (transfected or not with GFP constructs) were left unstimulated or were stimulated for 15 min at 37 °C with L-LICOS or L-B7.1 cells at a 1:2.5 ratio. The cells were lysed for 10 min with 0.5 ml of 1% NP-40 lysis buffer. ICOS, CD28 or GFP immunoprecipitates were performed from whole-cell lysates for 2 hrs at 4 °C, and washed with TNE buffer (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA and 0.1 mM sodium orthovanadate). The PI3K activity was determined using a PI3-kinase ELISA kit according to manufacturer's instructions (Echelon Biosciences, Salt Lake City, UT, USA). For each condition, the kinase reaction was run in triplicates.

#### Immunofluorescence labelling

*ICOS Capping.* Capping experiments were performed according to previously published procedures (30). Briefly,  $10^7$  T cells were incubated with anti-Myc antibody (Santa Cruz) at 4°C for 30 min followed by an additional 30 min incubation with Alexa 594 goat anti-mouse antibody (Sigma). Capping was performed at 37°C for 15 min. Cold PBS was added to stop the reaction, and cells were fixed and analyzed by confocal microscopy. *Jurkat/Raji conjugates.* To distinguish APCs from Jurkat cells, Raji B cells were loaded with Cell Tracker® Red CMTMR (Molecular Probes) for 30 min at 37°C, washed, and resuspended in RPMI 1640 with 10% FCS. Cells were then incubated for 15 min in the presence of 1 µg/ml of SEE Superantigen (Toxin Technology). Jurkat cells were mixed with an equal number of Raji cells in a final volume of 100 µl and were incubated at 37°C for 20 min. Conjugates were plated on poly-L-lysine-coated coverslips and fixed for 20 min in 4% formaldehyde. *T cells/L cells conjugates.* To distinguish APCs from T cells, L-LICOS or L-B7.1 cells were plated on poly-L-lysine-coated coverslips one day before and then loaded with Cell Tracker® Red CMTMR (Molecular Probes) for 30 min at 37°C, washed, and resuspended in RPMI 1640 with 10% FCS. T cells were added at a ratio of 1:3 for 20 min and fixed as above. *T cells/moDC conjugates.* DCs were plated, loaded with the cell tracker and pulsed with a cocktail of superantigens (SEA, SEB, SEC, SEE [final concentration, 1 ng/µl each], Toxin Technology) at the same time for 1 hr at 37°C. Before conjugation, receptor-ligand blockade was performed using Ig constant fragment fusion proteins at 10 µg/ml each for 1 hr on DC's. T cells were then added at a ratio of 1:3 for 20-45 min. Cells were fixed as described above.

The proportion of conjugates with GFP-p50 $\alpha$  or GFP-p85 $\alpha$  redistributed to the cell/cell contacts was calculated by randomly choosing 300 different conjugates. *For p50 $\alpha$ /ICOS colocalization experiments*, GFP-p50 $\alpha$  transfected T cells were mixed with DCs as previously described. T cells were labelled with indirect fluorescence staining using anti-ICOS antibody (# C398.4A, BioLegends) for 1 hr followed by Alexa Fluor 546 goat anti-hamster antibody (Molecular Probes) staining for 30 min. Confocal microscopy was carried out on a Zeiss LMS 510 META using a 63 x 1.4 oil Plan-Apochomat objective lens. For cytometry analysis, cells were labelled with CD28-PE and ICOS-PE antibodies (clones # CD28.2 and DX29 respectively, BD Biosciences Pharmingen).

#### Statistical analysis

Statistical analysis was performed with the Wilcoxon-Mann-Whitney test using Cytel's StatXact® software. Values of  $p < 0.05$  were considered significant.

## Results

The p50 $\alpha$  PI3K regulatory subunit is recruited by costimulatory receptors only on activated T cells.

Class IA PI3K members have been implicated in immune T cell signaling. However, little is known about the expression status of PI3K regulatory subunit isoforms in hematopoietic cell lines and in particular, in human T lymphocytes. Indeed, most studies involving PI3K signaling events focussed on p85 $\alpha$  expression and function neglecting the participation of other PI3K regulatory subunits. In order to determine the expression pattern of class IA PI3K regulatory subunits in resting and activated human T cells, Western blot analyses of cell lysates with a polyclonal antiserum that recognizes all regulatory alpha subunits were performed (Fig. 1A). In resting T cells, two bands corresponding to p85 $\alpha$  and p50 $\alpha$  were detected. An additional 55kDa band corresponding to p55 $\alpha$ , appeared in activated T cells. Like the CD28 costimulatory receptor, ICOS, whose expression is induced following T cell activation (Fig. 1B), is known to bind the p85 $\alpha$  regulatory subunit. Upon CD28 or ICOS ligation, the binding of the different PI3K regulatory subunits to CD28 or ICOS was next investigated. As shown in Fig. 1C, (left panel), in resting T cells, p85 $\alpha$  but not p50 $\alpha$  is able to interact with CD28 following receptor triggering. In activated T cells, a slight constitutive association of p85 $\alpha$  and p55 $\alpha$  with ICOS and CD28 was observed. However, ICOS triggering (alone or in combination with CD3 antibody) led to the recruitment of p50 $\alpha$  within the receptor. ICOS ligation also greatly enhanced its association with p85 $\alpha$  compared to unstimulated or CD3+CD28 stimulated T cells (Fig. 1C, middle panel). Thus, similar levels of p85 $\alpha$  and p50 $\alpha$  association to CD28 or ICOS were detected upon stimulation, but surprisingly, ICOS ligation promoted CD28 association “in trans” with the two regulatory subunit isoforms as efficiently as CD28 triggering (Fig. 1C, right panel). These results demonstrate a selective recruitment of p50 $\alpha$  to ICOS and CD28 receptors in activated T cells. They also suggest that ICOS triggering strengthens CD28 interactions with class IA alpha regulatory subunits.

ICOS costimulation shows a stronger ability to activate PI3K than CD28 costimulation (19). Using PI3K activity ELISA kit, we showed that ICOS ligation induces a higher PI3K activity in activated T cells compared to CD28 ligation (Fig. 1D). As it has been previously reported that p50 $\alpha$  and p85 $\alpha$  are carrying distinct PI3K activities (21-23), these observations

prompt us to investigate the dynamic of recruitment of these regulatory subunits at the plasma membrane of activated T cells.

The p50 $\alpha$  regulatory subunit localizes at the immunological synapse on activated T lymphocytes.

The p85 $\alpha$  and p50 $\alpha$  regulatory subunits are derived by alternative splicing of the same gene *pik3r1* (31). These molecules share two SH2 domains, the p110-binding inter-SH2 domain and a proline-rich region. They differ in their N-terminal domain: p50 $\alpha$  has a unique 6 amino-acid sequence and p85 $\alpha$  has a specific Bcr homology domain (BH) and a SH3 domain. We used GFP fusion proteins of p85 $\alpha$  and p50 $\alpha$  (Fig. 2A) in order to visualize the recruitment of these regulatory subunits in T cell/APC conjugates. It is now commonly accepted that class IA PI3K regulatory subunits stabilize the unstable and labile p110 catalytic subunits (32). To verify that our GFP-p50 $\alpha$  and GFP-p85 $\alpha$  constructs were functional, immunoprecipitation experiments were carried out in Jurkat T cells coexpressing a Myc-tagged form of the p110 $\delta$  catalytic subunit and either GFP-p50 $\alpha$  or GFP-p85 $\alpha$ . Unlike the GFP molecule used in the control, both GFP-p50 $\alpha$  and GFP-p85 $\alpha$  constructs bind the co-transfected p110 $\delta$  catalytic subunit molecule (Fig. 2B). We next evaluated p85 $\alpha$  and p50 $\alpha$  recruitment to the immunological synapse in a system of T-DC conjugates by mixing SAg pulsed DCs with either resting T cells or activated T cells transfected with GFP-p50 $\alpha$  or GFP-p85 $\alpha$  constructs (Fig. 2C). A similar GFP-p50 $\alpha$  or GFP-p85 $\alpha$  relocalization to the immunological synapse was observed at different times of cell conjugates formation (data not shown). Imaging analysis of the formed conjugates showed that p50 $\alpha$  and p85 $\alpha$  regulatory subunits were differentially recruited to the T-DC contact area depending on the activation state of T cells (Fig. 2D). Thus, p50 $\alpha$  was recruited exclusively at the IS in activated T cells, whereas p85 $\alpha$  was similarly recruited at the IS in resting or activated T cells.

ICOS overexpression brings p50 $\alpha$  to the immunological synapse through its YxxM motif.

As shown previously, the p50 $\alpha$  regulatory subunit preferentially localizes at the contact zone between an activated T lymphocyte and a dendritic cell (Fig. 2D), and the ICOS receptor seems to be a good candidate for recruiting the adaptor protein when triggered by its ligand (Fig. 1C). To further demonstrate the role of ICOS in recruiting p50 $\alpha$  at the IS, we analyzed

its localization in conjugates formed between Jurkat cells stably expressing ICOS (JICOS.1) or Jurkat parental cells (JA16) and SEE-pulsed Raji B cells, an APC known to express ICOS ligand (40). As shown in Fig. 3A, upper panel, the two cell lines only differ in ICOS expression levels. While JICOS.1 cells express high level of ICOS, JA16 cells express very low levels of the ICOS costimulatory receptor. Both T cell lines express the CD28 receptor at a similar level. p50 $\alpha$  was found to be recruited to the IS twice as much in JICOS.1/Raji cell conjugates in comparison to JA16/Raji cell conjugates, showing that the increase in ICOS expression led to greater p50 $\alpha$  accumulation to the immunological synapse. In contrast, p85 $\alpha$  was recruited similarly by both T cell lines (Fig. 3A). We next investigate the role of the ICOS YxxM intracellular motif in this process, a motif which has previously been shown to recruit p85 $\alpha$  (7). Tyrosine residue 180 in the YMF $\alpha$ M motif was mutated to phenylalanine in a Myc-tagged chimeric molecule composed of the extracellular domain of murine CD28 fused to the cytoplasmic domain of human ICOS (Fig. 3B, upper panel). Localization of this ICOS mutant together with GFP-p50 $\alpha$  or GFP-p85 $\alpha$  molecules was analyzed after anti-Myc-mediated capping. As shown in Fig. 3B, lower panel, the GFP-p50 $\alpha$  molecule is distributed throughout the cell before capping. Upon Myc cross-linking, GFP-p50 $\alpha$  was substantially recruited at the plasma membrane and identically localized with the ICOS receptor into caps. Tyrosine mutation into phenylalanine abrogated ICOS and GFP-p50 $\alpha$  colocalization at the membrane surface. While ICOS aggregated into caps, GFP-p50 $\alpha$  remained diffused in the whole cell. Taken together, these data provide evidence that ICOS expression drives p50 $\alpha$  relocalization at the IS via its YxxM motif.

ICOS and p50 $\alpha$  localize at the immunological synapse in activated T cells.

We showed that ICOS ligation induced p50 $\alpha$  association within the intracellular tail of the receptor (Fig. 1C), and that ICOS overexpression in Jurkat cells led to increased p50 $\alpha$  localization at the IS (Fig. 3A). As ICOS may have a specific role in recruiting the p50 $\alpha$  regulatory subunit to the plasma membrane in activated T cells, we directly investigated ICOS and GFP-p50 $\alpha$  localization to the IS between activated CD4<sup>+</sup> T cells and dendritic cells. Fig. 4A shows that ICOS and p50 $\alpha$  colocalized at the contact zone. To further analyse the contribution of ICOS in this recruitment of p50 $\alpha$ , we used Fc-fusion proteins containing the extracellular domain of ICOS or CTLA-4 to disrupt specific interactions between the receptors and their respective ligands (Fig. 4B). A PD-1-Fc fusion molecule was used as a

control. By disturbing the ICOS/ICOS-L interaction with an ICOS fusion protein, a slight decrease in p50 $\alpha$  recruitment at the IS was observed in activated T cells. In contrast, CTLA-4-Fc disruption of CD28/CD80 interactions affected predominantly p85 $\alpha$  IS localization. We also performed cell conjugates between L-LICOS or L-B7.1 cells and activated T cells which allowed the triggering of ICOS or CD28 with a limited contribution of other molecules expressed on T cells. The results showed that p85 $\alpha$  was similarly recruited at the contact area when ICOS or CD28 was engaged by its respective ligand. In contrast, ICOS-L was much more efficient than B7.1 in triggering p50 $\alpha$  localization at the cell-cell contact (Fig. 4C). **In order to selectively determine the PI3K activity associated to the different regulatory isoforms under ICOS ligation, activated T cells were transfected with GFP, GFP-p50 $\alpha$  or GFP-p85 $\alpha$  constructs for 24 hrs. Similar level of GFP proteins were detected by flow cytometry (data not shown). T cells were then stimulated with L-LICOS cells for 15 min as previously described. The PI3K assay was performed on GFP immunoprecipitates (Fig. 4D). Surprisingly, the GFP protein alone, used as a control, showed a basal PI3K activity. Nevertheless, the PI3K activity associated with both regulatory subunit isoforms (GFP-p50 $\alpha$  or GFP-p85 $\alpha$ ) was higher compared to our control (GFP alone). As previously described for the insulin receptor system in muscle homogenates (21), p50 $\alpha$  associated PI3K activity was higher compared to p85 $\alpha$  upon ICOS ligation in activated T cells. Together, these data provide strong evidence that ICOS receptor expression and engagement facilitate p50 $\alpha$  localization at the plasma membrane in activated T cells, thus probably leading to the strong PI3K activity associated to the receptor.**

ICOS ligation alone, but not CD28, is able to induce a strong phosphorylation of the PI3K effector Akt.

Although little is known about ICOS signaling pathways, it is now clearly established that ICOS is a major PI3K activator associated with strong kinase activity (7, 19), thus leading to strong subsequent phosphorylation of PI3K downstream effectors such as PDK1 and Akt/PKB (18, 33). To investigate the role of ICOS mediated PI3K activity in T cell conjugates, we performed ICOS triggering with ICOS ligand overexpressing L cells and analyzed the phosphorylation status of the serine/threonine kinase Akt/PKB (Fig. 5). Briefly, activated T cells remained unstimulated or stimulated either with a combination of CD3+CD28 antibodies or with L-LICOS or L-B7.1 cells. **In order to determine the activation status of Akt, a kinetic**

analysis of its phosphorylation was performed on activated T cells. As shown in Fig. 5A, ICOS ligation by its natural ligand induced a stronger Akt phosphorylation than CD28, at any tested time of stimulation from 5 to 60 min, reaching its maximum after 15 min. The same experiments were performed with blocking mAbs to analyse the specific contribution of ICOS in this event. In order to disrupt ICOS/ICOS-L or CD28/B7.1 interactions, L-cells were previously incubated for 15 min with anti-ICOS-L or anti-CD80 antibodies and used to stimulate activated T cells. Fig.5B showed that this very strong Akt phosphorylation induced by ICOS was specific as it was blocked after anti-ICOS-L pre-incubation. Following its activation, Akt phosphorylates the glycogen synthase kinase-3 (GSK-3), thus leading to its inactivation (34). A significant basal GSK-3 phosphorylation was detected in activated T cells that could reflect the activated status of these cells. However, amongst the different stimuli used to further activate the cells, ICOS ligation induced the strongest increase in GSK-3 phosphorylation which was partially blocked by the anti-ICOS-L antibody (Fig. 5B). Taken together, these data show that ICOS ligation leads to a strong activation of PI3K/Akt-dependent signaling pathways in activated T cells.

## Discussion

In T cells, Class IA PI3Ks are typically activated downstream of TcR and costimulatory molecule engagement. Mice lacking the major class IA regulatory subunits show selective impairments in T cell function and critical defects in costimulatory mediated events (35). In particular, *pik3r1* and *pik3r2* defective T cells have reduced helper functions for B cells. Due to genetic and functional redundancy of regulatory and catalytic PI3K subunits, and because of compensatory effects between all the class IA PI3K members, discrimination of individual regulatory subunit associated functions remains difficult to investigate (36-38). Previous studies investigating PI3K signaling events and functions mostly focussed on p85 $\alpha$  participation. However, there is now growing evidence that differences in the use of regulatory and/or catalytic subunits might occur through costimulatory receptors or adaptor proteins and that PI3K members probably display distinct biological and signaling functions (36).

ICOS and CD28, both members of the CD28 family of costimulatory receptors, recruit the p85 $\alpha$  regulatory subunit, thus leading to PI3K activation and signaling (7, 26). Expressed following T cell activation, ICOS shows much stronger PI3K activity compared to CD28 (Fig. 1D), as it is able to produce greatest amounts of PtdInsP<sub>3</sub> leading to strong subsequent phosphorylation of the serine/threonine kinase Akt/PKB, a key PI3K effector (18, 19). The mechanism of PI3K regulation has been well described in insulin signaling (39). All studies performed on the PI3K signaling pathway associated with the IRS-1 protein tend to show the same results and confer to the p50 $\alpha$ -p110 complex a stronger PI3K activity compared to p55 $\alpha$  or p85 $\alpha$  regulatory subunits (21). We postulated that ICOS could be a good candidate in differentially recruiting PI3K regulatory subunits within its intracellular YxxM binding motif and that the p50 $\alpha$  regulatory subunit could play an important role in ICOS mediated PI3K activity. Indeed, ICOS associated PI3K signaling pathway seems to be the “key” pathway involved in receptor functions. Consistent with previous studies (17), pulldown experiments performed with phospho-YxxM peptides, followed by mass spectrometry analysis showed that a unique PI3K signaling pathway is involved in ICOS mediated costimulation. The ICOS intracellular tail exclusively allows functional binding of the regulatory subunit isoforms of class IA PI3K who can in turn, recruit the p110 catalytic subunits (Camille Fos, Stéphane Audebert, data not shown). We also corroborate data showing that the same YxxM intracellular motif of CD28 is a scaffold for several signaling pathways, as it is able to recruit

proteins such as Sos1/2, Cbl, SLP-76, Grb2, and GRID (36). Unlike CD28 that is constitutively expressed, ICOS can only be detected on the surface of activated T cells (4) (Fig. 1B). Both p50 $\alpha$  and p85 $\alpha$  are expressed on resting T cells, but only p85 $\alpha$  binds to CD28 upon receptor ligation (Fig. 1A and 1C). On activated T cells, ICOS triggering leads to p50 $\alpha$  accumulation to the immunological synapse and association with the YMFV motif of the receptor (Fig. 3). In contrast to p85 $\alpha$  and p55 $\alpha$  that are associated with ICOS at a basal state, p50 $\alpha$  association with the receptor is an inducible event that occurs following ICOS ligation (Fig. 1C). Basal association of p85 $\alpha$  to the receptor is greatly increased upon ICOS ligation. This basal association of p85 $\alpha$  and p55 $\alpha$  is also found in CD28 immunoprecipitates and CD28 ligation significantly increases the association of regulatory subunits with the receptor. Surprisingly, ICOS ligation promotes and induces p50 $\alpha$  association with CD28 in a way that remains to be explored. However, one can imagine that ICOS brings p50 $\alpha$  to the immunological synapse, perhaps in membrane signaling microdomains, and thus provides a p50 $\alpha$  reserve to other transmembrane receptors involved in T cell activation (40-42). In the same way that CD28 has been shown to enhance and sustain TcR signaling, we can now consider that the ICOS receptor is able to cooperate with CD28 and to strengthen CD28 induced signaling pathways. A cross-talk between the two CD28 family members may occur at the immunological synapse and needs to be further investigated.

By using different T/APC cell conjugates, we show that ICOS is efficient in bringing p50 $\alpha$  to the immunological synapse. By using a T cell model overexpressing ICOS (Fig. 3A) or by triggering ICOS alone with limited contribution of other costimulatory molecules (Fig. 4C), we show that ICOS plays a critical role in recruiting p50 $\alpha$  to the plasma membrane. On T/DC cell conjugates, the participation of ICOS is more difficult to evaluate. When blocking ICOS/ICOS-L (or CD28/CD80) pathway, only a slight but significant decrease is observed in p50 $\alpha$  or p85 $\alpha$  relocalization to the immunological synapse (Fig. 4B). Indeed, in this physiological context, several PI3K adaptors and YxxM bearing receptors are involved in recruiting PI3K to the membrane and their relative contribution is difficult to evaluate. siRNA directed to p50 $\alpha$  or p85 $\alpha$  were designed in order to selectively extinguish these regulatory subunits and to investigate their role in ICOS mediated PI3K signaling and effector functions. Unfortunately, we were not able to downregulate PI3K gene expression in human T cells or in other T cell lines. p85 $\alpha$  and p50 $\alpha$  expression remained the same in all analysed conditions. Furthermore, when a downregulation was observed in heterologous cell lines, both regulatory subunits were touched in a non selective way (data not shown). Knock-out mice models of *pik3r* genes showed that class IA PI3K regulatory subunits are important for T cell functions

(35, 38). Great efforts have been made to better understand the participation of each regulatory subunit but there is real difficulty in evaluating the relative contribution of each subunit because of compensatory effects between class IA members. Since we show that p50 $\alpha$  accumulates to the IS in activated T cells only, the phenotype of *pik3r* knock-out mice should be reevaluated in secondary responses when receptors susceptible to associate with p50 $\alpha$  are expressed. Indeed, maintenance of T cell longevity has been shown to involve OX40 costimulation-regulated duration of Akt activation (43). Like OX40 that is induced following T cell activation, ICOS ligation provides a strong activation signal for the serine/threonine kinase Akt (Fig. 5), which can in turn regulate antiapoptotic molecules such as GSK-3. Following T cell activation, the PI3K/Akt/GSK-3 pathway might thus be involved in IL-10 production by ICOS expressing cells. Indeed, previous data showed that Akt mediated GSK3 inactivation promotes a great increase in IL-10 production following Toll-like receptor ligation (44). In contrast to CD28 that predominantly acts during the primary response, ICOS plays a role in restimulation and clonal expansion of T and B effector cells during secondary responses. ICOS, whose expression has been described not only on freshly activated T cells, but also on regulatory and memory T cells, can interact with its ligand on non-professional and non-hematopoietic “APCs” such as epithelial or endothelial cells under pro-inflammatory conditions (45, 46). ICOS ligation in inflamed peripheral tissues could thus induce a sustained PI3K activation thereby regulating T cell energy metabolism and allowing quick and efficient responses to antigen restimulation (47). Animal models targeting exclusively the p50 $\alpha$  regulatory subunit would be useful for further evaluating p50 $\alpha$  functions in metabolism and regulation of activated T cells.

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**Figure legends**

**Figure 1. p50 $\alpha$  regulatory subunit associates with YxxM bearing receptors in activated but not in resting T cells.** A. Immunoblot analysis of class IA PI3K regulatory subunits expression in human CD4<sup>+</sup> resting versus CD3<sup>+</sup>CD28 microbeads activated T cells for 48 hrs. Data represent 3 different donors. B. Flow cytometry analysis of resting or activated T cells stained with phycoerythrin (PE) labelled anti-CD28 or anti-ICOS antibodies. C. Binding of class IA regulatory subunits to YxxM costimulatory receptors. Resting or activated CD4<sup>+</sup> T cells were stimulated for 15 min with anti-CD3 plus anti-CD28, anti-CD3 plus anti-ICOS antibodies or LICOS or B7.1 expressing cells. Cell lysates were subjected to CD28 or ICOS immunoprecipitation and a class IA regulatory subunit (pan p85) Western blot was performed. Data are representative of 2 independent experiments. Molecular weight standards (kDa) are indicated on the left side of the panel. **D. ICOS and CD28 associated PI3-kinase activity. Following receptor ligation, cells were lysed and receptors immunoprecipitations were performed. The PI3-kinase activity was quantified in immunoprecipitates with an ELISA plate reader. Results are expressed in pmoles of produced PIP<sub>3</sub> per 10<sup>6</sup> T cells. Bars indicate means  $\pm$  SEM of triplicate determinations.**

**Figure 2. The p50 $\alpha$  regulatory subunit accumulates at the immunological synapse in activated but not in resting T cells.** A. Schematic representation of GFP-PI3K regulatory subunit constructs and their interaction with p110 catalytic subunit. B. Jurkat T cells were co-transfected with the p110 $\delta$ -myc and GFP-p50 $\alpha$  or GFP-p85 $\alpha$  constructs for 24hrs. Immunoprecipitation with anti-GFP antibodies was performed followed by a anti-GFP or anti-Myc antibody Western blot. C. Differential interference contrast (DIC) and green fluorescence images of GFP-p50 $\alpha$  or GFP-p85 $\alpha$  relocalization between transfected T cells and DC conjugates. Resting or activated human CD4<sup>+</sup> T cells transfected with GFP-p50 $\alpha$  or GFP-p85 $\alpha$  constructs were mixed with mature dendritic cells for 20 min at a ratio of 1:3. DCs were previously pulsed with a cocktail of SAg. D. The proportion of conjugates with GFP-p50 $\alpha$  or GFP-p85 $\alpha$  redistributed to the cell/cell contact was calculated by randomly choosing 300 different conjugates for each experiment. Results shown in diagrams correspond to 3 experiments  $\pm$  SD values. Asterisks indicate significant p-values ( $p < 0.05$ ).

**Figure 3. Upregulation of ICOS expression allows p85 $\alpha$  and p50 $\alpha$  association through its YxxM motif.** A. Upper panel. Flow cytometry analysis of Jurkat cells (JA16) or ICOS overexpressing Jurkat cells (JICOS.1) using PE labelled anti-ICOS antibody or PE labelled

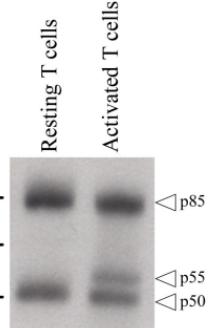
**anti-CD28 antibody.** Lower and right panels. p50 $\alpha$  and p85 $\alpha$  relocalization at the IS between JA16 or JICOS.1 and SEE pulsed Raji B cells. JA16 or JICOS.1 cells transfected with GFP-p50 $\alpha$  or GFP-p85 $\alpha$  constructs were mixed with Raji B cells for 20 min at a ratio of 1:1. Raji B cells were previously labelled with cell tracker CMTMR. The proportion of conjugates with GFP-p50 $\alpha$  or GFP-p85 $\alpha$  redistributed to the cell/cell contact was calculated by randomly choosing 300 different conjugates for each experiment. Results shown in diagrams correspond to 3 experiments  $\pm$  SD values. Asterisks indicate significant p-values ( $p < 0.05$ ). B. Capping experiments were performed on human activated T cells co-expressing MycICOS wt or MycICOS Y180F and GFP-p50 $\alpha$  or GFP-p85 $\alpha$  constructs. Briefly, the cells were incubated with anti-Myc antibody on ice for 30 min and then with goat anti-mouse Alexa 594 antibody for an additional 30 min. The capping of ICOS was performed at 37°C for 15 min. Cells were fixed on paraformaldehyde and analyzed by confocal microscopy.

**Figure 4. ICOS accumulates at the immunological synapse in T/DC conjugates and colocalizes with p50 $\alpha$  PI3K regulatory subunit.** A. Activated T cells were transfected with the GFP-p50 $\alpha$  construct and mixed with mature dendritic cells at a ratio of 1:3. DCs were previously pulsed with a cocktail of SAg. ICOS localization at the immunological synapse was investigated by cell labeling of T/DC conjugates with an anti-ICOS antibody followed by goat anti-hamster Alexa 546 antibody. B. Activated human T cells transfected with GFP-p50 $\alpha$  or GFP-p85 $\alpha$  construct were mixed with mature DC for 20 min at a ratio of 1:3 (B) or with L-LICOS or LB7.1 cells (C). The proportion of conjugates with GFP-p50 $\alpha$  or GFP-p85 $\alpha$  redistributed to the cell/cell contact was calculated by randomly choosing 300 different conjugates for each experiment. Results shown in diagrams are corresponding to 3 experiments  $\pm$  SD values. Asterisks indicate significant p-values ( $p < 0.05$ ). **In the panel B, the Y axis corresponds to the relative percentage of GFP constructs relocalization at the IS in presence of blocking reagents. The percentage obtained in cell conjugates formed without blocking fusion proteins was considered as 100%.** D. GFP-p50 $\alpha$  and GFP-p85 $\alpha$  associated PI3-kinase activity. Activated T cells were transfected with a GFP, a GFP-p50 $\alpha$  or a GFP-p85 $\alpha$  construct as previously described. Following ICOS ligation, cells were lysed and a GFP immunoprecipitation was performed. The PI3-kinase activity was quantified in immunoprecipitates with an ELISA plate reader. Results are expressed in pmoles of produced PIP<sub>3</sub> per 10<sup>6</sup> T cells. Bars indicate means  $\pm$  SEM of triplicate determinations.

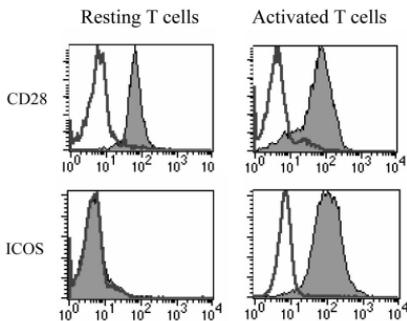
**Figure 5. ICOS ligation induces a strong activation of PI3K signaling dependent events.**

A. Time course analysis of Akt phosphorylation upon ICOS or CD28 ligation. Human activated CD4<sup>+</sup> T cells were left unstimulated (NS) or stimulated for 5 min with anti-CD3 plus CD28 antibodies, ICOS ligand expressing L cells (L-LICOS) or B7.1 ligand expressing L cells (L-B7.1) for indicated times. B. In order to disrupt receptor-ligand interactions, activated T cells were stimulated as above for 15 min with L-LICOS or L-B7.1 cells pre-treated with anti-ICOS-L antibody (B7-H2 Ab) or anti-CD80 antibody (2D10). L-cells were used as a negative control. Cell lysates were loaded to a 10% SDS-PAGE and Western blot analyses were performed with phospho-Akt (Ser<sup>473</sup>) or Akt specific antibodies and then reprobated with phospho-GSK-3 $\alpha/\beta$  (Ser<sup>21/9</sup>) and GSK-3 $\alpha$  antibodies. The relative positions of  $\alpha$  and  $\beta$  GSK-3 are indicated by arrows. Data are representative of 3 independent experiments.

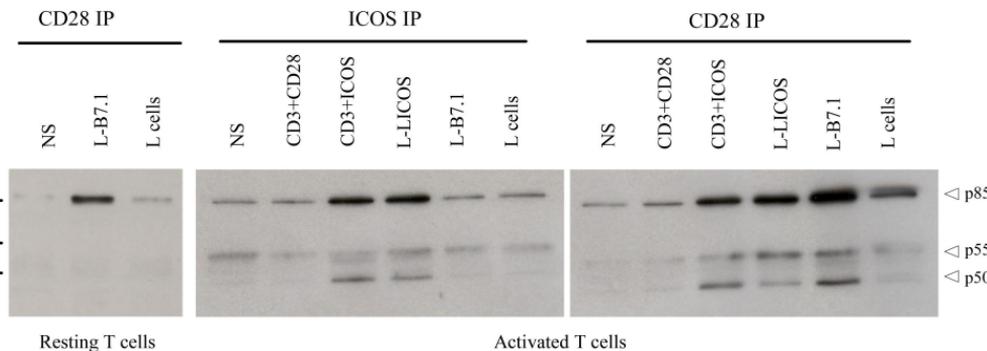
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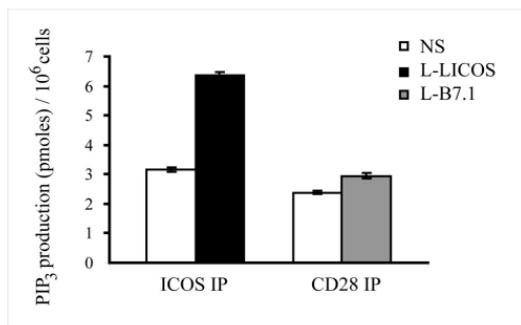
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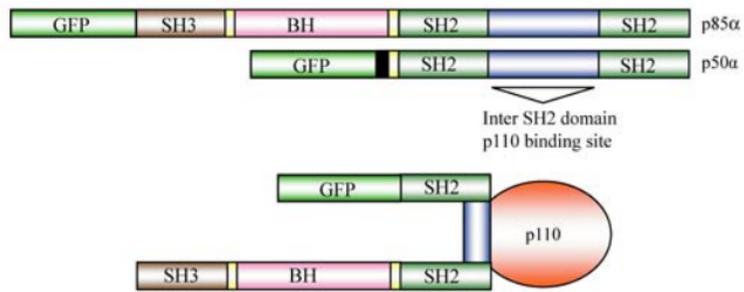
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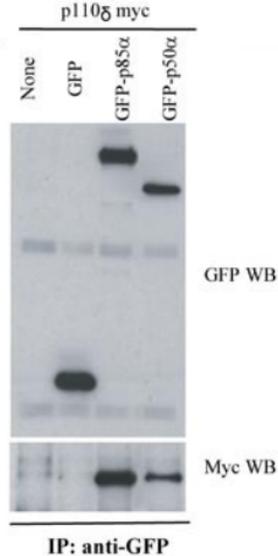
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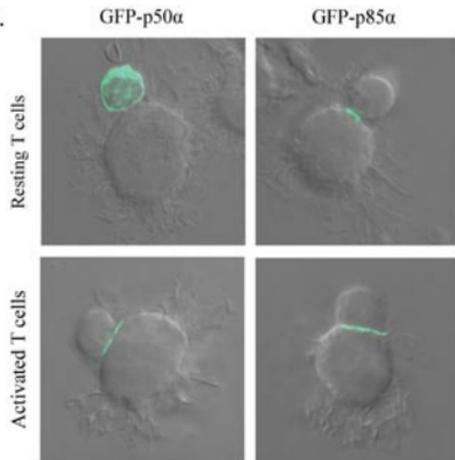
A.



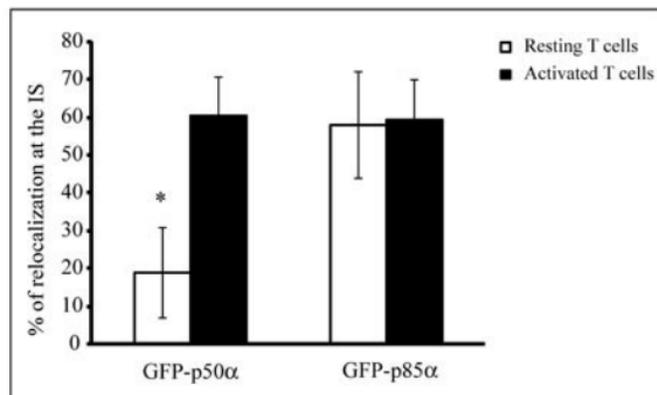
B.



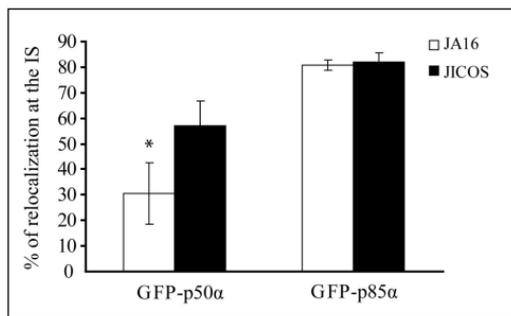
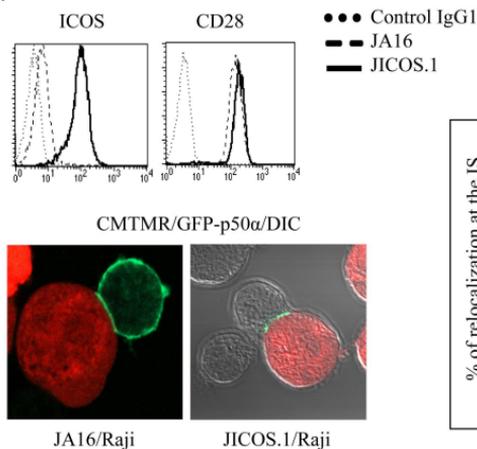
C.



D.



A.

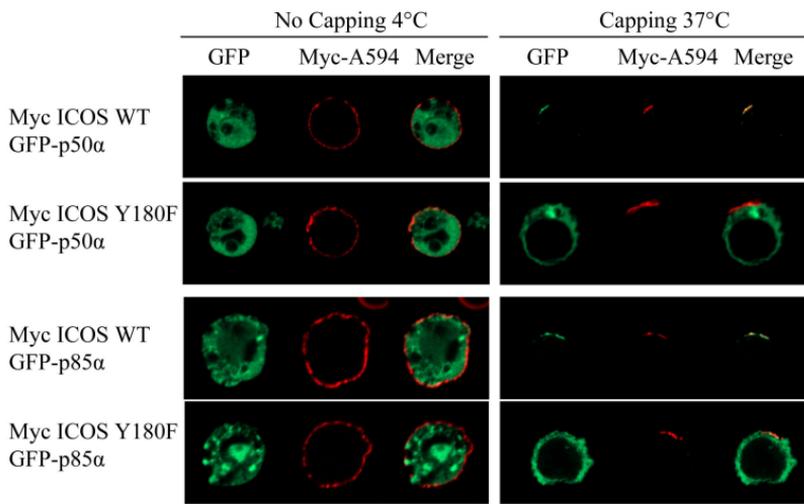


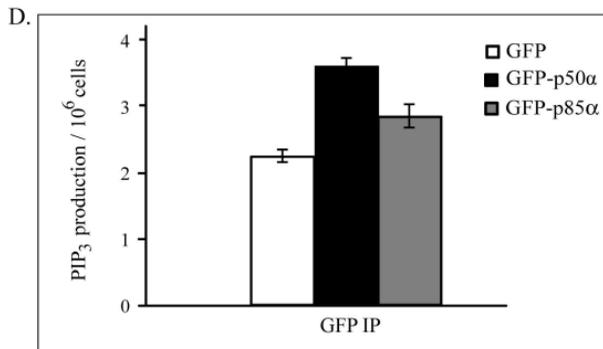
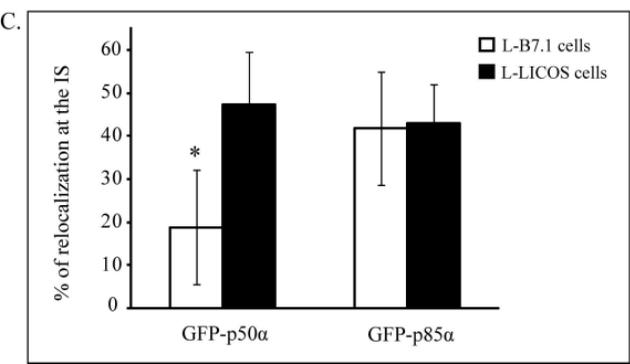
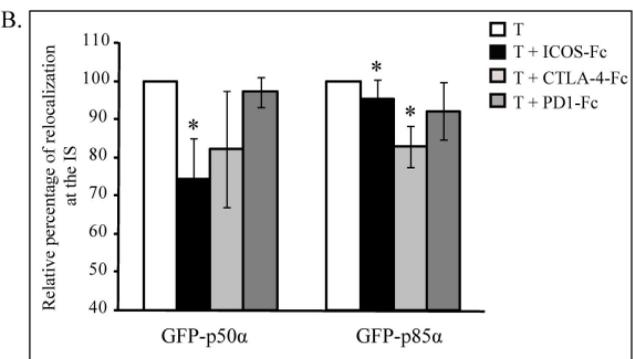
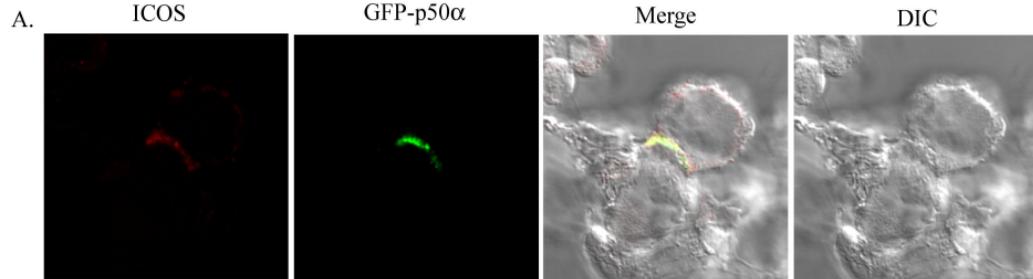
B.

muCD28

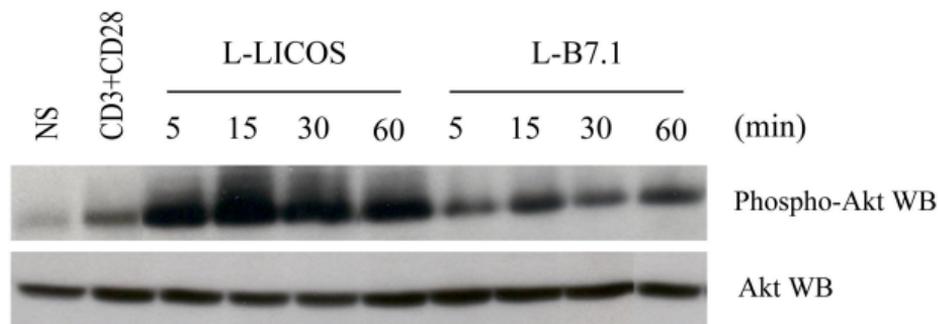
huICOS

SP	Myc-Tag	Extracellular	TM	[...]TKKKYSSSVHDPNGEY <b>MF</b> MRVNTAKKSRLTDVTL	Myc-ICOS WT
SP	Myc-Tag	Extracellular	TM	[...]TKKKYSSSVHDPNGE <b>F</b> MFMRVNTAKKSRLTDVTL	Myc-ICOS Y180F





A.



B.

