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Mechanism of Inducible Costimulator (ICOS)-mediated calcium signaling

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RÉSUMÉ

Le Costimulateur Inductible (ICOS) est un récepteur exprimé à la surface des cellules T CD4 auxiliaires et T CD8 cytotoxiques. Il fut démontré à l'aide de modèles murins de transplantation de moelle osseuse que ICOS joue un rôle important dans l'induction de la maladie du greffon contre l'hôte aigüe (GVHD). ICOS potentialise deux signaux médiés par le récepteur de cellules T (TCR) : l'activation de la phosphoinositide 3-kinase (PI3K) ainsi que la mobilisation interne de calcium. En conditions *in* vitro, dans les cellules CD4 et CD8, ICOS réussi à potentialiser le flux de calcium médié par le TCR indépendamment de PI3K. La voie de signalisation de ICOS impliquée dans la GVHD demeure inconnue. Cependant, en utilisant une lignée de souris 'knock-in' nommée ICOS-$Y^{181}F$, dans laquelle le cellules T ont sélectivement perdu la capacité d'activer PI3K par l'entremise d'ICOS, nous avons démontré que les cellules T peuvent utiliser un mécanisme ICOS indépendant de PI3K afin d'induire la GVHD.

La mobilisation interne du Ca^{2+} mène à l'activation de NFAT, un facteur de transcription clé régulant des gènes comme IFN-γ, qui exprime une des cytokines clés impliquées dans la GVHD. Nous émettons comme hypothèse que la capacité pathogénique intacte des cellules T ICOS-$Y^{181}F$ à induire la GVHD, repose sur la signalisation du Ca^{2+} indépendante de PI3K. Le but de mon projet est d'identifier les résidus responsables de cette signalisation de Ca^{2+} médiée par ICOS ainsi que le mécanisme par lequel ce récepteur fonctionne. À l'aide de la mutagénèse dirigée, j'ai généré des mutants d'ICOS et j'ai analysé par cytométrie en flux leur capacité à activer le flux de Ca^{2+}. J'ai ainsi identifié un groupe de lysine sur la queue cytoplasmique d'ICOS situé à proximité de la membrane comme étant essentiel à la fonction de potentialisation du flux de Ca^{2+}. Je fournis également des preuves de l'implication de la kinase Lck, membre de la famille de kinases Src, dans la voie de signalisation de ICOS médiant la potentialisation du flux de Ca^{2+}. Ainsi, ICOS s'associe à Lck et mène à une augmentation de l'activation de PLCγ1, la protéine effectrice clé causant la sortie de Ca^{2+} de la réserve intracellulaire.

En conclusion, notre étude permet de comprendre davantage une des voies de signalisation d'ICOS. L'influx de Ca^{2+} dans les cellules T implique la voie ICOS-Lck-PLCγ1. Une compréhension plus approfondie de cette voie de signalisation pourrait s'avérer bénéfique afin d'élaborer de nouvelles stratégies menant à la prévention de maladies reliées à ICOS, comme la GVHD.

ABSTRACT

The Inducible Costimulator (ICOS) is a receptor expressed on activated CD4 helper and CD8 cytotoxic T cells. It was previously shown that ICOS plays an important role in inducing acute graft versus host disease (GVHD) in murine models of allogeneic bone marrow transplantation (BMT). ICOS potentiates TCR-mediated phosphoinositide 3-kinase (PI3K) activation and intracellular calcium mobilization. In both $CD4^+$ and $CD8^+$ T cells, ICOS can potentiate TCR-mediated calcium flux in a PI3K-independent manner *in vitro*. However, the ICOS signal transduction pathway involved in GVHD remains unknown. Using a knock-in strain of mice (termed ICOS-$Y^{181}F$) in which T cells have selectively lost the ability to activate PI3K, we have recently shown that T cells can utilize PI3K-independent ICOS signaling pathways to induce GVHD.

The mobilization of intracellular Ca^{2+} leads to the activation of NFAT, a key transcription factor regulating genes such as IFN-γ, one of the key T cell cytokines involved in GVHD. Therefore, we hypothesize that the intact pathogenic capacity of ICOS-$Y^{181}F$ T cells to induce GVHD relies on ICOS-dependent, PI3K-independent calcium signaling. My goal is to identify the residue(s) responsible for this ICOS-mediated Ca^{2+} signaling and find the mechanism by which the receptor achieves its function. Through site-directed mutagenesis and flow cytometric analysis of calcium fluxing capacities of mutant ICOS proteins, I identified a membrane proximal cluster of lysine residues that is essential in inducing ICOS-mediated Ca^{2+} signaling. I also provide evidence for the involvement of the Src family kinase Lck in ICOS-mediated Ca^{2+}signaling. ICOS associates with Lck molecules, leading to the activation of PLCγ1, the key effector protein causing the release of Ca^{2+} from the intracellular pool.

Taken together, our study is beginning to unravel a complexity in ICOS signaling, and implicates the ICOS-Lck-PLCγ1 axis in T cell calcium signaling and potentially the induction of GVHD. Further understanding of this pathway could prove beneficial in designing new strategies to prevent ICOS-related diseases such as GVHD.

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LIST OF ABBREVIATIONS

3'-UTR	Three Prime Untranslated Region
Ab	Antibody
A/Ala	Alanine
Akt	Serine/Threonine-specific kinase
APC	Antigen Presenting Cell
ATCC	American Type Culture Collection
B7-1	B-lymphocyte activation antigen 7-1
B7-2	B-lymphocyte activation antigen 7-2
B7-H2	B7 homolog 2
Bcl-xl	B-Cell Lymphoma Extra Large
BMT	Bone Marrow Transplant
Brij-58	Polyethylene Glycol Hexadecyl Ether
BTLA	B- and T-Lymphocyte Attenuator
Ca^{2+}	Calcium
Ca^{2+}ATPase	Calcium Adenylpyrophosphatatse
$CaCl_2$	Calcium Chloride
CamK2	Calcium/Calmodulin-dependent Kinase 2
CD3	Cluster of Differentiation 3
CD4	Cluster of Differentiation 4
CD8	Cluster of Differentiation 8
CD28	Cluster of Differentiation 28
CD45	Cluster of Differentiation 45
CD80	Cluster of Differentiation 80
CD86	Cluster of Differentiation 86
CRAC	Calcium Release Activated Channel
Csk	C-terminal Src Kinase
cSMAC	Central SMAC

CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
CVID	Common Variable Immunodeficiency
cyTOF	Mass Cytometry Instrument
DAG	Diacyl-Glycerol
DAPP1	Dual Adapter for 3-Phosphotyrosine and 3-Phosphoinositide
DNA	Deoxyribonucleic Acid
dNTPs	DeoxyriboNucleotide tri-phosphates
dsDNA	Double Strand DNA
dSMAC	Distal SMAC
EDTA	Ethylene-Diamine-Tetraacetic Acid
EGTA	Ethylene-Glycol-Tetraacetic Acid
ER	Endoplasmic Reticulum
Erk	Extracellular signal-Regulated Kinase
F/Phe	Phenylalanine
FBS	Fetal Bovine Serum
Fyn	Proto-oncogene tyrosine-protein kinase
GADS	GRB2-related Adapter Protein 2
GATA-3	Trans-acting T-cell-specific transcription factor 3
GC	Germinal Center
Grb2	Growth factor Receptor-Bound protein 2
GTP	Guanosine Triphosphate
GVHD	Graft Versus Host Disease
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
Hrp	HorseRadish Peroxydase
ICOS	Inducible Costimulator
ICOSL	ICOS Ligand

Ig	Immunoglobulin
IgG	Immunoglobulin G
IgSF	Immunoglobulin Super Familly
IgV	Immunoglobulin V domain
IκB	Inhibitor of Kappa light polypeptide gene enhancer in B-Cells
IκK	IκB Kinase
ITAMs	Immunoreceptor Tyrosine-based Activation Motifs
IL-2	Interleukine 2
Itk	IL-2-inducible T-cell Kinase
K^+	Potassium
K/Lys	Lysine
kDA	Kylo Dalton
Lat	Linker of Activated T cells
Lck	Lymphocyte-specific protein tyrosine Kinase
LFA-1	Lymphocyte Function-associated Antigen 1
M/Met	Methionine
mAB	Monoclonal Antibody
MCD	Methyl-β-cyclodextrin
Mek	Mitogen-activated Protein Kinase Kinase
$MgCl_2$	Magnesium Chloride
$MgSO_4$	Magnesium Sulfate
MHC	Major Histocompatibility Ceomplex
N/Asn	Asparagine
NaCl	Sodium Chloride
NaF	Sodium Fluoride

$Na_4P_2O_7$	Sodium Pyrophosphate Tetrabasic
Na_3VO_4	Sodium Orthovanadate
NFAT	Nuclear Factor of Activated T cells
NFATc2	NFAT cytoplasmic 2
NFκB	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
NP-40	Nonidet P-40
ORAI1	Calcium release-activated calcium channel protein 1
P/Pro	Proline
p-X	Phosphorylated protein X
PAK1	Serine/Threonine-protein Kinase 1
PCR	Polymerase Chain Reaction
PD-1	Programmed cell Death Protein 1
PE	Phycoerythrin
PH (domain)	Pleckstrin Homology domain
PI3K	Phosphatidylinositide 3-Kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-triphosphate
PKCθ	Protein Kinase C Theta
PLCγ1	Phospholipase C gamma 1
PMSF	Phenylmethanesulfonylfluoride
pSMAC	Peripheral SMAC
PTEN	Phosphatase and Tensin Homolog
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
R/Arg	Arginine
Ras	Rat Sarcoma protein
RasGEF	Ras guanine nucleotide exchange factor

RasGRP1	Ras Guanyl-Releasing Protein 1
Raf	Proto-Oncogene Serine/Threonine-protein Kinase
RPMI	Roswell Park Memorial Institute Medium
SCID	Severe Combined Immunodeficiency
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SFK	Src Family Kinase
SH2	Src Homology domain 2
SLAM	Signaling Lymphocytic Activation Molecule
SLE	Systemic Lupus Erythematosus
SLP76	SH2 Domain Containing Leukocyte Protein of 76 kDA
SMAC	Supramolecular Activation Cluster
Sos1	Son Of Sevenless Homolog 1
Sos2	Son Of Sevenless Homolog 2
STAT6	Signal Transducer and Activator of Transcription 6
STIM1	Stromal Interaction Molecule 1
Syk	Spleen tyrosine kinase
TCR	T cell receptor
TGF-β	Transforming Growth Factor Beta
Th0	T helper cell 0
Th1	T helper cell 1
Th2	T helper cell 2
Th17	T helper cell 17
TNF	Tumour Necrosis Factor
TNFSF	TNF Super Family
Y/Tyr	Tyrosine

ZAP-70	Zeta-chain-Associated-Protein kinase of 70 kDa

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Chapter 1

INTRODUCTION

INTRODUCTION

1.1 Signal transduction pathways in T cells

1.1.1 General Overview

The immune system's main role in the body is to rid it of invading pathogens by mounting a protective immunity. It also serves to prevent harm to the host by inducing tolerance to self-tissues. T cells stand as key effectors in adaptive immune responses and calibration of their activities is a founding concept of the immune response[1]. T cells also play a large part in autoimmune diseases. To acquire specific effector functions, T cells must have their T cell receptor (TCR) engaged. This process requires the participation of multiple signals coming from the T cell environment. Understanding how the various T cell signals integrate to lead to appropriate functional outcome and dissecting the basis of T cell activation and regulation, can eventually help us to control infections and autoimmunity.

T cells only recognize foreign antigens that are displayed on the surface of the host's own cells. These antigens are derived from components of pathogens that are processed inside the cells; e.g. viruses, intracellular bacteria or pathogen products in the extracellular environment internalized by endocytosis. T cell activation can be broken down in four different stages: cell adhesion, antigen-specific activation, costimulation, and cytokine production or cytotoxicity (signaling). Adhesion between T cells and antigen presenting cells (APC) requires molecules such as integrins and selectins and is essential in coupling the cells together before ensuing cellular activation takes place. APCs acquire antigens through internalization (by receptor-mediated

endocytosis or phagocytosis) or infection and process the antigen-specific peptides. Next, they display the antigenic peptides on their surface in association with major histocompatibility complexes (MHCs) class I or class II molecules. These peptide-MHC molecules are to be recognized by T lymphocytes through their TCRs. The interaction between the MHC and TCR are not sufficient for full T cell responses, as TCR signal alone does not lead to sustained expansion and differentiation of T cells. To bolster the subsequent pathways, intercellular costimulatory signals are required. Costimulation promotes downstream signaling leading to proliferation, differentiation and survival of T cells. Cells involved in costimulatory signals need to communicate this way to determine if such course of actions goes through or terminates. Alongside perpetuating these T cell functions, costimulation can also mediate proinflammatory signals or generate immune tolerance in the periphery to avoid unwanted tissue damage due to staggeringly active proinflammatory effector signals[2].

The T cell program of proliferation and differentiation following MHC peptide presentation by APCs is controlled by signaling events that mainly take place in immunological synapse. The immunological synapse consists of an assembly of supramolecular activation clusters (SMACs) forming a ring-like structure[3]. The central SMAC (cSMAC) is composed of the TCR and its associated kinases. It represents the pivotal location where TCR signaling events take place. The peripheral SMAC (pSMAC) is composed of integrins (like lymphocyte function-associated antigen 1(LFA-1)[4]) stabilizing the interaction between the T cell and the APC and increasing the T-cell sensitivity to the antigen[5]. And lastly, the distal SMAC (dSMAC) comprises the molecules excluded from the central signaling center, e.g. phosphatases like CD45[6].

The first signaling molecules activated lead to a branched network of signaling cascades surrounding the TCR. A signaling balance exists in order to prevent activation of naïve T cells by self-antigens. The signal the cell receives must reach an established threshold in order for the cell to be activated. Such a strong signal can normally only occur from an interaction with an APC presenting a foreign antigen. However, autoimmune diseases ensue when a self-reactive T cells are erroneously activated.

In the recent years, knowledge of the molecules involved in early TCR signaling has improved greatly, due in part to advances in super-resolution microscopy (reviewed[7]). Also several new approach combine different techniques together to create powerful tools allowing better understanding of the events of TCR signaling. Coupling of mass spectrometry and cytometry created mass cytometry (cyTOF)[8] and a combination of imaging and flow cytometry saw the conception of Imaging flow cytometry[9]. These novel techniques now allow us to address molecular interactions in a dynamic manner. In the following sections, I will depict the events happening after the TCR is engaged and what key molecules are involved in that process (A schematic representation of TCR signaling events relevant to my project is in *Figure 1.1,* p.11*)*.

1.1.2 Src family kinases

Src family kinases (SFKs) are essential in providing the signals required for the survival of naïve T cells[10]. Regulation of SFK functions is central to the TCR signaling events. Since the TCR itself has no inherent enzymatic activity, it largely depends on the SFKs to trigger signaling after initial TCR engagement. SFKs are positively regulated by phosphorylation

of an activating tyrosine residue located in their catalytic domain. The activating tyrosine stabilizes an open conformation and promotes the full kinase activity of the molecule. The negative regulation happens by phosphorylation of an opposing tyrosine located in the carboxy-terminal domain. This negative phosphorylation is accomplished by the SRC kinase Csk[11]. This leads to a closed conformation of the kinase and an inhibition of its kinase activity[12-14]. Activation of SFKs is achieved by autophosphorylation and dephosphorylation is mediated by several phosphatases such as CD45 and PTPN22[15, 16].

1.1.3 Lck

Following TCR engagement, the SFK member lymphocyte-specific protein tyrosine kinase (Lck) is the first molecule that becomes activated. Lck binds to the cytoplasmic tail of the TCR co-receptors CD4 and CD8[17]. The co-receptors target the delivery of Lck into close proximity with its target: the TCR CD3 ζ-chains and their immunoreceptor tyrosine-based activation motifs (ITAMs)[18]. In naïve T cells, Lck is constitutively active in order to stabilize basal levels of CD3 ζ-chains phosphorylation[19].

Dynamic segregation of the signaling molecules is apparent as some transmembrane phosphatase like CD45 are forced out of the immunological synapse, enabling optimal downstream activation. The exclusion of CD45 could be either due to its larger size or the binding energy of the TCR-peptide-MHC interaction forcing it out[20, 21]. Abundance of Lck at the immunological synapse dictates the phosphorylation of subsequent targets: ITAMs, CD3 chains, and the ζ-chain associated protein kinase of 70 kDa (ZAP-70).

Recent evidence suggests that clustering in the early events of TCR activation is regulated by the conformational states of Lck[22]. However, the level of phosphorylation of Lck is relatively unchanged before and after T cell stimulation. Lck molecules are ≈40 % constitutively active[23]. This suggests that the local concentration of Lck rather then their phosphorylation state is more likely to direct TCR triggering.

1.1.4 Molecules downstream of Lck

Once Lck phosphorylates the Syk-family kinase Zap-70, the latter undergoes conformational changes as well[24]. This leads to the ensuing phosphorylation of its target molecule: the key adaptor molecule linker of activated T cells (LAT). Another molecule that is phosphorylated by ZAP-70 that associates with LAT is SLP76[25]. SLP76 colocalizes and connects with PLCγ1 and ITK.

The main role of LAT is to colocalize signaling molecules; to form a signalosome. LAT gets phosphorylated on several residues. These phosphorylated residues act as docking sites for kinases and other adaptors. For example, Lat recruits PLCγ1 through its tyrosine 136. LAT assures propagation of the TCR-mediated signals in a tightly regulated manner. Indeed, LAT-deficiency in Jurkat cells leads to impediment of TCR signals[26]. Also, partial loss-of-function mutation of LAT ($Tyr^{136}Phe$) accounts for LAT-signaling pathology as severe as a total loss of LAT[27]. The multiple phosphorylated sites of LAT eventually lead to the recruitment of downstream molecules and adaptors, which then branch to several different signaling pathways. One of the consequences of these signaling

pathways are the nuclear mobilization of key transcription factors crucial for gene expression responsible for T cell differentiation and function.

1.1.5 Fyn

Fyn is another SFK crucial for TCR signaling [28]. The vast majority of Fyn is not constitutively associated with other proteins. Biochemical and genetic evidence have shown a partial redundancy between Lck and Fyn[29]. Lck is has now been shown to induce Fyn activation[30]. Some studies have tried to show that in cell lines deficient for Lck, Fyn could replace the activity of Lck, but T cell activation was not sustainable[31, 32]. Fyn may in fact be rather more important for T cell anergy[33, 34] and may indicate that Fyn does not necessarily act downstream of Lck in a given signaling pathway.

1.1.6 Ras/Erk

The complex formed by PLCγ1 and SLP-76 is one that regulates Ras activation[35]. Ras is a small G protein central to numerous physiological conditions. In T cells, the Ras guanine exchange factor (RasGEF) RasGRP1 is phosphorylated on its tyrosine 184 by diacylglycerol (DAG), a secondary messenger generated by PLCγ1[36]. The RasGEFs Sos1 and Sos2 are constitutively associated with the adaptor Grb2. They are recruited to the membrane where they have basal RasGEF activity via Grb2/LAT interactions[37]. Sos proteins contain an allosteric Ras–GTP binding site. When that site is engaged, the RasGEF activity is greatly enhanced[38]. Ras-GTP binding to Sos allows the engagement of a positive feedback loop

between the two proteins[39, 40]. When Ras becomes activated, it induces multiple downstream pathways, including the Raf/MEK/ERK kinase cascade driving both T cell development and their effector functions[41, 42]. The Raf/MEK/Erk pathway can also be activated in a LAT-independent manner. It forms a complex with DAPP1, PLCγ1 and PAK1[43].

1.1.7 Lipid Rafts

Plasma membranes of several cell types contain microdomains enriched with cholesterol and sphingomyelin, called lipid rafts[44]. Sphingolipids allow ordered assembly beyond regular phospholipid bilayer. These rafts can be seen as organized platforms that mobilize in the plane of the plasma membrane. Their main function is to segregate molecules from one another[45]. Lipid rafts were initially discovered by their insolubility in non-ionic detergents as opposed to other areas of the plasma membrane[46, 47]. This facilitates their isolation from the rest of the membrane. In T cells, some molecules involved in TCR signaling are found in lipid rafts and disrupting these rafts can abrogate TCR signaling. Lipidation of Lck in its membrane anchoring N-terminal motif relocates the protein into lipid rafts[48]. Approximately 25-50% of total Lck molecules co-purifies with detergent insoluble fractions during lipid raft isolation[49]. Fyn is another molecule shown to be active in lipid rafts[50]. Some proteins such as ZAP-70 and PLCγ1[51, 52] are only transiently recruited into the lipid rafts. For the past two decades, numerous studies have been reported on lipid rafts without achieving a full comprehension. Not all studies are in accordance with the existence of the lipid rafts as some have questioned the approach to identify them[53, 54]. It's still up for debate regarding their true existence as well as their role in TCR signaling[55].

1.1.8 Costimulatory receptors

T cell co-signaling receptors (a broader concept accommodating positive and negative aspects of costimulation) positively or negatively modulate signaling pathways induced by TCR triggering. Co-signals consist of co-stimulatory and co-inhibitory signals[56, 57]. The crosstalk between co-signaling receptors and their respective ligand takes place in the T-APC contact area including the immunological synapse, where they influence the T cells for activation or inhibition[58]. Co-signaling receptors are broadly divided into those belonging to the immunoglobulin (Ig) superfamily (IgSF), the tumor necrosis factor (TNF) superfamily (TNFSF), and signaling lymphocytic activation molecule (SLAM) family. This classification is based on their phenotypic and signaling features. The CD28 and CD80 (B7-1)/CD86 (B7-2) costimulatory pathways represent prototypes of the Ig family of co-signaling receptors and ligands. The extended CD28 family includes CD28, CTLA-4, PD-1, ICOS, and BTLA.

1.1.9 CD28 and its ligands B7-1/B7-2

CD28 has been characterized as a homodimeric costimulatory receptor for the TCR complex and is responsible for providing signals required for T cell activation. During the very early stages of T-cell activation, CD28 is expressed on T cells and is ligated by B7-1/B7-2, which are constitutively expressed on dendritic cells (DCs) and inducible in other APCs such as B cells and monocytes[59-62]. CD28 stimulation has been shown to increase IL-2 production, promote survival of activated T cells and prevent T cell anergy [63]. The cytoplasmic tail of CD28 contains three signaling motif. The first motif contains an YMNM sequence. It serves as a binding site for the SH2-containg proteins (p85, GADS and Grb2). The

second motif contains a PRRP sequence. It interacts with the SH3 domain of Itk. The third motif contains a PYAP sequence. It associates with the SH3 domain of Grb2, GADS, Lck, and filamin-A. The association of Grb2 to the motifs YMNM and PYAP is crucial for the recruitment of PKCθ and RASGRP to the immunological synapse and its ensuing activation[64, 65]. The B7-1/B7-2/CD28 pathway decreases the threshold for T cell activation. Subsequently, this results in T cell proliferation, the upregulation of anti-apoptotic proteins like Bcl-xL, and the increase of IL-2 production[66-68].

In contrast, B7-1 and B7-2 also deliver a co-inhibitory signal to activated T cells through CTLA-4, the CD28 antagonist. The cytoplasmic tail of CTLA-4 contains the immunoreceptor tyrosine inhibitory motif (ITIM) recruiting SHP-1 and SHP-2[69, 70]. CTLA-4 possesses a higher affinity to B7 ligands and competes out CD28 for their binding leading to signal-independent T cell suppression by sequestering the ligands from the APC surface[71]. Contrary to the CD28 pathway, the B7/CTLA-4 pathway increases the threshold to reduce T cell activation and ultimately terminate it. B7-1 and B7-2 are considered to deliver bidirectional signaling critical for the downregulation of T cell response and induction of T cell tolerance.

Once CD28 is engaged in TCR-mediated signaling, it is thought to bind to phosphatidylinositde-3 kinase (PI3K). CD28 brings PI3K to the membrane where it will generate phosphatidylinositol (3,4,5)-triphosphate (PIP3) and anchor proteins containing pleckstrin homology (PH) domains. The catalytic PI3K helps to convert phosphatidylinositol (4,5)-bisphosphate (PIP2) into PIP3. The excess of PIP3 is removed by phosphatase and tensin homolog (PTEN). PI3K is required to activate Akt, the regulator of many downstream targets. PI3K binding alone doesn't account for all the CD28 effects. Indeed, recent data from knock-in mouse models show that it is

the PYAP motif that plays most important role in CD28-mediated costimulation whereas the contribution of YMNM motif is minimal[72]. Nuclear factors of activated T cells (NFAT), as well as Nuclear Factor KappaB (NFκB), play a crucial role in the regulation of gene transcription, a function triggered by CD28 stimulation, independently of PI3K.

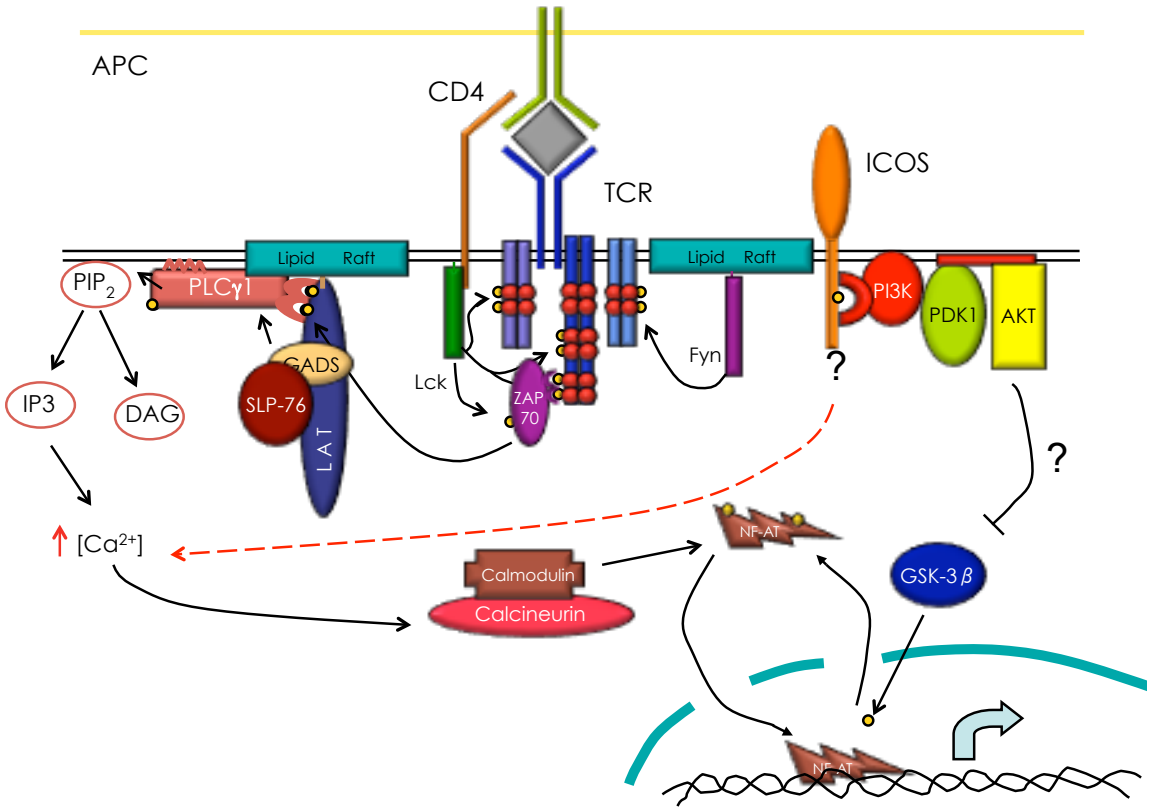

Figure 1.1 ***Simplified overview of TCR signaling leading to activation of the Ca^{2+} signaling pathway***. Once an MHC molecule presents an antigenic peptide and engages the TCR, signaling pathways are activated leading to effector functions of the cell. One of the downstream signaling events that are activated is the nuclear relocalization of NFAT that induces the transcription of several genes, e.g. IL-2 and IFN-ϒ. During the recognition of antigens by TCR complexes Lck is delivered nearby through association with coreceptors (CD4/CD8) and phosphorylates its target molecules: ITAM motifs on CD3 ζ-chains. Phosphorylated ITAMS recruit ZAP-70 kinases that activate LAT/GADS/SLP-76 signalosomes. Phosphorylated LAT recruits PLCγ1. Activated PLCγ1 generates secondary messengers DAG and IP3; IP3 in turn leads to the activation of calcineurin and eventually the nuclear relocalization of NFAT. It has been unclear how ICOS achieves potentiation of TCR-mediated Ca^{2+} flux and if the activation of PI3K is required in regulation of the downstream Ca^{2+} signaling pathway.

1.2 The Inducible Costimulator (ICOS)

1.2.1 General Description

It has almost been 15 years since the Inducible Costimulator (ICOS) was discovered[73-75]. ICOS is a costimulatory receptor of the CD28 family of costimulatory receptors and was found to be selectively 'induced' on activated T cells, hence its given name. It was later discovered that it was expressed on T cells in the germinal centers[76, 77]. ICOS also displayed a structural similarity with the prototypical costimulatory receptor CD28. However, ICOS binds to its ligand B7-H2, but not other members of B7 family proteins[78]. Costimulation of ICOS enhances mainly cytokine production but promotes proliferation minimally. Both mice and humans, ICOS-deficiency leads to reduced antibody production due to impaired germinal center reaction. We and others have shown that this is due to its crucial role in the development of Tfh cells[79].

1.2.2 ICOS Structure

Structurally, ICOS is similar to CD28. Both are type I transmembrane glycoproteins. The amino acid sequence of ICOS is divided in three distinctive sections: a signal peptide, a single IgV-like domain, a 23 amino-acid transmembrane region and a 35 amino-acid cytoplasmic tail[80]. The whole ICOS sequence is composed of numerous amino acid clusters that are conserved through evolution between species. The two conserved cysteine residues at position 42 and 109 within the Ig domain are supposed to stabilize the Ig fold by forming disulfide bonds. On the surface, ICOS assembles as a homodimeric receptor. A conserved cysteine residue at position 136 is predicted to help forming the disulfide bridge between the homodimeric chains. In mice, ICOS has an apparent relative

molecular mass of 47-57 kDa[75, 81].

In mice and humans, the amino-acid sequence of ICOS shares closely to 40% of sequence similarity with CD28[78, 80]; a significant number as 26% amino-acid identity is found between CD28 and CTLA-4[82]. The human and mouse amino-acid sequences of ICOS share approximately 70% identity, which is comparable to identity between human and mouse CD28 (69%)[78]. The similarities of sequences between species imply that the mechanism of action for ICOS in both mice and humans should be conserved, indicating that studies in mouse models are likely reflecting what would happen in humans.

1.2.3 ICOS signaling mechanisms and functions

ICOS is known to potentiate two TCR-mediated signaling pathways: the PI3K pathway[83-85] and the Ca^{2+} pathway[85, 86]. While ICOS binds PI3K directly, the mechanism of Ca^{2+} mobilization by ICOS remains unclear. At first, it was assumed that ICOS-mediated Ca^{2+} potentiation was an indirect result of PI3K activity, as PI3K induces Ca^{2+} mobilization in T cells[87], but our lab showed that this function was occurring in a PI3K-independent manner[79].

Similar to CD28's YMNM motif, the cytoplasmic tail of ICOS contains a YMFM motif. Once the tyrosine in the motif is phosphorylated, it can recruit the SH2 domain- containing PI3K regulatory subunits p85α and p50α, but not Grb2[83, 88]. Compared to CD28, ICOS has a much more potent capacity to activate PI3K[79, 85]. This may be due to a competition for binding between PI3K and Grb2 for the p-Tyr motif that happens in CD28 cytoplasmic tail does not occurring ICOS. ICOS augments T cell effector functions, but not through augmenting proliferation of naïve T cells. It is

rather through enhancement of Th1 and Th2 cytokine production[89-91]. ICOS also regulates humoral immune responses by enabling germinal center T cells to achieve cognate interaction with B cells, providing a signal leading to a germinal center reaction and consequently, antibody maturation[79, 92, 93]. In preactivated CD4 T cells, ICOS is constitutively bound to PI3K and ICOS ligation further increases PI3K recruitment[94]. Co-ligation of the TCR and ICOS gives rise to a maximal PI3K signaling. It was highlighted by imaging and biochemical studies that ICOS is in complex with TCR complexes, and ICOS may get recruited into the immunological synapses. This supports the view that ICOS probably functions in conjunction with the TCR[75, 88, 95]. Moreover, inactivation of the p110δ isoform of PI3K also leads to impaired humoral immunity, reduced generation of Tfh cells, and impaired germinal center reaction [96, 97]. Therefore, ICOS-PI3K signaling axis play critical role to support generation of Tfh cells.

Although ICOS-mediated Ca^{2+} flux can take place independently of ICOS-mediated PI3K activation, the molecular mechanisms and its biological significance were not known at the beginning of this project (depicted in *Figure 1.1,* p.11*)*.

1.2.4 Regulation of Expression

ICOS is expressed at low levels on naïve T cells and is significantly upregulated after TCR and CD28 costimulation[78, 80]. Fyn and Erk can regulate ICOS expression at the transcriptional level after T cell activation[98]. Fyn activates calcineurin, which in turn dephosphorylates NFATc2 and induces its nuclear translocation[99]. In the nucleus, NFATc2 and Erk bind independently to the *Icos* promoter and activate its

transcription.

ICOS is expressed on Tfh cells, Th1, Th2, Tregs, Th17 and unpolarized activated CD4 T cells (Th0)[100]. Recent studies have shown that ICOS is regulated differently depending on the T cell subsets. In Th1 cells, the master regulator of Th1 cells, T-bet, binds to the *Icos* promoter and synergizes with NFATc2 to upregulate ICOS transcription. In Th2 cells, NFATc2 also binds to the ICOS promoter, but it is GATA-3 that operates via an *Icos* 3'UTR elements[101]. This proves relevant as Th2 cells express higher levels of ICOS than do Th1 cells[83].

1.2.5 B7-H2 (ICOSL or CD275)

B7-H2 is a co-stimulatory ligand that only binds ICOS on the T cell surface[73, 78, 102]. B7-H2 is detected on the surface of APCs including B cells, DCs, and macrophages and a subset of CD3 T cells, but as well on non-hematopoietic cells such as endothelial cells and some epithelial cells[103, 104]. B7-H2 mRNA is constitutively expressed in several non-hematopoietic tissues like the liver, kidney, testes and lung[74, 105]. Anatomically, B7-H2 is expressed in areas where B cell are present like the lymph nodes and the spleen[76]. It was recently discovered that human B7-H2, but not mouse B7-H2, also binds to CD28 and CTLA-4. Thus, ICOS, CD28 and CTLA-4 may compete for a similar binding site on human B7-H2[106]. The questions regarding the physiologic role of the B7-H2 interaction with CD28 and CTLA-4 in vivo remain unresolved.

1.2.6 Disease relevance

ICOS defect in humans has been reported to cause common variable immunodeficiency (CVID), a disease characterized by a severe reduction in class-switched antibodies, a failure to mount specific antibody responses to vaccination or natural infection and hypogammaglobulinemia and recurrent bacterial infections[93, 107, 108]. Consistently, mice with ICOS or ICOSL deficiencies also have severe defects in Tfh cell generation and GC reactions[92, 109-113]. On the contrary, mice with increased surface expression levels of ICOS have increased Tfh cell numbers and are prone to autoimmunity[114, 115]. ICOS was found highly expressed on activated CD4+ T cells in patients with autoimmune conditions such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and inflammatory bowel disease[116-118]. ICOS-deficient mice were noted to have reduced total IgG and anti-dsDNA production[119]. ICOS-L blockade of a mouse cardiac allograft model was shown to enhance cardiac graft survival where CD28 costimulation was absent and while CD8 T cells, CTLA-4, and the STAT-6 pathway were functionally active[120].

Recent studies published in collaboration with our lab showed that ICOS plays an important role in inducing acute graft-versus-host disease (GVHD) in murine models of allogeneic bone marrow transplant (BMT)[121]. Furthermore, using the ICOS-Y^{181}F mouse strain, we showed that this contribution was mediated by PI3K-independent mechanisms[122]. However, the mechanism by which ICOS triggers the onset of the disease remains unknown. GVHD is a major complication following allogenic hematopoietic stem cell transplantation, in which the grafted cells recognizes allogeneic antigens on host tissue cells leading to subsequent inflammation and tissue damage. Standard treatments are 1-2 mg/day of prednisone with continued administration of calcineurin inhibitor for

steroid sparing. However, the general prognosis remains weak[123]. Several players are hinted as being potentially involved in the acute manifestation of the disease, with TGF-β[124], T regulatory cells (Tregs)[125] and Th1/Th2/Th17 cytokine[126-128] having potential roles. It becomes apparent that further research on the pathophysiology of GVHD may facilitate the establishment of novel strategies leading to prevention and cure of this disease. With the promising results observed with ICOS[122] and the favourable effects of blocking Ca^{2+} signaling in standard treatments, understanding in depth how ICOS-mediated Ca^{2+} signaling functions might prove beneficial in preventing GVHD.

1.3 Calcium signaling pathway in T cells

1.3.1 General overview

A rapid increase in intracellular Ca^{2+} concentration is crucial for T cell activation and modulation of TCR signal intensity[129, 130]. A remarkable variety of Ca^{2+} signals in T cells, ranging from infrequent spikes to sustained oscillations and plateaus, derived from the interactions of multiple Ca^{2+} sources and sinks in the cell. By depleting the stores prolonged Ca^{2+} influx is triggered through calcium release activated calcium channels (CRAC) in the plasma membrane. The range and dynamics of Ca^{2+} signals are shaped by the action of several intertwining mechanisms. These different events include potassium (K^+) channels and membrane potentials, mitochondria that buffer Ca^{2+} preventing the activation of CRAC channels and plasma membrane Ca^{2+}-ATPase (PMCA).

The initial steps of TCR-mediated calcium signaling are well established[129]. As previously described, upon TCR engagement, SFKs is

activated, resulting in the phosphorylation of, amongst other CD3 modules, the ζ chain of the TCR complex. This leads to the recruitment and activation of ZAP70, and then LAT. Tyrosine- phosphorylated LAT then recruits several SH2-containing proteins, including PLC-γ1.

1.3.2 PLCγ1

Upon activation, PLCγ1 interacts with the phosphorylated tyrosine 136 of LAT. Stabilization of this interaction by the adaptors GADS (that bind LAT p–Y175 and p-Y195) and SLP-76 allows phosphorylation of PLCγ1 on residues critical for its activation[37]. Once activated, PLCγ1 cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) to generate two distinct secondary messengers: inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG).

DAG activates RasGRP1 by either directly binding its C1 domain of RasGRP1 or indirectly by activating novel protein kinase C (PKC) isoforms (notably PKCθ). RasGRP1 is phosphorylated at the tyrosine 184 and its RasGEF activity is increased. PKCθ along with calmodulin and calcium/calmodulin-dependent protein kinase-2 (CalmK2) regulates the phosphorylation state of the inhibitor of kappa light polypeptide gene enhancer in B-Cells (IkB) kinase (IκK) complex through direct and indirect interactions. Activated IκK induces phosphorylation and degradation of IkB, and nuclear factor-kappaB (NF-κB) can be released from IkB and translocate into the nucleus.

The other second messenger IP3 travels through the cytoplasm and induces the release of intracellular Ca^{2+} by binding to the IP3 receptor located on the outer membrane of the endoplasmic reticulum

(ER). Ca^{2+} activates the phosphatase calcineurin, which dephosphorylates the transcription factor nuclear factor of activated T cells (NFAT) in the cytoplasm. This induces the translocation of NFAT to the nucleus and the transcription of a large array of genes such as IL-2 and IFN-γ. The relationship between PLCγ1 and ICOS, as well as the role of ICOS-mediated Ca^{2+} flux potentiation has yet to be fully examined (depicted in *Figure 1.2* along with a summary of TCR-mediated Ca^{2+} signaling, p.20).

CRAC Channels

In T lymphocytes, CRAC channels constitute the only pathway for Ca^{2+} entry following TCR engagement. Their function is essentially to drive the program of gene expression that underlies T-cell activation by antigen. Prolonged Ca^{2+} entry through CRAC channels is essential to activate transcription factors such as NFAT initiating many of the changes in gene expression[131]. Abrogated signaling through CRAC channels results in a lethal severe combined immunodeficiency (SCID) syndrome in human patients, characterized by defective T-cell activation and proliferation[132, 133]. Studies have established that CRAC channels are the primary Ca^{2+}-influx pathway that is activated upon TCR engagement in T cells and their essential role in T-cell function and human health[133-135]. Since the discovery of CRAC channels no mechanism had been proposed to explain how their function is regulated. In the last few years, the first molecular components of this pathway have been identified. The first one is the ER Ca^{2+} sensor, the stromal interaction molecule 1 (STIM1)[136, 137]. The second one is Orai1, a pore-forming subunit of the CRAC channel[138, 139]. Recent work shows that CRAC channels are activated in a complex fashion that involves co-clustering of STIM1 in junctional ER near

the plasma membrane[140, 141]. They align to face opposite to Orai1 molecules. These studies reveal an abundance of sites where Ca^{2+} signaling might be controlled to modulate the activity of T cells during the immune response. To balance the concentration of Ca^{2+} inside the cells, PMCAs provide the dominant mechanism though which clearance of the ions occurs[142, 143]. At the beginning of this project, there was no information as to where ICOS fits in these general Ca^{2+} signaling pathways in T cells.

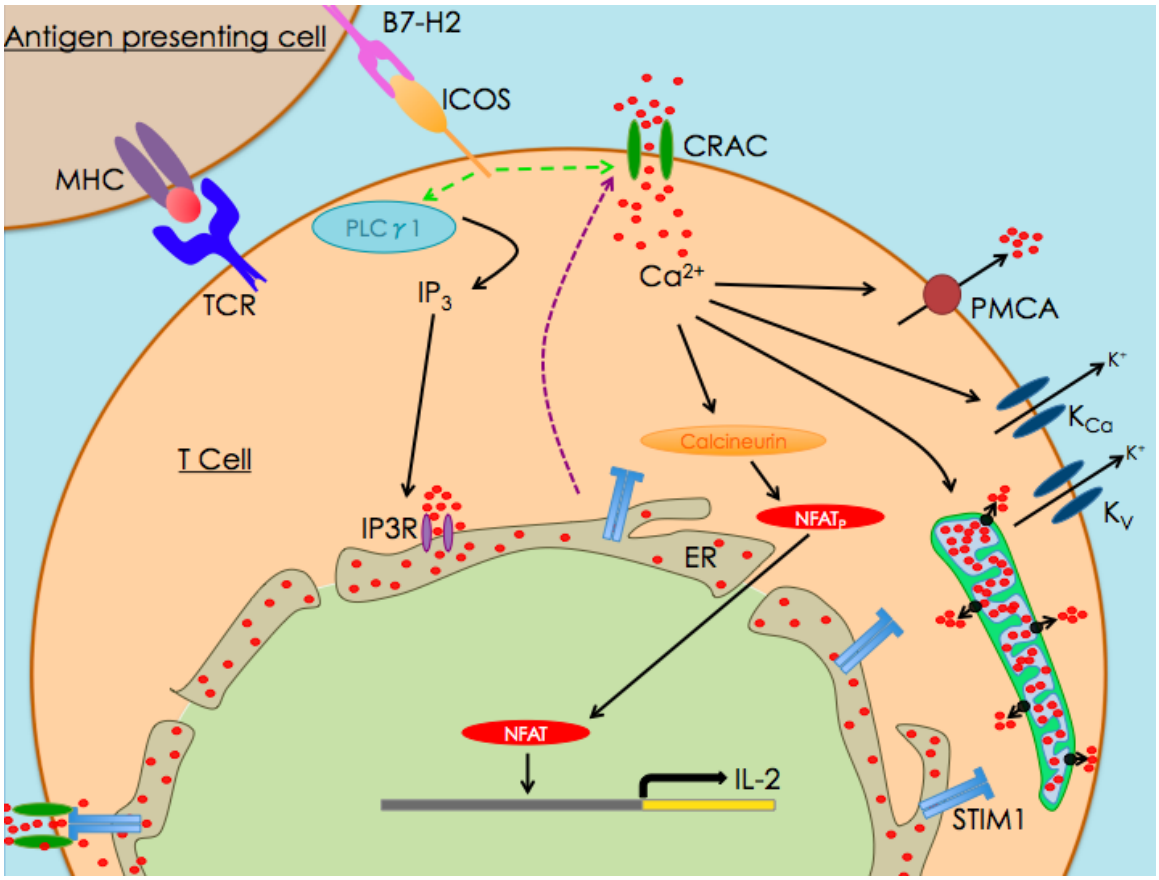

Figure 1.2: ***Overview of Ca2+ signaling pathways in T cells***. IP3 releases Ca^{2+} from the ER. Depletion of the intracellular Ca^{2+} stores activates plasma membrane CRAC channels. Ca^{2+} depletion is detected by Ca2+ sensor STIM1 on the ER membrane. It then associates with the CRAC channels (purple dashed line). Ca^{2+} entering the cell activates the K^+/Ca^{2+} channels and upregulates PMCA activity. Calcineurin is activated and it leads to dephosphorylation of NFAT allowing its translocation into the nucleus. Mitochondria take up Ca^{2+} near the CRAC channels to prevent their auto-inactivation by negative feedback-loop, redistributing it elsewhere in the cell. The route by which ICOS achieves its function in mediating Ca^{2+}-flux needs to be investigated (green dashed lines).

Chapter 2

MATERIALS AND METHODS

MATERIALS AND METHODS

Mice

C57BL/6 mice (WT) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). ICOS-KO (*Icos*$^{-/-}$) or ICOS-YF knock-in (*Icos*$^{yf/yf}$) mouse strains were previously described[79, 111]. FYN-KO (*Fyn*$^{-/-}$) mice were provided by Dr. A. Veillette (IRCM, Montreal, QC, Canada) and were previously described[34, 145]. All the mice were in C57BL/6 background (minimum N10) and were housed in the IRCM Animal Facility under specific pathogen-free conditions. Animal experiments were performed according to animal use protocols approved by the IRCM Animal Care Committee.

Antibodies and cytokines

For T cell stimulation, the following functional grade purified Armenian hamster anti-mouse antibodies were used: antibodies against ICOS (mAb C398.4A), CD3 (145.2C11) and CD28 (37.51)(eBioscience). For flow cytometry, the following Armenian hamster anti-mouse antibodies were used: PE-conjugated anti-ICOS (mAb 15F9), biotinylated anti-ICOS (mAb C398.4A), biotinylated anti-CD3ε (mAb 145.2C11), biotinylated anti-CD28 (mAb 37.51)(eBioscience). Goat anti-Armenian hamster IgG (Jackson Immunoresearch) or avidin (Calbiochem) were used to crosslink primary antibodies. For immunoblots, the following antibodies were used: goat anti-mouse ICOS (Santa Cruz, sc-5748), rabbit anti-mouse PLCγ1 (Santa Cruz, sc-81), mouse anti-human/mouse GAPDH (Santa Cruz, sc-32233), mouse anti-human/mouse pTyr (4G10; Millipore), rabbit anti-mouse pY505-Lck (Cell Signaling, 2751P), rabbit anti-mouse pY783-PLCγ1 (Cell Signaling,

2821S). HRP-conjugated goat anti-rabbit or sheep anti-mouse secondary antibodies were purchased from BioRad. Rabbit antisera against mouse Lck, Fyn or ZAP-70 were kind gifts from Dr. A. Veillette (IRCM, Montreal, QC, Canada). Recombinant IL-2 was purchased from Peprotech.

Reagents

The PLCγ1 inhibitor U73122 and its non-specific analog U73343 were from Calbiochem. Hexadimethrine bromide (polybrene), sucrose, calcium chloride, magnesium chloride, EGTA, Brij58, methylcyclodextrin and *n*-Dodecyl β-D-maltoside were purchased from Sigma. Nonidet p-40 was purchased from Calbiochem.

In vitro CD4 T cells isolation, activation and restimulation

CD4 T cells were isolated from splenocytes and superficial lymph nodes (popliteal, axillary, inguinal and submandibular) using Mouse CD4 T cell Enrichment Kit™ (EasySep) according to the manufacturer's instructions. T cells were cultured in RPMI1640 medium supplemented with 10 % FBS, 300 mg/ml glutamine, 1 Unit/ml penicillin, 1 μg/ml streptomycin, 55 mM β-mercaptoethanol and 10 mM HEPES. Purified CD4 T cells were activated by culturing with plate-bound anti-CD3 (3 μg/mL) and soluble anti-CD28 (2 μg/mL) for 2 days and were subsequently expanded in media containing 100 U/mL IL-2 (Peprotech) for 3 days. For restimulation experiments, CD4 T cell blasts were harvested and incubated for 1 min at room temperature with primary antibodies: anti-CD3 (1 μg/mL) alone or anti-CD3 (1 μg/mL) plus anti-ICOS (2 μg/mL). Immediately after addition of

anti-hamster IgG (20 μg/mL) for crosslinking, the cells were transferred to a water bath at 37 °C and incubated for 1–5 min depending on experimental settings.

Jurkat cells

Jurkat, JCam1 (Lck-deficient Jurkat), P116 (ZAP70-deficienct Jurkat), and their reconstituted counterpart cell lines were generous gifts from Dr. A. Weiss (UCSF, San-Francisco, CA, USA). Jgamma1 (PLCγ1-deficient Jurkat) and its reconstituted counterpart cell lines were purchased from ATCC, All Jurkat-derived cell lines were cultured according to ATCC guidelines in RPMI1640 medium supplemented with 10 % FBS, 300 mg/ml glutamine, 1 Unit/ml penicillin, 1 μg/ml streptomycin, 55 mM β-mercaptoethanol and 10 mM HEPES. For reconstituted cell lines P116_WT and Jgamma1_WT geneticin (G418) (Sigma) was added (2 mg/ml) to maintain the ectopic expression of the reconstituted genes. The Jurkat-Eco (Jurkat derivative expressing ecotropic receptor) cell line was obtained from Dr. Linda Penn (OCI, Toronto, Canada).

Ca^{2+} flux

For Indo-1 loading, CD4+ T blasts (1 X 10^7 cells/ml) were incubated for 30 min with Indo-1 AM (Life Technologies) in Ca^{2+} buffer (HBSS buffer supplemented with 0.1 % BSA, 1 mM $CaCl_2$ and 1 mM $MgCl_2$). After washing, cells were stained with anti-ICOS-PE (15F9). For stimulation, Indo-1 loaded ICOS stained cells (1 X 10^6 cells in 50 μl) were incubated for 1 min at room temperature with biotinylated antibodies: 0.2 μg/ml of anti-CD3

+/- 2 μg/ml anti-ICOS. When needed, EGTA was added to the media to chelate Ca^{2+} ions (at a concentration of 2.5 mM). After diluting in 500 μl of total Ca^{2+} buffer, cells were run in LSR II flow cytometer (BD). After recording baseline for 30 sec, avidin (28 μg/ml) was added and the mobilization of intracellular Ca^{2+} was monitored by measuring FL4/FL5 ratio. Equal loading of Indo-1 was confirmed by releasing intracellular Ca^{2+} by ionomycin (Sigma-Aldrich, 1 μg/ml). The same procedure was performed for Jurkat cells, except that the stimulation was achieved with biotinylated anti-ICOS antibody without anti-TCR antibody.

Immunoprecipitation and immunoblot analysis

Restimulation was stopped by adding ice-cold Ca^{2+} buffer with 10 % FBS, 1 mM Na_3VO_4, 1 mM EDTA pH 8.0. Cells were lysed in NP-40 lysis buffer (1 % NP-40 in 10 mM Tris pH 7.5, 5 mM $Na_4P_2O_7$, 100 μM Na_3VO_4, 5 mM NaF, 150 mM NaCl, 1 mM PMSF and protease inhibitor cocktail (Sigma)) or digitonin lysis buffer (the same recipe with 1 % Digitonin instead of NP-40) for 20 min on ice. Cell debris was removed by centrifugation at 16,000 x g for 10 min at 4 °C and the cleared lysates were collected. For immunoprecipitation, the cleared lysates were incubated for 1 hour on ice after addition of indicated antibodies (2 μg/ml). Immune complexes were recovered with protein A beads (Pierce) (1 hour on rocker at 4 C) and then washed in NP-40 lysis buffer (two times in 1 ml). Cleared lysates or immunoprecipitates were boiled in SDS-PAGE sample buffer. The samples were run on 8 % (for PLCγ1) or 12 % (all other proteins) SDS-PAGE gels and transferred to Amersham Hyperfilm ECL nitrocellulose membranes (GE Healthcare). Blocking was performed in either 5 % fat-free skim milk powder (Lck, ZAP-70, Fyn and ICOS) or 5 % BSA (PLCγ1, pY783-PLCγ1, pY394-Lck, pY505-Lck,

GAPDH), both in TBST. Detection was achieved with Amersham ECL Plus™ Western Blotting Detection Reagents (GE Healthcare), membranes were revealed using ChemiDoc™ MP Imaging System (BioRad) and protein bands were quantified using the Image Lab™ software (BioRad).

Plasmid construction and cell transfection

Mutant ICOS constructs were generated by site-directed mutagenesis using the GeneArt Site-Directed Mutagenesis System (Invitrogen) according to the manufacturer's instructions with modifications: Platinium Pfx® high capacity DNA polymerase (Life technology) was used instead of Accuprime Pfx® and 50mM $MgSO_4$ and 10mM dNTPs were added to the PCR mixture for each mutagenesis reaction as normally these components are present in the Accuprime Pfx® reaction mix. . Methylation of the template DNA was accomplished in a 37°C water bath prior to mutagenesis PCR reaction. Primers are detailed in *Appendix (Table 1, p.67)*. Template DNA used was pBMN_ICOS_IRES_GFP plasmid. ICOS mutant constructs were expressed in ICOS KO CD4 T cells or Jurkat-Eco (ICOS negative) cells through retroviral transduction[146]. In case of Jurkat and their derivatives, endofectin-mediated lentiviral transduction method was used according to the manufacturer's instructions to express murine ICOS (Ex-Mm07236-Lv81; Genecopoeia).

Sucrose density gradient and lipid raft isolation

Stimulated cells were lysed in Brij 58 Lysis Buffer (1 % Brij 58, 25 mM Tris [pH 7.6], 150 mM NaCl, 5 mM EDTA, 1mM Na_3VO_4, protease/ phosphatase inhibitor mix) for 30 min on ice. Lysate was mixed in 1:1 ratio of 80% Sucrose (in Brij 58 lysis buffer) and poured in polyallomer centrifuge tubes (Beckman Coulter). Column was assembled with subsequent layers of 40 %, 30 % and 5 % sucrose/lysis buffer. Columns were spun at 39, 000 rpm for 20 hours with a SW 41 Ti rotor (Beckman Coulter). 1 ml fractions were collected and 1 % maltoside was added to each tube to solubilize proteins. A portion of the fractions were then boiled with sample buffer and analyzed by immunoblot.

Chapter 3

RESULTS

RESULTS

Four membrane proximal residues in the cytoplasmic tail of ICOS are sufficient for Ca^{2+} flux

I sought to determine the residues in the cytoplasmic tail of ICOS responsible for the potentiation of TCR-mediated Ca^{2+} release in a PI3K-independent way. To this end, I generated a series of ICOS mutants through site-directed mutagenesis on ICOS-$Y^{181}F$ backbone cDNA in a retroviral vector (pBMN-IRES-GFP). The murine ICOS cytoplasmic tail has several evolutionarily conserved regions as shown in Figure 3.1A (p.31). In order to delineate the minimal segment of the ICOS tail required for Ca^{2+} flux, I first made truncation mutants by introducing stop codons right after Ala185 or Tyr170 (Figure 3.1B, p.31). Once these mutants were generated, I expressed them in either Jurkat-Eco cells, which are known to be negative for the expression of ICOS[88, 147], or in activated primary CD4 T cells isolated from ICOS-KO mice. I then tested the ability of these ICOS mutants to induce Ca^{2+} flux. Importantly, I found that ICOS was able to induce Ca^{2+} flux upon ligation without co-ligation with TCR in Jurkat-Eco cells. This is in sharp contrast with CD4 T cells in which ICOS can only potentiate TCR-mediated Ca^{2+} flux but cannot function by itself (Figure 3.1C; first panel, p.31). This "autonomous" nature of ICOS-mediated Ca^{2+} flux in Jurkat-Eco cells facilitated biochemical and genetic analyses that can be limited or impossible in primary T cells. Through this approach, I found that the membrane distal clusters beyond Ala-185 were dispensable for ICOS-autonomous Ca^{2+} flux in Jurkat cells as well as potentiation of TCR-mediated Ca^{2+} flux in primary CD4 T cells (Figure 3.1C; third panel, p.31). Remarkably, when most of the cytoplasmic tail was truncated except the four membrane proximal residues, ICOS still maintained its Ca^{2+} fluxing

capacities in both cell types (Figure 3.1C; fourth panel, p.31). Therefore, I conclude that the membrane proximal cluster KKKY in the cytoplasmic tail of ICOS is sufficient to induce Ca^{2+} flux.

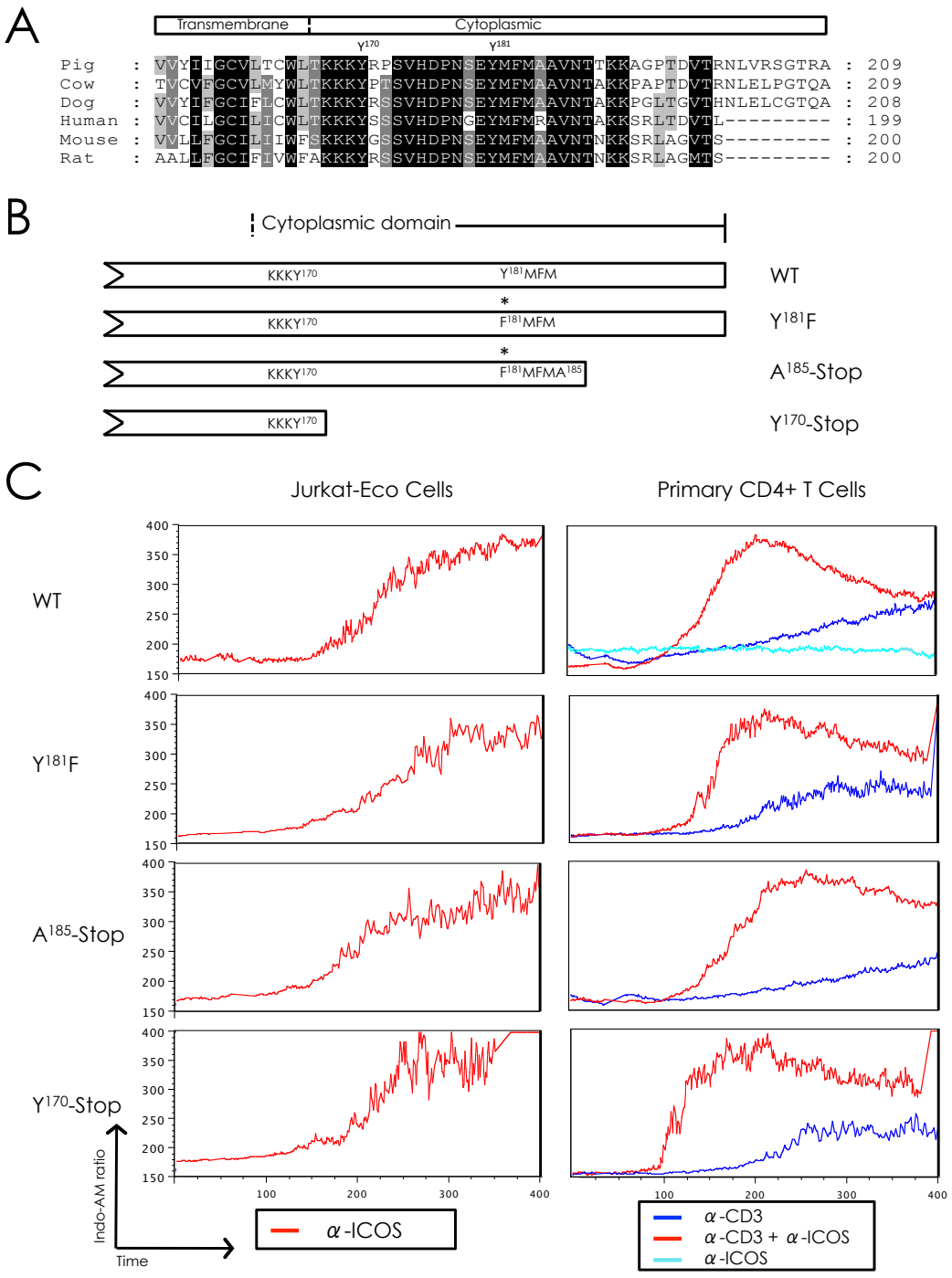

Figure 3.1: ***Mapping the minimal segment of ICOS cytoplasmic tail sufficient for Ca^{2+} flux.*** **(A)** Schematic representation of the transmembrane and cytoplasmic sequence of ICOS in multiple species. Evolutionarily conserved regions are highlighted in black. **(B)** Schematic representation of ICOS mutants **(C)** Autonomous calcium fluxing capacities (Jurkat-Eco) or potentiation of TCR-mediated calcium flux by ICOS mutants. Data shown are representative of two independent experiments.

The triple lysine motif is necessary for Ca^{2+} fluxing capacity of ICOS

To determine whether or not the KKKY motif is essential in mediating the Ca^{2+} fluxing ability of ICOS, I mutated the single tyrosine or the triple lysine residues in the ICOS-$Y^{181}F$ context sequence (Figure 3.2A, p.33). When the tyrosine at position 170 was mutated to phenylalanine, I observed a striking decrease in Ca^{2+} flux in Jurkat-Eco cells as well as in primary CD4 T cells (Figure 3.2B; second panel, p.33). Similar results were obtained when Tyr170 was mutated to alanine (data not shown). Mutation of the triple lysine residues ($KKK^{167\text{-}169}AAA\_Y^{181}F$) led to substantial reduction of cell surface ICOS level compared to the $Y^{170}F\_Y^{181}F$ mutant (Figure 3.2C, p.33). In order to overcome these differential expression levels, I took a dual staining strategy: a non-agonist staining antibody (clone 15F9-PE) to gate on cells expressing similar levels of ICOS mutants and an agonist antibody (C398.4A-biotin) to stimulate the cells with avidin-mediated crosslinking. In both cell types, Ca^{2+} fluxing ability of ICOS was completely abrogated when the triple lysine residues were changed to alanine (Figure 3.2B; third panel, p.33). I conclude that the triple lysine residues are crucial for ICOS-mediated $Ca2^{+}$ mobilization as well as maintaining ICOS on the cell surface. The tyrosine residue at position 170 is also involved in Ca^{2+} mobilization but its contribution to cell surface expression of ICOS is minimal.

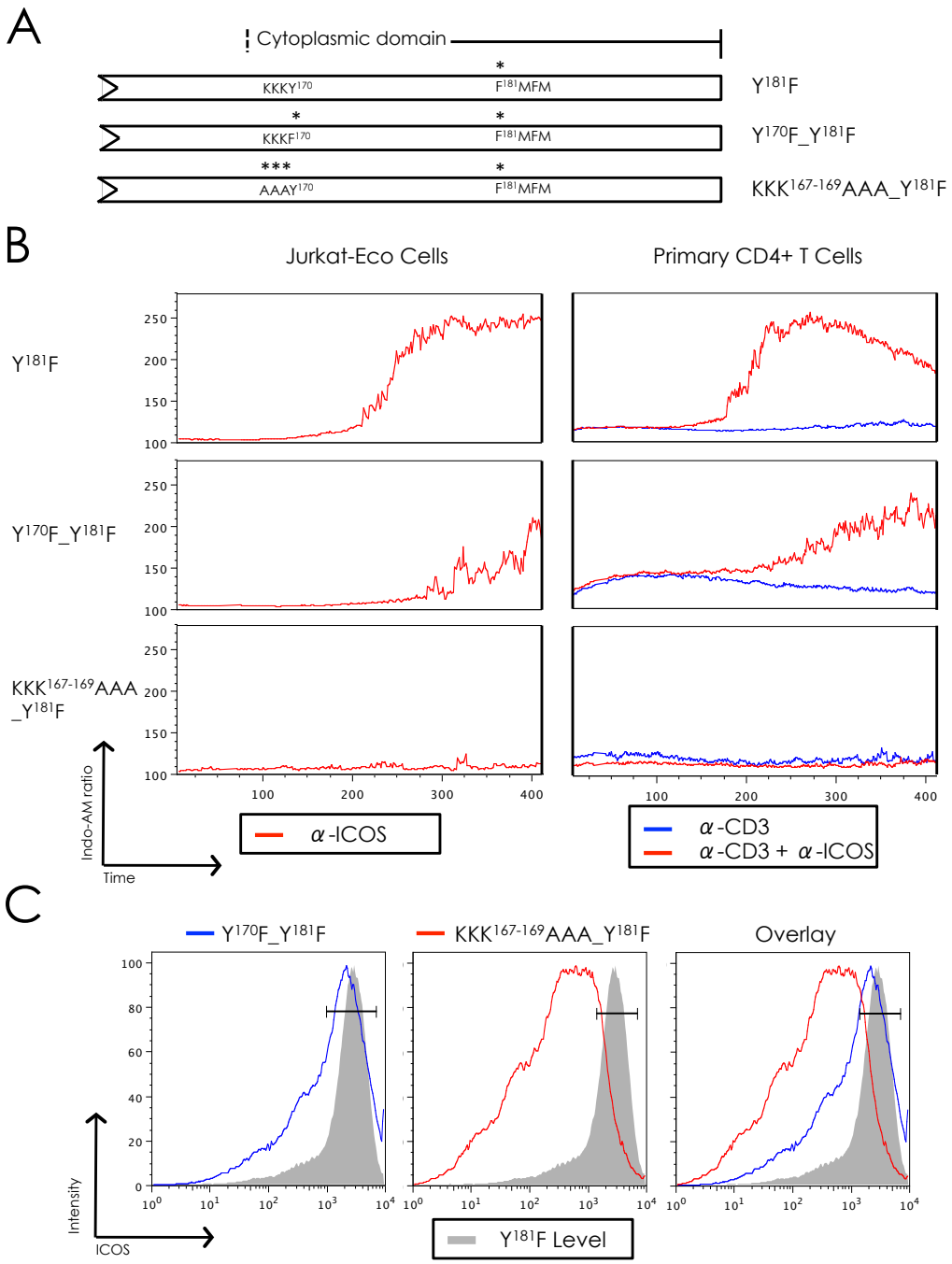

Figure 3.2: ***Membrane proximal motif KKKY is crucial for ICOS-mediated Ca^{2+} flux.*** **(A)** Representation of ICOS tail mutants in the context of full length ICOS-$Y^{181}F$. **(B)** Jurkat-Eco cells and preactivated ICOS KO CD4 T cells were transduced by retroviral vectors encoding indicated ICOS mutant constructs. Both cell types were then examined for their ICOS-Ca^{2+} signaling capacities. **(C)** Transduced cells were stained with anti-ICOS-PE to access surface levels. Data shown are representative of two independent experiments.

Interruption of the triple lysine stretch affects Ca^{2+} flux initiation

In order to find ICOS mutants that selectively lose Ca^{2+} fluxing ability without affecting surface expression level, I carried out individual alanine mutations of the triple lysine residues in the ICOS-Y^{181}F backbone (Figure 3.3A, p.35). These single lysine mutations did not affect the surface expression of ICOS on the surface (data not shown). However, the mutation of a single lysine residue did not lead to an abrogation of the signal. Nonetheless, there were clear delays in the initiation of Ca^{2+} flux (Figure 3.3B, p.35). The delay was biggest when the middle lysine residue (K168) was targeted and the impacts of changing the flanking lysine residues (K167 or K169) were smaller. These results suggest that an uninterrupted stretch of positive charges can be important for ICOS to induce Ca^{2+} flux. It remains to be seen if mutations of the lysine residues for a similar stretch of positive residues, such as three arginine residues, would occasion the receptor function to become ineffective or keep it intact.

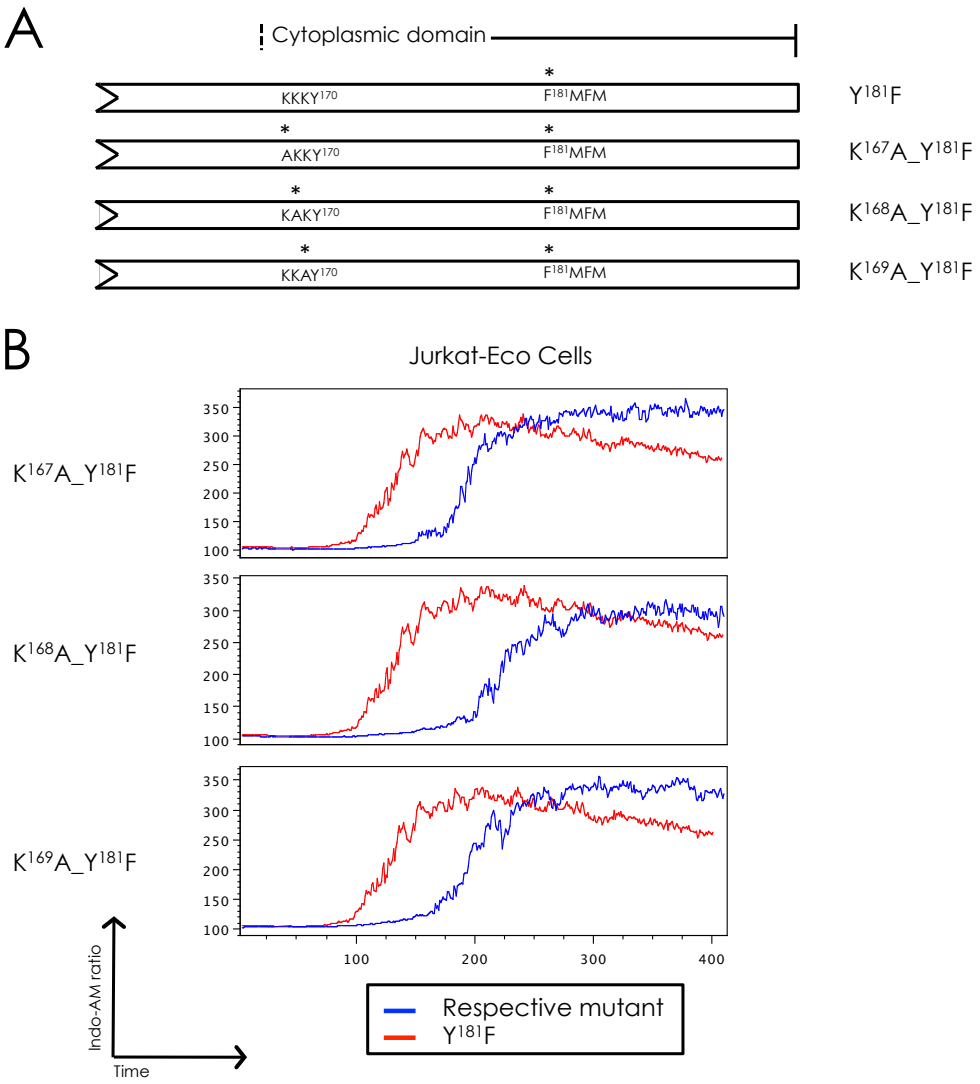

Figure 3.3: ***Single mutations in the membrane proximal triple lysine cluster of ICOS lead to delayed Ca^{2+} initiation.*** **(A)** Representation of single lysine ICOS mutants in the context of ICOS-$Y^{181}F$. **(B)** Jurkat-Eco cells were transduced by retroviral vectors expressing indicated ICOS mutants and were examined for their ICOS-Ca^{2+} signaling capacities.

ICOS induces intracellular Ca^{2+} release from the intracellular pool

ICOS could be involved in TCR-mediated Ca^{2+} flux possibly through the release of the intracellular pool of Ca^{2+} from the ER or in the later stages by interacting with plasma membrane CRAC channels. I tested if ICOS can induce calcium release from the intracellular pool by analyzing Ca^{2+} flux capacities of ICOS-Y^{181}F expressed in Jurkat-Eco cells in the presence or absence of Ca^{2+} chelator ethylene glycol tetraacetic acid (EGTA) in the buffer. I observed that despite the deprivation of extracellular Ca^{2+}, the initial Ca^{2+} release peak was maintained (Figure 3.4A, p.37). Consistent with this, I have preliminary data showing that when the cells are treated with U73122, an inhibitor of PLCγ1[148, 149], ICOS-mediated Ca^{2+} induction is compromised in comparison with treatment with U73343, an inactive analog of the inhibitor (Figure 3.4B, p.37). In this setting, cell viability was reduced, as the concentration of the inhibitor might have been higher than optimal for Jurkat-Eco cells [150]. Although it needs to be further substantiated, these results suggest that ICOS is involved in the earlier events of intracellular Ca^{2+} release in T cells possibly through PLCγ1.

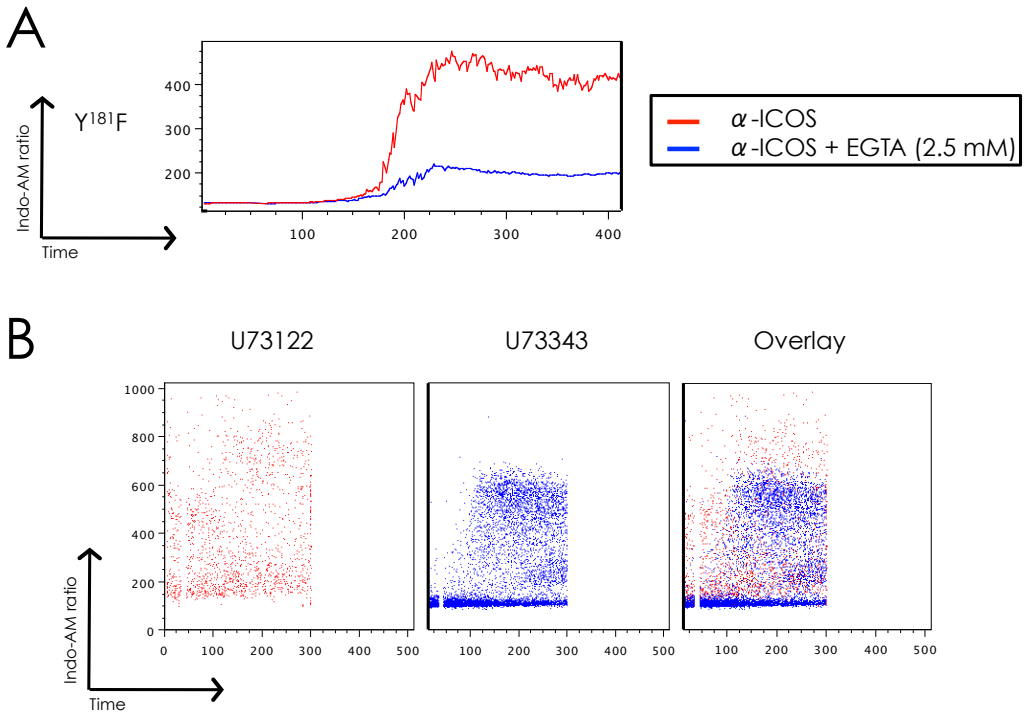

Figure 3.4: ***ICOS is involved in the release of intracellular Ca^{2+}.*** Jurkat-Eco ICOS_$Y^{181}F$ cells were examined for their ICOS-Ca^{2+} signaling capacities **(A)** in the presence of Ca^{2+} chelator EGTA, or **(B)** after treatment with PLCγ1 inhibitor U73122 or it's inactive analog U73343. Data shown are representative of two independent experiments.

ICOS-mediated Ca^{2+} flux depends on Src family kinases

TCR-mediated calcium signaling involves Src family kinases. To test whether ICOS-mediated Ca^{2+} flux relies on Src family kinases, I treated ICOS-$Y^{181}F$ expressing Jurkat-Eco cells with PP2, an inhibitor whose main targets are two of the most documented Src family kinases associated with TCR signaling: Lck and Fyn. In the presence of PP2, ICOS-mediated

Ca^{2+} flux was completely abrogated (Figure 3.5A, p.39). I then investigated the potential involvements of Fyn and Lck in ICOS-mediated Ca^{2+} flux. I tested the fluxing abilities of primary CD4 T cells isolated from $Fyn^{-/-}$ mice compared to those from WT littermates. I found that Fyn-deficient T cells had largely intact ICOS-mediated potentiation of TCR-mediated Ca^{2+} flux (Figure 3.5B, p.39). Next, I examined the specific contribution of Lck using Lck-deficient Jurkat cell line JCam1 and its reconstituted derivative JCam1_Lck^{151} (Figure 3.5C, p.39). After expressing murine ICOS in these cells through endofectin-mediated lentiviral transduction, I analyzed their Ca^{2+} flux capacities. In these preliminary experiments, I observed a total abrogation of Ca^{2+} flux in JCam1 cells whereas JCam1_Lck cell had an intact Ca^{2+} flux (Figure 3.5D, p.39). Based on these, I conclude that ICOS-mediated Ca^{2+} flux depend on the Src family kinase Lck whereas Fyn is dispensable.

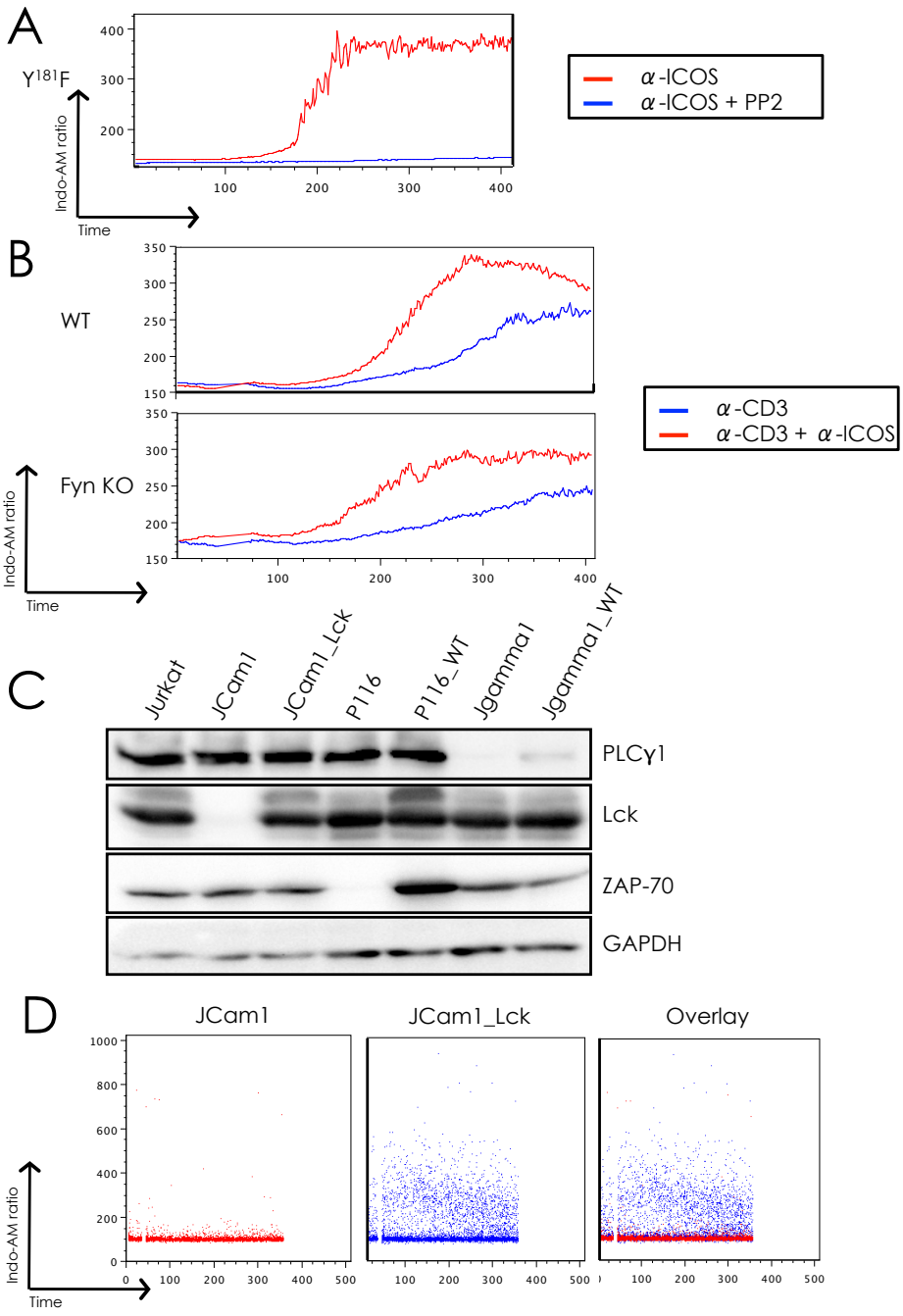

Figure 3.5: ***ICOS mediates its Ca^{2+} flux function through Src family kinases.*** **(A)** Jurkat ICOS_$Y^{181}F$ cells were examined for their ICOS-Ca^{2+} signaling capacities after treatment with Src family kinases inhibitor PP2. **(B)** Preactivated CD4 T cells were prepared from WT or Fyn-KO mice and were examined for their ICOS-Ca^{2+} signaling capacities. **(C)** Western Blot depicting complete loss of specific proteins in respectively depleted Jurkat mutants and their reconstituted counterparts. **(D)** JCam1 and JCam1_Lck cells were given ICOS-$Y^{181}F$ expressing plasmids by retroviral transduction and were then examined for their ICOS-Ca^{2+} signaling capacities. Data shown are representative of four independent experiments.

Following stimulation, ICOS associates with Lck and ZAP70 and increases activation of PLCγ1

In an effort to understand the mechanism by which ICOS interacts with Lck and PLCγ1 during Ca^{2+} flux potentiation, I performed in-depth biochemical analysis of different TCR signaling components following stimulation of the cells. I stimulated activated primary WT CD4 T cells with anti-CD3 without or with anti-ICOS and prepared cell lysates to analyze protein interactions and phosphorylation status. First, to see whether ICOS was associated with known TCR proximal kinases, I lysed the stimulated cells with a mild detergent digitonin to maximize the recovery of protein complexes. After immunoprecipitation of ICOS, I noticed that without costimulation the co-receptor was already associated with significant levels of Lck and some ZAP-70 molecules (Figure 3.6A, p.41). Remarkably, after costimulation the abundance of Lck molecules increased by 60 % and ZAP-70 by 80 %. To specifically look at Lck phosphorylation status[13, 152], I immunoprecipitated Lck directly following lysis in NP-40 lysis buffer. No significant differences were detected for either Tyr505 (Lck inhibitory tyrosine) or Tyr394 (Lck activating tyrosine)(Figure 3.6A, p.41). Total tyrosine phosphorylation (p-Tyr) didn't show any differences as well ruling out potential changes at other sites on the protein. PLCγ1 is known to be activated through phosphorylation of Tyr793. By immunoprecipitating PLCγ1, I aimed to look at whether or not after stimulation, ICOS augmented PLCγ1 activation through the phosphorylation of Tyr793[153, 154]. Strikingly, costimulation of ICOS significantly increased PLCγ1 activation compared to TCR stimulation alone (Figure 3.6B, p.41). Thus, ICOS associates with Lck and ZAP-70 and increases PLCγ1 activation in the context of CD4 T cell costimulation.

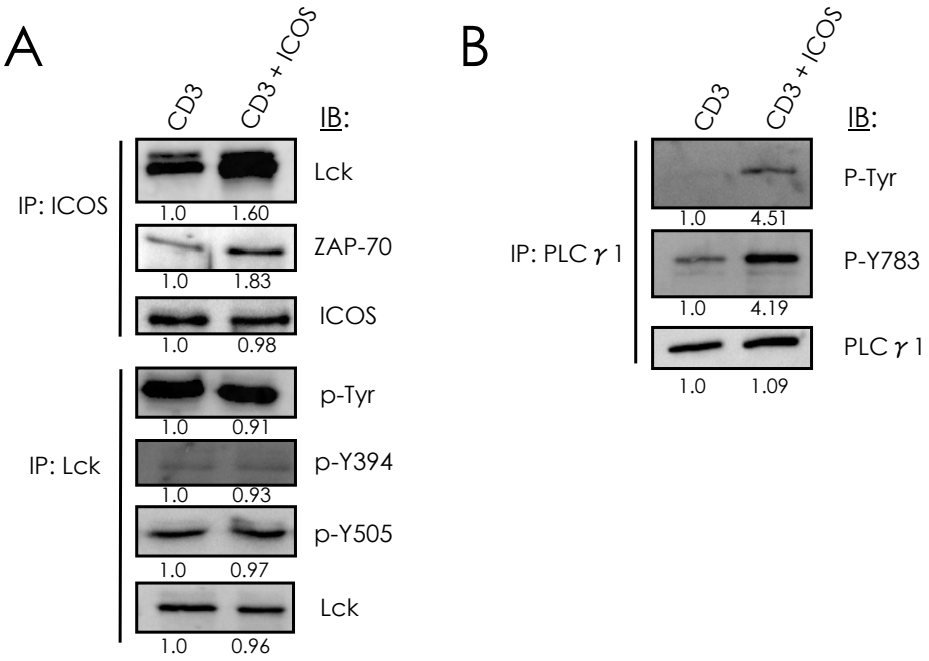

Figure 3.6: ***ICOS associates with Lck and ZAP-70 and increases PLCγ1 activation.*** **(A)** Western Blot revealing the effect of ICOS costimulation on the association of Src family kinases with ICOS and changes in their activation status. **(B)** Western Blot portraying changes in PLCγ1 phosphorylation status upon ICOS costimulation.

ICOS does not relocalize into lipid rafts upon stimulation

Several studies have showed that the ICOS homolog, the prototype costimulatory receptor CD28, translocate into lipid rafts following stimulation[155, 156]. Furthermore, other groups have demonstrated that Fyn was present in those lipid rafts and that Lck was recruited to the area upon T cell stimulation[34, 157, 158]. I wanted to determine whether ICOS needed to relocalize into lipid rafts in order to carry out its Ca^{2+} flux functions. As a preliminary experiment, I first examined the effects of disrupting the lipid rafts by treating the cells with different doses of methyl-

β-cyclodextrin (MCD), a compound forming inclusion complexes with cholesterol, therefore disrupting the lipid rafts[159]. The ability of ICOS-Y^{181}F expressing Jurkat-Eco cells to induce Ca^{2+} flux through ICOS was significantly compromised by MCD in a dose-dependent manner (Figure 3.7A, p.43). Next I tried to determine if ICOS was indeed relocalizing into the membrane lipid rafts. For this purpose I isolated lipid rafts by sucrose density from Jurkat-Eco cells expressing ICOS-Y^{181}F following stimulation to trigger autonomous Ca^{2+} flux induction. The fractionation method was first confirmed in unstimulated cells by blotting the different fractions of the gradient for Fyn, which is known to be mostly present in lipid rafts[157, 160](Figure 3.7B, p.43). Then, following ICOS stimulation, I isolated and pooled the raft-containing fractions along with the soluble fractions and blotted each of them for the presence of ICOS, to judge whether or not ICOS relocalized after stimulation. I observed no relocalization of ICOS into lipid rafts after stimulation (Figure 3.7C, p.43). Therefore, I found no evidence that ICOS relocalizes into lipid rafts upon ligation and the impact of MCD on ICOS-mediated Ca^{2+} flux is probably due to a general toxic effect caused by disturbance in membrane structure.

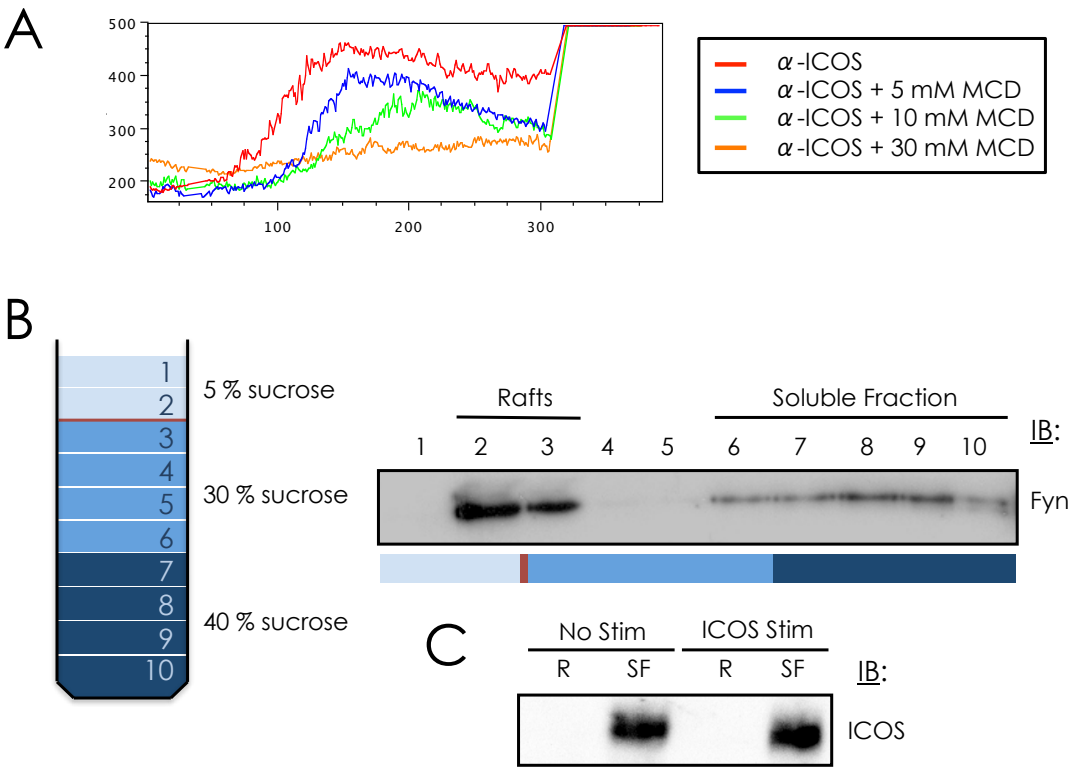

Figure 3.7: ***ICOS doesn't relocalize into lipid rafts upon stimulation*** **(A)** Jurkat ICOS_Y^{181}F cells were examined for their ICOS-Ca^{2+} signaling capacities after treatment with the indicated doses of methyl-β-cyclodextrin (MCD). **(B)** Jurkat ICOS_Y^{181}F cell lysate was fractionated by ultracentrifugation on a sucrose gradient. Collected fractions were then analyzed by Western Blot to confirm isolation of lipid raft fractionations. **(C)** Jurkat ICOS_Y^{181}F cells were stimulated with α-ICOS, lysed and fractions representing lipid rafts and soluble fractions were pooled. ICOS was immunoprecipitated from these pools and the IP products were quantified by Western blot.

Chapter 4

DISCUSSION & SUMMARY

DISCUSSION

As discussed in the opening chapter, ICOS potentiates TCR-mediated PI3K activation and intracellular calcium mobilization. Our group previously showed that the ICOS-Y^{181}F mutation selectively abrogated the capacity of ICOS to recruit PI3K but still led to TCR-mediated Ca^{2+} flux potentiation[79]. In collaboration with Dr. Yu's group, we also demonstrated that ICOS plays an important role in inducing acute GVHD in murine models through PI3K-independent manner[121 122]. However, at this point, the ICOS signal transduction pathway(s) involved in GVHD remain unknown. We hypothesized that the intact pathogenic capacity of ICOS-Y^{181}F T cells to induce GVHD relies on ICOS-mediated calcium signaling. Our initial goals were to identify the residue(s) responsible for this ICOS-mediated Ca^{2+} signaling and find the mechanism by which the receptor achieves this function. Through site-directed mutagenesis and flow cytometric analysis of calcium fluxing capacities of mutant ICOS proteins, I sought to identify the cytoplasmic residues essential for ICOS-mediated Ca^{2+} signaling.

In the early stages of this study, I realized that ICOS, when transduced in Jurkat-Eco cells, enacted its Ca^{2+} flux function independently of TCR co-ligation. In primary CD4 T cells, this feature remained unachievable as ICOS depends on TCR stimulation. The "autonomous" nature of ICOS-mediated Ca^{2+} flux in Jurkat-Eco cells facilitated biochemical and genetic analyses that can be either limited or impossible in primary T cells. Specifically, targeted disruption of genes encoding Lck, ZAP-70, or PLCγ1 in mice leads to defects in T cell development[161, 162]. However, Jurkat mutants lacking these components proved to be viable with defects in TCR signaling. [163-165]. It is important to note that every time a mutant form of ICOS was expressed in either system

(Jurkat or primary CD4+ T cells), Ca^{2+} flux patterns were congruent suggesting these two systems use the same biochemical signaling mechanisms. Also, all the mutants tested never displayed an ICOS autonomous Ca^{2+} flux phenotype in primary CD4+ T cells. Although the lack of autonomous Ca^{2+} fluxing capacity by ICOS in primary T cells presumably prevent inappropriate activation of autoreactive T cells, it remains elusive how this can be achieved at molecular level. In primary T cells, ICOS could be repressed until initial TCR-mediated signal(s) lift the repression mechanism. In Jurkat cells, ICOS could be circumventing the initial requirements to lift this repression due to the increase amount of Itk and its chronically elevated activity[166]. Indeed, Itk is known to interact with Fyn in the early stages of TCR signaling[167]. It is possible that the abundance of Itk could lead to a bypass of TCR-mediated ICOS licensing, allowing it to directly potentiate, or in this case induce, Ca^{2+} flux. It is still unclear if ICOS directly utilizes the TCR machinery in Jurkat-Eco cells or if it uses an alternative mechanism. One simple way to investigate this hypothesis would be by looking at Ca^{2+} flux capacity of ICOS in Jurkat cells lacking the TCR ζ chain. It should help us unfold the reasons behind ICOS autonomous Ca^{2+} flux induction in Jurkat cells.

For the identification of the cytoplasmic cluster(s) of residues essential in inducing ICOS-mediated Ca^{2+} signaling, I examined the evolutionary conserved clusters in the cytoplasmic tail of ICOS. ICOS possesses three conserved clusters in its cytoplasmic tail: the KKKY cluster spanning positions 167 to 170, the SVHDPN from positions 173 to 178 and the AVNTNKK cluster from positions 186 to 192. Triple lysine motifs have been documented as having various applications for different protein functions. One of them is for extracellular matrix adhesion by peripheral blood T cells with cyclophilins (cyclosporine A-binding proteins)[168] that contain the KKK motif as a binding site for glycosaminoglycans (GAGs). In

this protein, the lys-lys-lys cluster forms a binding site for GAGs in association with another cluster in the protein's sequence[169]. A triple lysine cluster also represents a positively charged binding site. Negatively charged triple glutamic acid clusters (EEE) are known binding sites present in calmodulin[170, 171]. Common posttranslational modifications of lysine, e.g. methylation of the ε-amino group NH_3^+, as it occurs in calmodulin, allows for participation in hydrogen bonding and as a general base in catalysis. The co-receptor CTLA-4, also bears a lysine rich motif essential to interact with the enzyme PP2A[172-174]. PP2A is essential to control cell signaling by timely inactivation of kinase signaling cascades. Although there is no evidence for direct binding of PP2A and calmodulin to ICOS in our mass-spectrometry-based screening experiments these examples of Lys cluster in protein-protein interaction suggest its role in signal transduction. The second conserved cluster SVHDPN contains a SXXD motif that is a well known consensus phosphorylation site for the serine/threonine selective protein kinase Casein kinase 2 (CK2)[175]. CK2 is a pleiotropic protein kinase known to be targeting several different substrates[176]. CK2 is notably implicated in cell signaling and proliferation[177, 178]. CK2 is already recognized as being implicated in Ca^{2+} signaling in T cells through association with CD45[179]. The D2 domain of CD45 contains four CK2 phosphorylation sites and is implicated in the regulation of the calcium/NFAT T cell activation pathway[180]. And finally, TXK motifs are consensus phosphorylation sites for protein kinase C (PKC). PKC are activated by increases of DAG and Ca^{2+}[181]. PKC activation leads to downstream NFAT and NFκB activation[182]. These three key consensus sites present in the cytoplasmic tail sequence of ICOS possibly relate to signaling molecules closely or remotely involved in the signaling pathways downstream of TCR triggering. I predicted that the key-signaling motif of ICOS-mediated Ca^{2+} flux to be one of these three clusters. My data

indicated that the ICOS mutants targeting the SVHDPN and AVNTNKK conserved regions still maintaining ICOS Ca^{2+} flux abilities (data not shown; primer sequences in Table 1; -A*ppendix Table 1,* p.67). Results from truncated ICOS mutants (A^{185}-Stop, Y^{170}-Stop) are consistent with these data (depicted in Figure1C, p.31). The data from Y^{170}-Stop mutant indicate that the membrane proximal KKKY region is sufficient for ICOS-mediated Ca^{2+} flux. Furthermore, mutating the KKKY region to alanine residues in the full-length context led to total abrogation of the Ca^{2+} fluxing capacities of ICOS, an effect not observed for the other conserved clusters of the cytoplasmic tail. Therefore, I identified a membrane proximal cluster of lysine residues both sufficient and necessary for ICOS-mediated Ca^{2+} flux.

Importantly, this KKK cluster is also important to maintain ICOS on the T cell surface as triple Ala mutation drastically reduces cell surface ICOS level. This dual function of KKK cluster was not easily separable. However, the fact that single Ala mutation can delay Ca^{2+} flux with minimal impact on cell surface levels imply that mediators of Ca^{2+} flux may rely on the triple lysine residues more heavily than those involved in ensuring ICOS on the cell surface. It remains as a challenge to identify a mutant ICOS that selective loses the ability to flux Ca^{2+} while maintaining normal ICOS surface level. A systematic combinatorial mutagenesis approach in which two residues of the three lysine residues in the KKK cluster are replaced by alanine or glutamic acid residues may prove fruitful.

It is puzzling how ICOS is licensed by TCR signaling to potentiate Ca^{2+} flux in primary T cells. One tempting hypothesis is that the highly positive KKK cluster of the cytoplasmic tail of ICOS is normally bound to the negatively charged membrane through ionic interactions preventing its

function, a phenomenon reported for TCR CD3 chains[183]. A local influx of Ca^{2+} near the TCR may release ICOS tails from the membrane so that it can activate downstream signaling cascade. To test this, I added strontium ions (Sr^{2+}) in the buffer used to carry out our Ca^{2+} flux experiments. I proceeded with Ca^{2+} flux analysis of primary WT CD4 T cells treated with that modified buffer (Figure S1; *Appendix,* p.68). Under these conditions, ICOS was still unable to achieve autonomous ICOS-mediated Ca^{2+} flux in primary cells. Thus, it remains to be seen what is mediating the repression of ICOS-Ca^{2+} pathway in the absence of TCR signaling in primary T cells. Another tantalizing hypothesis would be that mechanism mediating ICOS licensing would be the same mechanism observed for the lysine rich motif in CTLA-4. In the case of CTLA-4, the key signaling kinases involved downstream are binding this lysine repeat site leading to modifications of the phosphorylation state of a tyrosine residue located upstream of its the cytoplasmic tail[172-174]. In ICOS, a tyrosine residue follows the lysine rich repeat. It is fair to suggest that this tyrosine residue could be involved in TCR-mediated ICOS licensing.

I provided preliminary evidence for the involvement of the Src family kinase Lck as the potential key signaling molecule downstream of the initial events triggering ICOS-mediated Ca^{2+} flux. Jurkat mutants lacking Lck (JCam1) were unable to induce Ca^{2+} flux by ICOS stimulation while their reconstituted counterparts were (JCam1_Lck). Jurkat mutant experiments need to be revisited as the preliminary ones involving lentivirus-mediated transduction of Jurkat cells led to reduced cell viability. This made impossible the rendition of analysis through kinetics as too few cells were responding to the signals. A new approach through electroporation gave better results and should be able to provide conclusive data. Supplemental experiments were performed to further demonstrate the crucial role of Lck in this process. Lck is known to be

constitutively active in T cells and its maintenance relies on the HSP90-CDC37 chaperone complex[23, 184, 185]. Tanespimycin (17-AAG) is an analog of geldanamycin, a proven HSP90 inhibitor[186, 187]. I treated the cells with this compound in order to test the effects on ICOS-mediated Ca^{2+} flux. In my experimental settings, the compound's efficiency wasn't optimal, but even with ~30% reduction of activated Lck I still observed a delay in Ca^{2+} flux initiation (Figure S2; *Appendix,* p.68). Altogether, these data strongly correlate in showing that Lck is crucial in carrying out ICOS signal downstream to mediated Ca^{2+} flux induction. I also found no evidence that ICOS relocalizes into lipid rafts upon ligation. Therefore the potential interaction with Lck is probably not occurring in lipid rafts. Next aim is to define signaling components downstream of Lck. A logical extension is to perform Ca^{2+} flux assays using Jurkat mutants lacking ZAP-70 and PLCγ1. I predict the outcome to be consistent with the results obtained from Lck-mutant Jurkat cells.

I showed that ICOS stimulation led to the elevated activation of PLCγ1 and that the release of Ca^{2+} originated from the intracellular pool. Whether it is directly involved in the release from the ER through the IP3 receptor remains to be proven. Another possibility is that it could involve mitochondria since they are known to be involved in Ca^{2+} signaling[188], but since their involvement concerns the regulation of CRAC channels rather than the release from the ER, it is unlikely that ICOS governs their activation. The results on PLCγ1 activation were shown in an ICOS-WT context. The next step is to confirm it in an ICOS-Y^{181}F context and determine if mutations that affect Ca^{2+} flux also compromise or disrupt Lck association and PLCγ1 activation.

I still don't know what role ICOS might play downstream of PLCγ1. Downstream of the Ca^{2+} signaling pathway, NFAT is relocalized to the

nucleus after activation. The activation of AKT by PI3K can lead to an inhibition of the GSK3β kinase[189, 190]. In the recent years, GSK3β has been showed to mediate NFAT phosphorylation in the nucleus leading to its nuclear export and therefore, its inactivation[191]. This allows the cell to dictate the occupancy time of transcription factors inside the nucleus[192]. Although, we have shown that ICOS-PI3K pathway is dispensable for early Ca^{2+} flux, where we measure the release of Ca^{2+} from the intracellular pool, later signaling events might require the involvement of PI3K. This can be relevant to distinct behavior of CD4 vs. CD8 T cell subsets in GVHD[122]. Whereas CD8 T cells can induce GVHD independently of ICOS-PI3K pathway, CD4 T cells seem dependent on PI3K to set off the disease symptoms. One possible explanation can be that in CD4 T cells, NFAT nuclear retention could be achieved through PI3K-mediated GSK3β suppression. Thus, in the absence of ICOS-PI3K activation pathway, ICOS can induce initial Ca^{2+} flux but NFAT may not be able to stay in the nucleus long enough due to insufficient suppression of NFAT export mechanism. In this scenario, CD8 T cells may not need to operate ICOS-PI3K pathway to maintain NFAT nuclear residence. It is interesting to see if CD8 T cells rely on other kinases such as DYRK1A and DYRK2 rather than GSK3β to achieve NFAT nuclear export[193].

To fully understand the dynamics of costimulation in TCR-mediated Ca^{2+} flux, one cannot underestimate to highly probable involvement of CD28[194]. I have preliminary data showing that ICOS and CD28 act synergistically to further potentiate TCR-mediated Ca^{2+} flux (Figure S3; *Appendix,* p.69). Since the concentrations of anti-ICOS Ab I used in these experiments are already saturated, this synergy between ICOS and CD28, which goes beyond each receptor's costimulatory capacities, might indicate that the two costimulatory molecules activate distinct pathways to accomplish their functions. Further work is required to test this idea and

understand the potential collaboration of these to costimulatory receptors in T cell biology.

SUMMARY

While most studies involving ICOS in GVHD have confirmed its critical role in the disease onset, it is yet to be described how it works.

During the course of my Master's study, I identified the membrane proximal cluster of lysine residues as essential in inducing ICOS-mediated Ca^{2+} signaling. Furthermore, I provided evidence for the involvement of the Src family kinase Lck in ICOS-mediated Ca^{2+}signaling. With the added insight of preliminary results, I propose a model in which ICOS utilizes Lck-ZAP70-PLCγ1 signaling axis to potentiate TCR-mediated Ca^{2+} flux and possibly PI3K-independent pathogenic function of T cells in GVHD.

Thus, my study is beginning to unravel a complexity in ICOS-induced calcium signaling. Further understanding of this pathway could prove beneficial in designing new strategies to prevent ICOS-related diseases such as GVHD.

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APPENDIX

APPENDIX

Table 1: *List of primers used for site-directed mutagenesis*	
A^{185}-Stop	TTCATGTTCATGGCGTAATAGGCAGTCAACACAAAC
Y^{170}-Stop	TCAAAAAAGAAATACTAATGAGGATCCAGTGTGCAT
$Y^{170}F\_Y^{181}F$	TTTTCAAAAAAGAAATTCGGATCCAGTGTGCAT
$KKK^{167\text{-}169}AAA\_Y^{181}F$	ATCATCTGGTTTTCAGCGGCGGCGTACGGATCCAGTGTG
$K^{167}A\_Y^{181}F$	ATCATCTGGTTTTCAGCAAAGAAATACGGATAA
$K^{168}A\_Y^{181}F$	ATCTGGTTTTCAAAAGCAAAATACTAATGAGGA
$K^{169}A\_Y^{181}F$	TGGTTTTCAAAAAAGGCATACTAATGAGGATCC
$K^{167}A\_K^{168}A\_Y^{181}F$	ATCATCTGGTTTTCAGCAGCAAAATACGGATCCAGTGTG
$K^{167}A\_K^{169}A\_Y^{181}F$	ATCATCTGGTTTTCAGCAAAGGCATACGGATCCAGTGTG
$K^{168}A\_K^{169}A\_Y^{181}F$	ATCATCTGGTTTTCAAAAGCAGCATACGGATCCAGTGTG
$K^{167}E\_K^{168}E\_Y^{181}F$	ATCATCTGGTTTTCAGAAGAAAAATACGGATCCAGTGTG
$K^{167}E\_K^{169}E\_Y^{181}F$	ATCATCTGGTTTTCAGAAAAGGAATACGGATCCAGTGTG
$K^{168}E\_K^{169}E\_Y^{181}F$	ATCATCTGGTTTTCAAAAGAAGAATACGGATCCAGTGTG
$DPN^{176\text{-}178}AAA\_Y^{181}F$	GGATCCAGTGTGCATGCAGCAGCGAGTGAATTCATGTTC
$S^{173}A\_DPN^{176\text{-}178}AAA\_Y^{181}F$	AAGAAATACGGATCCGCAGTGCATGCAGCAGCG
$T^{189}A\_Y^{181}F$	ATGGCGGCAGTCAACGCAAACAAAAAGTCTAGA
$K^{191}A\_K^{192}A\_Y^{181}F$	GCAGTCAACACAAACGCAGCATCTAGACTTGCAGGT
$IWF^{163\text{-}165}VAL\_Y^{181}F$	GGATGCATACTTATCGTGGCGCTGTCAAAAAAGAAATAC

Sense primer sequences are listed (5'-3'). Orange–highlighted bases represent the mutation in the target sequence.

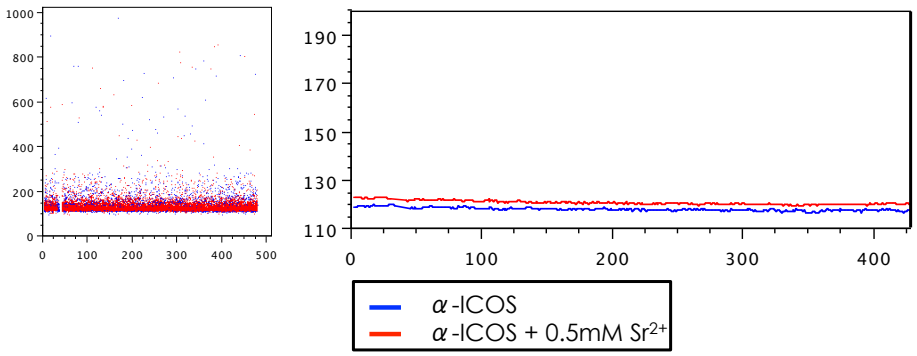

Figure S1: ***Addition of Sr^{2+} is not sufficient to allow autonomous ICOS-mediated Ca^{2+} flux in primary $CD4^{+}$ T cells***. Preactivated CD4 T cells were prepared from WT mice and were then examined for their autonomous ICOS-Ca^{2+} signaling capacities after addition of Sr^{2+} in the media during Indo-1 loading and stimulation processes.

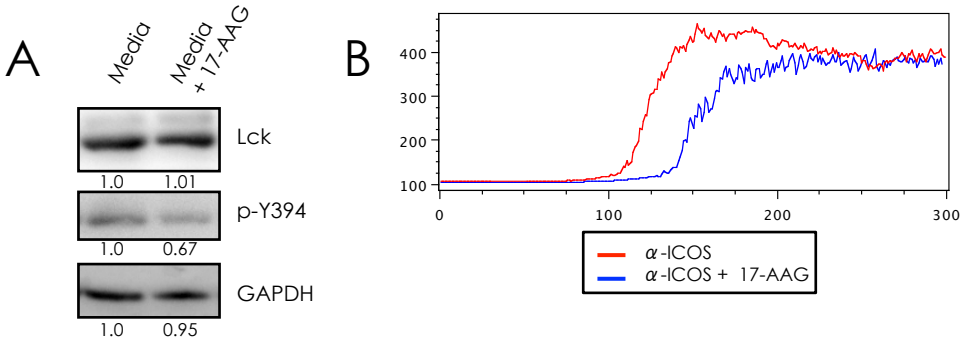

Figure S2: ***Inhibition of HSP90 by Tanespimycin treatment leads to delayed ICOS-mediated Ca^{2+} flux initiation***. **(A)** Western Blot illustrating the effect of 17-AAG on the phosphorylation status of Lck after a 3-hour treatment on Jurkat ICOS-Y^{181}F cells. **(B)** Cells were examined for their ICOS-Ca^{2+} signaling capacities with or without a 17-AGG treatment.

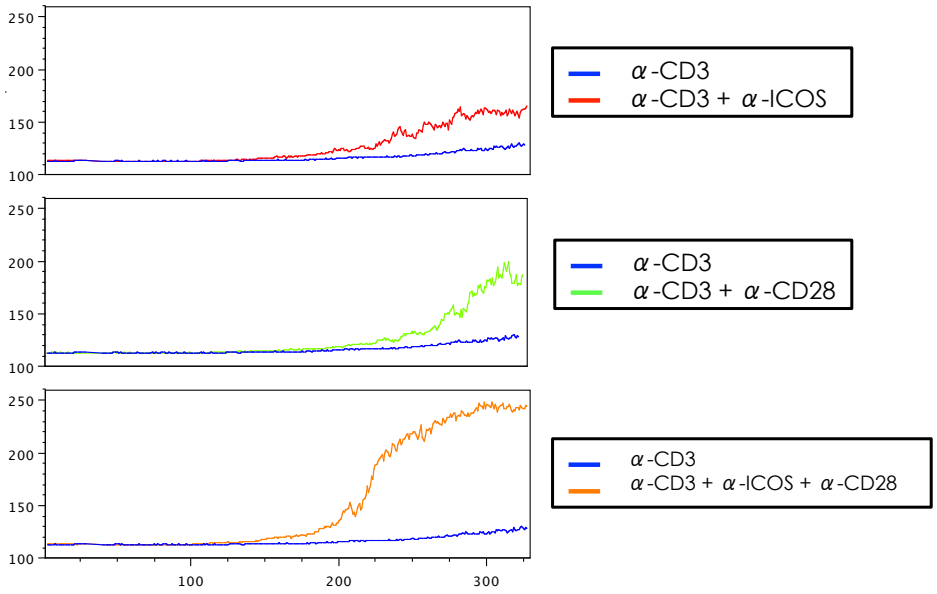

Figure S3: ***ICOS and CD28 act synergistically to further potentiate TCR-mediated Ca^{2+} flux***. Preactivated CD4 T cells were prepared from WT mice and were examined for their TCR-Ca^{2+} signaling capacities in combination with costimulatory signals coming from either ICOS and CD28 or both.

Curriculum vitae

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EDUCATION

2011-2014 M.Sc. Dept. of Microbiology and Immunology
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2007-2010 B.Sc. Microbiology, Dept. of Biology
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ACADEMIC WORK EXPERIENCE

2010 Developing projects on microorganism detection methods (for *Campylobacter sp.* and *Listeria monocytogenes)*
Third B.Sc. Internship, Health Products and Food Laboratory, Health Canada, Longueuil, QC, Canada

2009 Laboratory Technician in drinkable water analysis
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2009 Research project on Human Papilloma Virus (HPV)
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EXTRA ACADEMIC ACTIVITIES

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-Organization of Annual IRCM Student Summer Retreat

2010 Vice-President, Student Association, Dept. of Biology
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-Organization of Graduation Prom (Fund Raising & Celebrations)

2010 Columnist, Le Collectif, School Journal
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AWARDS

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PUBLICATIONS

Gigoux, M., Lovato, A., **Leconte, J.,** Leung, J., Sonenberg, N., and Suh, W.K. (2014) Inducible Costimulator facilitates T-dependent B cell activation by augmenting IL-4 translation. *Mol Immunol*. May;59(1):46-54.

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ABSTRACTS AND POSTERS

Leconte, J., Bagherzadeh-Yazdchi, S., Suh, W.-K. (2014) Mechanism of the inducible costimulator (ICOS)-mediated calcium signaling. Abstract/Poster. Université de Montréal Immunology and Microbiology Research Day, Montréal, QC, Canada; and Abstract/Oral presentation. IRCM Research Day, Montréal, QC, Canada.

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Li, J., **Leconte, J.**, Semple, K., Heinrichs, J., Anasetti, C., Suh, W.-K., and Yu, X.-Z. (2012) PI3K-independent signaling pathways contribute to ICOS-mediated T-cell costimulation in acute graft-versus-host disease in mice. Abstract/Poster. ASH Meeting, Atlanta, GA, USA.

Li, J., Semple, K., **Leconte, J.,** Suh, W.-K., and Yu. X.-Z. (2012) ICOS promotes T cell activation and function independently of PI3K signaling. Abstract/Poster/Oral Presentation. AAI Meeting, Boston, MA, USA.

Leconte, J., Gigoux, M., Suh, W.-K. (2012) Mechanism of the inducible costimulator (ICOS)-mediated calcium signaling. Abstract/Poster. IRCM Research Day, Montréal, QC, Canada

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