The role of Inducible Costimulator-mediated phosphoinositide 3-kinase activation in the differentiation and function of follicular helper T cells

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DEDICATIONS

To my family and to Marissa

À ma famille et à Marissa

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ABSTRACT

Antibodies are crucial components of the adaptive immune arsenal against invading pathogens. Production of high-affinity class-switched antibodies relies on follicular helper T (Tfh) cells, a distinct subset of CD4 helper T cells that migrate into B cell follicles and promote B cell differentiation into plasma cells during germinal center (GC) reactions. The CD28-like costimulatory receptor Inducible Costimulator (ICOS) is expressed on the surface of activated T cells and is crucial for the generation of Tfh cells in mice and humans, but the molecular mechanisms remained unknown. ICOS had been known as a potent activator of phosphoinositide 3-kinase (PI3K), but the role of ICOS-mediated PI3K activation in T cells has been poorly understood. The work presented here is a compilation of two studies that highlight the unique role of PI3K in ICOS-mediated Tfh cell differentiation and function.

In the first study, presented in Chapter II, I analyzed a knock-in strain of mice possessing a point mutation in the cytoplasmic tail of ICOS that prevents binding of PI3K (ICOS-YF). I show that ICOS-mediated PI3K activation is crucial for the generation of Tfh cells, and in turn, GC formation, antibody class-switch, and affinity maturation. The ICOS-PI3K axis was crucial for the potentiation of T cell receptor (TCR)-mediated expression of IL-21 and IL-4, key cytokines involved in T cell-mediated B cell help. I also show data that strongly suggests that ICOS and CD28 have differential roles in the multistep process of Tfh cell differentiation, where CD28 is mainly involved in the early expansion of CD4 T cells through non-PI3K signaling mechanisms, while ICOS is involved in the later stages of Tfh cell differentiation in a PI3K-dependent manner.

In the study presented in Chapter III, I show that ICOS costimulation enhances TCR-mediated activation of the key translation mediators 4E-BP1 and S6K, in a manner dependent on PI3K. Consistently, I show that the ICOS-PI3K axis enhances the formation of polysomes on IL-4 mRNA. Using an *in vitro* T-B cell co-culture system, I provide evidence that ICOS mutant CD4 T cells have impaired ability to induce B cell differentiation due to a limited production of IL-4. These findings suggest that ICOS-PI3K signaling facilitates targeted delivery of IL-4 from helper T cells to cognate B cells during T cell-B cell interactions in the GC.

Thus, I demonstrate that PI3K is a key downstream signaling component in ICOS signaling during Tfh cell generation. I also show that ICOS-PI3K signaling can alter translational efficiency of pre-existing mRNAs suggesting ICOS' potential role in regulating the function of Tfh cells.

RÉSUMÉ

Les anticorps sont des composantes cruciales de l'arsenal que le système immunitaire adaptatif utilise contre les pathogènes invasifs. La production d'anticorps de haute-affinité réarrangés par commutation isotypique nécessite l'apport des lymphocytes T auxiliaires folliculaires (Tfh), un sous-type de lymphocytes T auxiliaires CD4+ qui migrent dans les follicules nodules lymphatiques et y promeuvent la différentiation des cellules B en cellules plasmatiques, le tout durant les réactions du centre germinatif (GC). Le récepteur de costimulation de type CD28 Costimulateur Inductible (ICOS) est exprimé sur la surface des cellules T activées et joue un rôle critique dans la génération de cellules Tfh autant chez la souris que l'humain, cependant les mécanismes moléculaires demeurent inconnus. Jusqu'à présent, ICOS était reconnu comme un puissant activateur de la phosphatidyl inositol-3 kinase (PI3K), mais le rôle de l'activation de PI3K médiée par ICOS dans les cellules T demeure mal compris. Le travail présenté dans cette thèse retrace deux études qui décrivent le rôle unique de PI3K dans la fonction et la différentiation des cellules Tfh médiées par ICOS.

Dans la première étude, présenté dans le Chapitre II, j'ai analysé une ligné de souris 'knock-in' possédant une mutation ponctuelle dans la région cytoplasmique de ICOS empêchant ainsi la liaison de PI3K (ICOS-YF). Je démontre que l'activation de PI3K médiée par ICOS demeure cruciale pour la génération de cellules Tfh, ainsi qu'en conséquence la formation des GC, la commutation isotypique d'anticorps et la maturation d'affinité. L'axe ICOS-PI3K s'avère critique pour la potentialisation de l'expression médiée par le récepteur de cellules T (TCR) de IL-21 et IL-4, des cytokines clés impliquées dans l'aide aux cellules B médiée par les cellules T. J'illustre également des résultats qui prouvent que ICOS et CD28 exercent des rôles distinct dans le processus complexe de la différentiation des cellules Tfh, où CD28 est principalement impliqué dans l'expansion précoce des cellules T $CD4^+$ par l'entremise de mécanismes de signalisation indépendants de PI3K, tandis que ICOS s'engage plutôt de façon PI3K-dépendante dans les étapes tardives de la différentiation des cellules Tfh.

Dans la seconde étude au Chapitre III, je révèle que la costimulation par ICOS augmente l'activation médiée par le TCR de médiateurs clés de la traduction de 4E-BP1 ainsi que S6K de façon dépendante à PI3K. De façon cohérente, je démontre que l'axe ICOS-PI3K augmente la formation de polysomes sur l'ARN messager d'IL-4. En utilisant un système *in-vitro* de co-culture

de cellules T et B, je fourni des preuves que les cellules T $CD4^+$ mutantes pour ICOS ont une capacité détérioré d'induire la différentiation de cellules B due à une production limitée d'IL-4. Ces découvertes suggèrent que la signalisation par ICOS-PI3K facilite l'acheminement dirigé d'IL-4 d'une cellule T auxiliaire à une cellule B apparentée durant une interaction entre les deux types cellulaires dans le GC.

En conclusion, je démontre que PI3K est une composante de signalisation clé en aval de la signalisation par ICOS durant la génération de Tfh. De plus, je prouve que la voix ICOS-PI3K peut modifier l'efficacité de traduction d'ARN messager préexistant suggérant un rôle potentiel d'ICOS dans la régulation de la fonction des cellules Tfh.

PREFACE

In accordance with the "Guidelines for Thesis Preparation", the candidate has chosen to present the results of his research in manuscript format. A general introduction is presented in Chapter I. A general discussion is presented in Chapter IV. The materials and methods as well as results are described in Chapters II and III and appear in the following published, or soon to be submitted, manuscripts:

1. **Gigoux, M.**, Shang, J., Pak, Y., Xu, M., Choe, J., Mak, T.W., and Suh, W.K. (2009). **Inducible costimulator promotes helper T-cell differentiation through phosphoinositide 3-kinase.** *Proceedings of the National Academy of Sciences of the United States of America* 106, 20371-20376.

2. **Gigoux, M.**, Lovato, A., Leung, J., Leconte, J., Sonenberg, N., and Suh, W.K. (2013). **Inducible Costimulator (ICOS) facilitates T-dependent B cell differentiation by augmenting IL-4 translation**. *To be submitted*.

AUTHORS' CONTRIBUTION

Described in Chapters II and III are as follows:

Jijun Chang helped generate the ICOS-YF mice used in Chapter II and Chapter III. He performed the ELISA assays, as well as performed some immunizations, dissections and cheek-bleeds for Chapter II. He also did extensive genotyping for Chapter II.

Dr. Young-Shil Pak was involved in interpreting some of the data for Chapter II.

Dr. Minghong Xu performed some immunohistology experiments for Chapter II.

Dr. Jongseon Choe was involved in performing preliminary experiments for Chapter II.

Dr. Tak Mak provided the ICOS-KO mice used for Chapter II and Chapter III.

Amanda Lovato optimized the polysome experiments protocol for Chapter III

Julien Leconte performed cloning for the luciferase experiments, as well as some qPCRs for Chapter III

Joanne Leung performed some qPCRs for Chapter III

Dr. Nahum Sonenberg was involved in the conception of the project for Chapter III, as well as contributed the immortalized MEFs for Chapter III.

Dr. Woong-Kyung Suh was involved in the conception of the project, interpretation of the data, and writing of the manuscripts for both Chapter II and Chapter III. He also helped generate the ICOS-YF mice used in Chapter II and Chapter III, as well as performed calcium flux experiments and proliferation assay for Chapter II. He wrote most of the manuscript for Chapter II and part of the manuscript for Chapter III. He also did extensive genotyping for Chapter II and Chapter III.

I, **Mathieu Gigoux,** performed dissections, *in vitro* CD4 T cell experiments, Western blots, flow cytometry and qPCRs for Chapter II, and *in vitro* CD4 T cell experiments, Western blots, polysome experiments, qPCRs, *in vitro* T-B cocultures and luciferase experiments for Chapter III. I was also involved in the analysis of the data for Chapter II and Chapter III, as well as involved in the conception of experiments for Chapter III. I wrote a small part in the manuscript for Chapter II and part of the manuscript for Chapter III. I also did extensive genotyping for Chapter II and Chapter III.

The candidate was also involved in collaborations with other laboratory, which resulted in the following publications:

1. Gao, X., **Gigoux, M.**, Yang, J., Leconte, J., Yang, X., and Suh, W.K. (2012). **Anti-Chlamydial Th17 Responses Are Controlled by the Inducible Costimulator Partially through Phosphoinositide 3-Kinase Signaling. PloS one** 7, e52657.

2. Li, J., Heinrichs, J., Leconte, J., Haarberg, K., Semple, K., Liu, C., **Gigoux, M.**, Kornete, M., Piccirillo, C.A., Suh, W.K., and Yu, X.Z. (2013). **Phosphatidylinositol 3-Kinase-Independent Signaling Pathways Contribute to ICOS-Mediated T Cell Costimulation in Acute Graft-Versus-Host Disease in Mice. J Immunol.** 191 (1): p200-7

TABLE OF CONTENT

LIST OF FIGURES

Chapter I: Introduction

Chapter II: Inducible costimulator promotes helper T cell differentiation through phosphoinositide 3-kinase

Chapter III: Inducible Costimulator (ICOS) facilitates T-dependent B cell differentiation by augmenting IL-4 translation

Chapter IV: General Discussion

Appendix II: Increased PI3K signalling results in more Tfh cells

LIST OF ABBREVIATIONS

4E-BP:	eIF4E-binding protein
4get:	IL-4/GFP-enhanced transcript
γ_c:	Common cytokine-receptor γ-chain
Ab:	Antibody
AID:	Activation induced cytidine deaminase
AITL:	Angioimmunoblastic T cell lymphoma
APC:	Antigen presenting cell
ATP:	Adenosine triphosphate
ATR:	Ataxia telangiectasia and Rad3-related protein
Bcl:	B cell lymphoma
BCR:	B cell receptor
BiP:	Binding immunoglobulin protein
Blimp-1:	B lymphocyte-induced maturation protein 1
BSF1:	B cell stimulating factor 1
BTB/POZ:	Broad complex, tramtrack and brick-à-brack/Pox virus and Zinc finger
CCR:	C-C chemokine receptor
CD:	Cluster of differentiation
Cre:	Causes recombination
CSR:	Antibody class-switch recombination
CTL:	Cytotoxic T lymphocyte
CTLA-4:	Cytotoxic T lymphocyte antigen-4
CVID:	Common variable immunodeficiency
CXCR:	C-X-C chemokine receptor
DAG:	Diacyl glycerol
DC:	Dendritic cell
DZ:	Dark zone (of germinal center)
EAE:	Experimental autoimmune encephalomyelitis
EBI2:	Epstein-Barr virus-induced G-protein coupled receptor 2
EBV:	Epstein-Barr virus
eIF:	Eukaryotic initiation factor

ER:	Endoplasmic reticulum
GAP:	GTPase-activating protein
GDP:	Guanosine diphosphate
GTP:	Guanosine triphosphate
GVDH:	Graft-versus-host disease
FTCL:	Follicular T cell lymphoma
GC:	Germinal center
Grb2:	Growth factor receptor-bound protein 2
GPCR:	G protein coupled receptor
ICOS:	Inducible costimulator
ICOSL:	ICOS ligand
IL:	Interleukin
Ig:	Immunoglobulin
IRES:	Internal ribosome entry site
IP3:	Inositol-(1,4,5)-trisphosphate
ITIM:	Immunoreceptor tyrosine-based inhibitory motif
ITK:	Interleukin-2-inducible T-cell kinase
ITSM:	Immunoreceptor tyrosine-based switch motif
IFN:	Interferon
JAK:	Janus Kinase
KO:	Knock out
LAT:	Linker for activated T cells
LCK:	Lymphocyte-specific protein tyrosine kinase
LCMV:	Lymphocytic Choriomeningitis Virus
LN:	Lymph node
LPS:	Lipopolysaccharides
LZ:	Light zone (of germinal center)
MAP:	Mitogen-activated protein
MEF:	Mouse embryonic fybroblasts
Met:	Methionyl
MHC:	Major histocompatibility complex

mRNA:	Messager ribonucleic acid
mRNP:	Messager ribonucleoprotein
mTOR:	Mammalian target of rapamycin
NFAT:	Nuclear factor of activated T cells
NK:	Natural killer (cell)
NKT:	Natural killer T (cell)
NP:	4-Hydroxy-3-nitrophenylacetyl
ORF:	Open reading frame
PABP:	Poly-A binding protein
PC:	Plasma cell
PCC:	Pigeon cytochrome C
PD-1:	Programed death 1
PDK1:	Phosphoinositide-dependent kinase 1
PDL:	PD-1 ligand
PFA:	Paraformaldehyde
PH:	Pleckstrin homology
PI3K:	Phosphoinositide 3-kinase
PI(3,4)P_2:	Phosphoinositide-(3,4)-biphosphate
PI(4)P:	Phosphoinositide-(4)-phosphate
PI(4,5)P_2:	Phosphoinositide-(4,5)-biphosphate
PI(3,4,5)P_3:	Phosphoinositide-(3,4,5)-triphosphate
PIC:	Pre-initiation complex
PLC-γ:	Phospholipase C-γ
PMA:	Phorbol 12-myristate 13-acetate
PP:	Peyer's patch
PRTE:	Pyridime-rich translational element
PTCL:	Peripheral T cell lymphoma
PtdIns:	Phosphatidylinositide
PTEN:	Phosphatase and tensin homologue
RF:	Ribosome footprinting
Rheb:	Ras homolog enriched in brain

rpS6:	Ribosomal protein S6
S6K:	S6 kinase
SAP:	SLAM-associated protein
SH2:	Src homology 2
SHIP:	SH2-domain-containing inositol polyphosphate 5-phosphatase
SHP:	SH2-domain containing tyrosine phosphatase
SHM:	Somatic hypermutation
SLAM:	Signaling lymphocyte activation molecule
SM:	LCMV-specific TCR transgenic (SMARTA) CD4 T cells
STAT:	Signal transducer and activator of Transcription
TC:	Ternary complex
TCR:	T cell receptor
TD:	T cell dependent response
Tfh:	Follicular helper T (cell)
Tfr:	Follicular regulatory T (cell)
Th:	T helper (cell)
TI:	T cell independent response
TLR:	Toll-like receptor
TOP:	5’ terminal oligopyrimidine
Treg:	Regulatory T (cell)
TSC:	Tuberous sclerosis complex
UTR:	Untranslated region
WT:	Wild type
XLP:	X-linked lymphoproliferative disease
ZAP70:	Zeta-chain-associated protein kinase 70

Chapter I

Introduction

1. FOLLICULAR HELPER T CELLS

The exposure to pathogens and foreign antigens triggers the production of high affinity antibodies as well as lifelong immunity. Antibodies produced by B cells bind to pathogens with high affinity and remarkable specificity, and through neutralization, opsonization or activation of the complement pathway, an infection can be controlled. Every subsequent round of exposure to that same pathogen induces the production of even higher affinity antibodies. This is one of the main mechanisms by which the immune system prevents reinfections and this forms the basis of vaccination.

There exist several mechanisms that allow B cells to produce antibodies without help from any other cell types, but these usually yield low affinity antibodies and the B cells that produce them apoptose after only a few days (Fairfax et al., 2008). In order for B cells to produce high-affinity, long-lasting, class-switched antibodies, they require the help of a specific subset of T cells called follicular helper T (Tfh) cells. These ensure the differentiation of a B cell into an antibody-producing plasma cell (PC) that produces high affinity antibodies and ensure life-long immunity against a particular pathogen.

We have known for decades that T cells are required for the generation of antibody producing PCs and an effective humoral response (Miller et al., 1965), but the nature of that relationship remained elusive for many years. Early studies suggested that the Th2 subset provided help to B cells, as they produce high levels of interleukin (IL)-4, a cytokine which induces isotype switching and antibody secretion in B cells (Howard et al., 1982; Paul and Ohara, 1987). However, mice lacking a key regulator for the development of Th2 cells are still able to produce high levels of class-switched antibodies *in vivo* (Kaplan et al., 1996). In 2000, two groups independently discovered a subset of CD4 helper T cells in human tonsils, which localize to the B cell follicles (Breitfeld et al., 2000; Schaerli et al., 2000). B cell follicles are the sub-compartment of secondary lymphoid organs where B cells reside. T cells on the other hand, reside in another sub-region called the T cell zone and at the time, it was believed that each cell type did not wander over into the other's region. It was therefore fascinating that these B cell follicle-residing T cells had high levels of CXCR5 (a B cell follicle homing receptor) and low levels of CCR7 (a T cell zone homing receptor). Importantly, when sorted and co-cultured with B cells *in vitro*, these cells induced high

levels of class-switched antibody production by the B cells. These two groups, whose findings were published in the same issue of the *Journal of Experimental Medicine*, jointly coined the term "follicular B helper T cell" (later shortened to follicular helper T cell). It is also important to note that these Tfh cells also had high surface levels of CD40 ligand (CD40L), a costimulatory molecule required for B cell activation, and a novel costimulatory receptor that had just been discovered the year before: ICOS, or the Inducible Costimulator. ICOS proved to have a crucial role in both the development and function of Tfh cells.

At first, Tfh cells proved to be an oddity and were not immediately accepted as a distinct subset of helper T cells. Many groups observed that under different immune conditions, the canonical cytokine expression profiles of other T helper (Th) cell subsets such as Th1, Th2 and Th17 could be detected in CD4 T cells expressing CXCR5 (King et al., 2008). However, these cells could clearly display different gene expression profiles (Chtanova et al., 2004; Nurieva et al., 2008). Therefore, there was a debate over whether these $CXCR5^{+}$ CD4 T cells were an extension of the other subsets or a distinct subset in their own right. It was not until 2009 that three groups identified the transcriptional repressor Bcl6 as a master regulator of Tfh cells (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009), solidifying the model that Tfh cells were a distinct Th subset.

Tfh cells reside in the anatomical structure called the germinal center (GC), which is a secondary follicle within the primary B cell follicle, and is induced upon T cell-dependent (TD) antigen exposure. GCs are sites of B cell clonal expansion, antibody affinity maturation and class-switch recombination (CSR) (MacLennan, 1994; Shlomchik and Weisel, 2012; Victora and Nussenzweig, 2012). Tfh cells are necessary for both the induction and the maintenance of GCs. They ensure that only the B cells with the highest affinity for a specific antigen continue to become differentiated and long-lasting PCs that secrete high-affinity class-switched antibodies. However, it is only a small number of CD4 T cells that differentiate to become this specialized class of Th cells.

My work focuses on characterizing the role of ICOS in the development and function of Tfh cells. This chapter will describe much of what is known regarding Tfh cells as a distinct cell type, as well as Tfh cell differentiation and function. I should note that the past five years have seen an acceleration in papers published regarding Tfh cells and the field in general has evolved

at an exceptionally fast pace over that time. Therefore, throughout this chapter I will outline major outstanding questions that we faced during the conception of our early work that have since been resolved by us and other groups.

1.1 GERMINAL CENTER REACTION

It is impractical to discuss Tfh cells without first understanding GC reactions. As previously mentioned, GCs arise in the B cell follicles of secondary lymphoid organs, which include the spleen, lymph nodes (LNs) and Peyer's patches (PPs), in response to foreign antigens. They are absent in the spleen and LNs of uninfected mice and develop dynamically upon antigenic stimulation, beginning to take shape around 4-6 days, peaking at 10-14 days and detectable up to 8 weeks post-antigenic stimulation (MacLennan, 1994; Kitano et al., 2011; Shlomchik and Weisel, 2012; Victora and Nussenzweig, 2012). PPs on the other hand feature ongoing GC reactions due to the immune response against the gut microflora.

In outward appearance, GCs appear as a mass of highly proliferative B cells that form within an otherwise quiescent B cell follicle in response to foreign antigens. But conceptually, they are incubators of long-term immunity. Their ultimate purpose is to generate long-lasting plasma cells that make high-affinity antibodies or alternatively, memory B cells which can induce a much quicker immune response in case of re-infection. In many cases, small infections can be cleared well before the GC reaction peaks. Therefore, it is often considered that GC reactions are more a tool of long-lasting immunity rather than immediate pathogen clearance. In this section, I will describe the cellular mechanisms that lead to GC development, as well as the structure of an ongoing GC reaction. Finally, I will explain the distinct role that Tfh cells play in the maintenance of GCs.

1.1.1 GERMINAL CENTER DYNAMICS

Germinal center dynamics can be divided in any number of stages, but I will divide them into three stages for simplicity: 1) priming, 2) effector function and 3) GC reaction (Figure 1). Also, for the sake of simplicity I will not go into the details of Tfh differentiation in this section. Rather, Tfh differentiation will be covered extensively in *Section - 1.3 Tfh cell differentiation*, while this section will focus on GC dynamics.

The first stage in a GC reaction consists of initial antigen exposure and the priming of both T and B cells. During the first days of an immune response, antigen binding to the B cell receptor (BCR) triggers the upregulation of the T cell zone homing receptors CCR7 and Epstein-Barr virus-induced G-protein coupled receptor (EBI)2, allowing the B cell to migrate to the T cell zone border (Garside et al., 1998; Okada et al., 2005). B cells can encounter antigen through many different mechanisms that largely depend upon the nature and size of the antigen, as well as the cellular context and location in which antigen presentation occurs. For example, small soluble antigens can directly pass into B cell follicles by simple diffusion, while larger particulate antigens are captured and presented to B cells by subcapsular sinus macrophages (Batista and Harwood, 2009). Alternatively, antibody-bound antigens (in the form of antigen-immune complexes) are deposited on the surface of follicular dendritic cells (FDCs), which are found in B cell follicles, through binding of Fc receptors. However, this latter mechanism is probably not the initial source of antigens for B cells. Antigen-specific naïve CD4 helper T cells are activated by antigen-presenting dendritic cells (DCs) in the T cell zone. Some of these CD4 T cells upregulate factors associated with Tfh cells, such as the transcriptional repressor Bcl6, the B cell follicle homing receptor CXCR5, the costimulatory receptor ICOS and others, and downregulate factors associated with other Th cell effector subsets (Crotty, 2011). This is the first stage of Tfh cell differentiation and makes what I will call the pre-Tfh cell subset (the nomenclature of these cells is still a subject of debate – see *Section - 1.2 Defining Tfh cell subsets*). At this stage, all DC-engaged CD4 T cells upregulate Bcl6, the master regulator of Tfh cells (Baumjohann et al., 2011; Kitano et al., 2011). However, only a subset of these will retain expression of this transcription factor and engage in a second wave of Bcl6 expression mediated by B cell engagement (Baumjohann et al., 2011). It is important to note that although both the B cells and pre-Tfh cells in question upregulate the homing receptor for the other compartment, they retain the homing receptor for their own compartment. This allows both cells types to migrate to the T-B border, but generally the cells do not cross-over into the other compartment.

The second stage in a GC reaction involves the seeding of the GC and the full differentiation of Tfh cells. Once the antigen-specific B cells and pre-Tfh cells migrate to the border, B cells become the predominant antigen-presenting cell (APC) for pre-Tfh cells (Deenick et al., 2010). After cognate interaction between the antigen-specific B cells and the pre-Tfh cells, the Tfh cells will provide the appropriate stimuli to the highest affinity B cells, inducing the

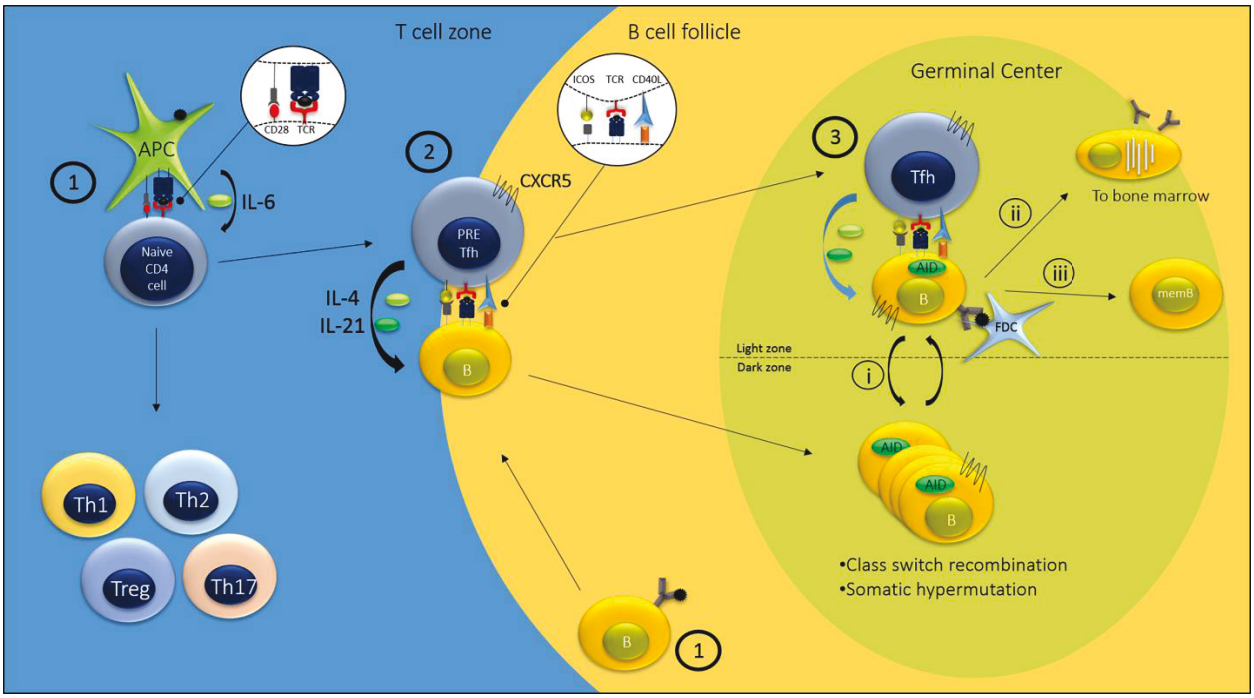

Figure 1. A model of Tfh cell differentiation. 1) During initial antigen exposure, DCs in the T cell zone induce naïve CD4 T cell activation through TCR and CD28-mediated signals. High affinity CD4 T cells that receive IL-6 preferentially differentiate into pre-Tfh cells, which express CXCR5 and migrate to the T-B border. Simultaneously, antigen-mediated BCR crosslinking induces B cell migration to the T-B border. 2) Interactions between antigen-specific pre-Tfh cells and cognate B cells, leads to the formation of a GC in a manner dependent on IL-4, IL-21, CD40L and possibly ICOS. At the same time, costimulation of ICOS is crucial for the full differentiation of pre-Tfh cells to Tfh cells, which migrate into the light zone of GCs. 3) During B cell clonal expansion in the dark zone (centroblasts), B cells undergo AID-dependent CSR and SHM, then proceed to migrate into the light zone (centrocytes) and acquire antigens from the surface of FDCs. B cells undergo selection in the light zone based on their BCR affinity: high affinity B cells survive by interacting with cognate Tfh cells and others die. Surviving B cells take one of three paths: i) return to the dark zone and undergo another round of clonal expansion and SHM ii) differentiate into antibody producing PC or iii) become memory B cells.

migration of B cells to migrate back into the B cell follicle to seed a GC (Okada et al., 2005; Schwickert et al., 2011). Alternatively, some B cells will migrate to the extrafollicular region, bypassing the GC, and differentiate into short-lived plasma cells producing low-affinity Abs (Crotty et al., 2010; Yu and Vinuesa, 2010b). This likely gives a boost to the early immune response, as well as increases the amount of available antigen-immune complexes that can later be deposited on the surface of FDCs to ensure antigen availability during the ongoing GC reaction. On the other hand, GC-bound B cells begin to proliferate rapidly and undergo somatic hypermutation (SHM). Due to the highly proliferative nature of these B cells, they have been dubbed centroblasts. For their part, pre-Tfh cells receive further differentiation cues from B cells, downregulate CCR7 and migrate into the B cell follicle. There is evidence that the B cells may even "drag" the now fully differentiated Tfh cells into the follicle (Okada et al., 2005; Kerfoot et al., 2011). Although many factors are involved at the stage of Tfh cell differentiation, one important interaction is that of ICOS-ICOSL, as a loss of ICOS-ICOSL interaction in humans and mice leads to severe defects in Tfh cell differentiation and GC development (Grimbacher et al., 2003; Akiba et al., 2005; Bossaller et al., 2006; Warnatz et al., 2006; Nurieva et al., 2008; Reinhardt et al., 2009).

The third stage involves the ongoing GC reaction. Mature GCs are subdivided into two zones: the light zone (LZ) and the dark zone (DZ). The DZ consists mainly of the highly proliferative centroblasts. These cells proliferate as a dense cluster, which appear darker under a light microscope; thus the source of the name "dark zone" (MacLennan, 1994). Here centroblasts upregulate the transcription of genes associated with mitosis (Victora et al., 2010), as well the gene coding for activation induced cytidine deaminase (AID), which introduces point mutations in the variable region and in the switch region of the BCR (Rush et al., 2005). The resulting mutations lead to SHM and CSR in the B cells. Centroblasts also express the chemokine receptor CXCR4 which allows them to localize specifically to the DZ (Allen et al., 2004). This is partnered with the downregulation of p53 and ataxia telangiectasia and Rad3-related protein (ATR), which contributes to the GC B cell's ability to tolerate DNA damage, occurring as a result of rapid proliferation and AID activity (Phan and Dalla-Favera, 2004; Ranuncolo et al., 2007). In contrast, the less proliferative GC B cells, termed centrocytes, as well as Tfh cells and FDCs, populate the LZ. These centrocytes downregulate genes associated with cell cycle progression and cell survival, such as Bcl2, and upregulate genes associated with lymphocyte activation and cell-surface

molecules (Victora et al., 2010). In contrast with an older model proposed by Ian MacLennan, stipulating a unidirectional migration of B cells from the DZ to the LZ, recent live imaging experiments show that B cells in the GC shuttle continuously between the LZ and the DZ, but that there is a net migration of B cells from the DZ to the LZ (MacLennan, 1994; Victora et al., 2010; Victora and Nussenzweig, 2012). The relevance of this update to the older model is significant as it strongly suggests that B cells can undergo multiple rounds of SHM and selection.

The current model proposes that after undergoing clonal expansion and SHM as centroblasts, the daughter B cells differentiate into centrocytes and migrate to the LZ. There they acquire deposited antigen, either through FDCs or by other means, and present that antigen to Tfh cells via the major histocompatibility complex class-II (MHC-II). The relative scarcity of Tfh cells means that centrocytes undergo a Darwinian-like competition for cognate Tfh cell availability. Only the B cells with the highest affinity BCR receive stimulatory cues from Tfh cells, which include CD40L, IL-21 and IL-4 (Tangye et al., 2013). B cells with low affinity will not receive survival cues from Tfh cells and, as they are prone to apoptosis due to low expression of Bcl2 (Saito et al., 2009), die by neglect. The few B cells that have managed to acquire the appropriate cues will follow one of three paths. They will either: 1) migrate back to DZ and undergo another round of clonal expansion and somatic hypermutation, 2) differentiate into antibody producing PCs or 3) differentiate into memory B cells (Goodnow et al., 2011). This cycle of clonal expansion, SHM and affinity selection (ie: affinity maturation), ensures long-term production of high-affinity antibodies alongside short-term help of lower affinity antibodies, with some of the B cells becoming PCs after every cycle. However, it remains unclear how these decisions of cell fate are made at the molecular level.

The older model of GC dynamics had suggested that direct BCR-affinity for antigen on the surface of FDCs was the main mechanism for affinity maturation (MacLennan, 1994). However, more recent findings have suggested that availability of cognate Tfh cells is the key to B cell selection (Victora et al., 2010). Therefore, in order to better understand humoral immunity, we must further investigate the mechanisms of Tfh cell development and function.

1.1.2 ROLE OF Tfh CELLS IN NASCENT AND ONGOING GC REACTIONS

Tfh cells execute a necessary role as both inducer and gatekeeper of GC reactions. During the first few days of a TD immune response, Tfh cells (or their precursors) are crucial for the seeding of the GC by antigen-specific B cells (Schwickert et al., 2011). Once established, Tfh cells are found inside the GC and select the highest affinity B cells for PC differentiation (Vinuesa et al., 2010; Crotty, 2011). Importantly, competition for Tfh cells is the limiting factor for survival, differentiation and affinity maturation for B cells.

Antigen binding to the BCR alone is not sufficient to induce cell cycle progression through G1 in B cells, but does lead to changes in the expression of chemokine and chemokine receptor genes implicated in initiating a T-dependent response, as well as accentuating later responsiveness to CD40 signalling (Damdinsuren et al., 2010). Importantly, antigen binding leads to antigen uptake and ultimately to antigen presentation. The ability of Tfh cells to provide CD40L and cytokines such as IL-4 and IL-21 is what allows the B cells to begin differentiation. CD40 stimulation induces B cell entry into the cell cycle, while IL-4 and IL-21 synergize with CD40 stimulation to drive AID expression, SHM and CSR (Victora and Nussenzweig, 2012).

The importance of Tfh cells in the maintenance of an ongoing GC reaction was underscored by studies that demonstrated that some T-independent (TI) antigens such as 4-hydroxy-3-nitrophenylacetyl(NP)-Ficoll are able to give rise to GC reactions that have all the hallmarks of a standard early GC, including the expression in B cells of Bcl6, GL-7 and Fas, downregulation of IgD and induction of SHM (de Vinuesa et al., 2000; Lentz and Manser, 2001; Gaspal et al., 2006). Interestingly, CD40 stimulation to B cells was acquired through FDCs in combination with complement proteins (Gaspal et al., 2006). However, the GCs in this TI context were unable to survive past the initial burst of centroblast growth and the generation of the first wave of centrocytes. After five days, the GC B cells underwent massive apoptosis and the GC aborted synchronously (de Vinuesa et al., 2000). These studies emphasizes the essential role of Tfh cells for the maintenance of ongoing GCs and for centrocyte selection.

1.2 DEFINING Tfh CELLS SUBSETS

As with any area of study, it is important to accurately define the terms that we use in order to avoid confusion. In the case of Tfh cells, many different research groups use slightly different

nomenclature to define Tfh cells. There are at least three sets of nomenclature for Tfh cells and variations continue to arise (McHeyzer-Williams et al., 2009; Yu and Vinuesa, 2010a; Crotty, 2011). This stems from the fact that Tfh cell research has evolved rapidly over the years and nomenclature has yet to catch up. Most researchers in the field agree on the major points of Tfh cell development and the variations in definitions are more of a sign of an immature field rather than a sign of major disagreements. For the purpose of this thesis, I will divide Tfh cells based exclusively on their state of differentiation and argue that further sub-dividing them by cytokine expression disregards recent studies that finds striking plasticity on behalf of Th subsets (Oestreich and Weinmann, 2012). Therefore, with regards to Tfh cells, I will divide all CD4 T cells into three categories.

First, non-Tfh cells are the easiest to define. These cells express neither CXCR5 nor Bcl6, and are not found in the B cell follicle or near the T-B border. This includes naïve CD4 T cells, which are either in the T cell zone or circulating in the blood stream, and effector T helper cells such as Th1, Th2, Th17 and Treg, which are activated in the T cell zone and usually migrate to the site of infection.

Pre-Tfh cells are the hardest to define. They constitute developing Tfh cells whose Tfh cell phenotype and gene expression profile ($CXCR5^{hi}Bcl6^{hi}PD\text{-}1^{hi}ICOS^{hi}$) has yet to stabilize. They express low levels of Bcl6 and CXCR5, may express CCR7, and are generally considered to be at the T-B cell border. What makes them hard to define is the fact that all CD4 T cells have been observed to upregulate Bcl6 (Baumjohann et al., 2011) and CXCR5 (Ansel et al., 1999) upon initial DC-engagement. Importantly, after the initial burst of Bcl6 expression in all DC-engaged CD4 T cells, Bcl6 expression diminishes slightly before undergoing a second wave of Bcl6 upregulation. This second wave of Bcl6 upregulation occurs only in some cells and with varying ranges of expression. There is no clear distinction between $Bcl6^{+}$ and $Bcl6^{-}$ cells, but rather a spectrum of Bcl6 expression where high Bcl6 expression does correlate with CXCR5 expression (Baumjohann et al., 2011). CCR7 expression also broadly decreases in most DC-engaged CD4 T cells, further complicating the distinction between pre-Tfh cells and Tfh cells (Baumjohann et al., 2011). Finally, most studies analyze Tfh cells at the peak of a GC reaction, with only a handful of groups studying early Tfh cell development dynamics. Almost none has looked at the combined range of surface markers, transcription factors, early time points and *in vivo* localization that have

just been discussed. Therefore, one of the main reasons we conceptualize this loosely defined "pre-Tfh cell" subset is because it helps us separates DC-mediated activation and B cell-mediated "stabilization" of the Tfh cell phenotype. As I will discuss in *Section 1.3 – Tfh cell differentiation*, DCs are crucial for the naïve to pre-Tfh cell development but B cells are dispensable, and B cells are crucial for the pre-Tfh cell to Tfh cell development, or possibly stabilization.

Finally, there are fully differentiated Tfh cells, which are $CXCR5^{+}CCR7^{-}Bcl6^{hi}$, as well as programmed death-1(PD-1)^{hi}$ICOS^{hi}$ and are found strictly inside B cell follicles. They also express high levels of the cytokines IL-21 and IL-4, which are important for B cell activation and CSR (King and Mohrs, 2009). Some Tfh cells are found inside the GC (as opposed to outside of the GC, but still inside the B cell follicle) and express even higher levels of Bcl6 and CXCR5, as well as other activation markers such as ICOS and PD-1. Importantly, they also express slightly higher levels of IL-21, as well as dramatically higher levels of IL-4. Interestingly, they also stain positive for α2,6-linked N-acetylneuraminic acid on glycan chain (labeled as GL-7 positive from the name of the antibody used for staining), suggesting that they are metabolically active (Yusuf et al., 2010). Some groups have suggested that these "GC Tfh cells" are a distinct subset that is more developed, or rather an elite version of Tfh cells, but there is insufficient data to support this notion. It is possible that these may just be Tfh cells that have most recently been in contact with B cells and have upregulated activation markers. I will therefore consider Tfh cells and GC Tfh cells as the same cell type. Furthermore, many studies have observed that Tfh cells are able to express high levels of canonical cytokine expression profiles of other helper T cell subsets depending on the type of immune response. For example, in infection models that induce strong Th1, Th2 or Th17 responses, $CXCR5^{+}Bcl6^{hi}$ cells have been observed to express IFN-γ, IL-4 or IL-17, respectively (Yu and Vinuesa, 2010a), the main cytokines associated with the respective effector subset. This had led some groups to propose further subdividing Tfh cells into more specific subsets such as Tfh1, Tfh2 and Tfh17 (Fazilleau et al., 2009a). However, as recent studies have shown, Th cells are able to change their canonical cytokine expression profiles under artificial immune conditions (Oestreich and Weinmann, 2012). In other words, there is insufficient data to suggest that Tfh cells which express different canonical cytokine profiles are inherently different from each other beyond cytokine patterns.

1.3 Tfh CELL DIFFERENTIATION

There are many stages of Tfh cell differentiation that separate naïve CD4 T cells found in the T cell zone and the Tfh cells found inside the GC. Unlike the other effector Th subsets (ie: Th1, Th2 and Th17) which are believed to differentiate entirely in the T cell zone (Zhu et al., 2010), Tfh cells require at least two rounds of differentiation cues in two different compartments. These differentiation cues are given first in the T cell zone and the second at the T-B border, and with the help of DCs and then B cells. In this section, I will broadly describe these two stages of Tfh cell differentiation (Figure 1).

1.3.1 STAGE ONE: DC-MEDIATED POLARIZATION OF PRE-Tfh CELLS

The first round of Tfh cell differentiation consists of DC-mediated activation, which takes place in the T cell zone. Antigen-presenting DCs provide antigen-specific CD4 T cells with three crucial components for initial Tfh cell differentiation: 1) TCR-mediate signals, 2) costimulatory signals and 3) STAT3-activating cytokines.

First, high TCR affinity seems to dictate preferential Tfh cell differentiation. Using an I-E^k-restricted Th cell response of B10.BR mice to pigeon cytochrome c (PCC) and a tractable protein vaccination model that allows for the identification of TCR affinity, McHeyzer-Williams' group elegantly showed that strong TCR affinity induces the differentiation of $CXCR5^+CCR7^-Bcl6^+$ Tfh cells that reside inside B cell follicles. Conversely, intermediate TCR strength induces an "emigrant" $CXCR5^{lo}Bcl6^-Blimp1^+$ effector population that migrates out of the lymphoid organs into the periphery; in essence, Th1, Th2 and Th17 cells (Fazilleau et al., 2009b). However, a more recent study was published by Mark Jenkins' group, using two TCR transgenic cell lines that bind to a set of I-A^b-bound peptides, all with known TCR-peptide:MHC equilibrium affinity and TCR-peptide:MHC interaction half-life. This group found that TCR affinity had no effect on Tfh cell differentiation, but that increased TCR-peptide:MHC dwell time and MHC surface density had a positive influence on Tfh cell differentiation (Tubo et al., 2013). The reasons for the discrepancies between these studies are not clear, but the general trend is the same: increased TCR "strength" further induces Tfh cell differentiation.

Second, DCs provide CD28 costimulation to T cells, which is crucial for initial T cell activation and ultimately for Tfh cell differentiation as well. TD humoral immunity is significantly

diminished in CD28-deficient mice (Shahinian et al., 1993), indicating that CD28 costimulation is necessary for differentiation of all Th cell subsets, including Tfh cells. These CD28-deficient mice completely lack T cell-mediated GCs and Tfh cells (Mittrucker et al., 1999; McSorley and Jenkins, 2000). DCs may also provide a necessary round of ICOS costimulation during early priming, as Tfh cell differentiation defects are seen after two to three cell divisions in ICOS- or ICOSL-deficient mice (Fazilleau et al., 2009b; Choi et al., 2011). However, more convincing studies are needed to demonstrate this, as ICOS costimulation is widely believed to be important during the second round of Tfh cell differentiation, mediated by B cells, which shall be discussed in the next section.

Finally, cytokines are essential for the generation of many of the known CD4 T cell subsets (Th1, Th2, Th17 and iTreg) (Zhu et al., 2010). In the case of Tfh cells, DC-engaged T cells require STAT3-activating cytokines to develop into Tfh cells, specifically IL-6 or IL-21 (Nurieva et al., 2008; Suto et al., 2008; Vogelzang et al., 2008; Nurieva et al., 2009; Eto et al., 2011; Choi et al., 2013b). IL-6 is provided by DCs upon toll-like receptor (TLR) induction (Kishimoto, 2005) and IL-6 is able to upregulate IL-21 production by T cells (Nurieva et al., 2007b; Suto et al., 2008; Dienz et al., 2009; Eddahri et al., 2009). This T-cell mediated IL-21 production likely also plays a role in Tfh cell differentiation (Nurieva et al., 2008; Vogelzang et al., 2008; Eto et al., 2011). Most importantly however, eliminating either IL-6 or IL-21 individually does not significantly inhibit Tfh cell differentiation, but eliminating both does (Nurieva et al., 2008; Vogelzang et al., 2008; Eto et al., 2011). STAT3-deficient mice have defects in Tfh cell differentiation (Nurieva et al., 2008), but this data conflicts with another study which suggests that IL-6-mediated Tfh cell differentiation is STAT1-dependent and that STAT3 is required to downregulate IL-2Rα (Choi et al., 2013a), confirming findings in three papers that demonstrate that IL-2 inhibits Tfh cell differentiation (Ballesteros-Tato et al., 2012; Johnston et al., 2012; Nurieva et al., 2012). Nevertheless, IL-6 and IL-21 are likely redundant, probably via STAT3 activation. Finally, it has also recently emerged that IL-12, IL-23 and STAT4 can also be important specifically in human, but not mouse, Tfh cell differentiation (Schmitt et al., 2013). However, the molecular mechanisms for this difference between human and mouse are still unclear.

To summarize, DCs act as the first initiator of the Tfh cell genetic program during the first 2-3 days of antigen exposure. Both Bcl6 and CXCR5 have been shown to be directly induced in

CD4 T cells after DC-engagement in the T cell zone (Baumjohann et al., 2011; Kitano et al., 2011; Liu et al., 2012) and importantly, early pre-Tfh cell differentiation is B cell-independent. The combination of CXCR5 and CCR7 expression forces the pre-Tfh cell to migrate to the T-B border, where the cells come into contact with their cognate B cell partners. It is there that pre-Tfh cells begin their second stage of differentiation.

1.3.2 STAGE TWO: COGNATE B CELL-MEDIATED DIFFERENTIATION

Cognate B cells are crucial for ongoing Tfh cell survival, as mice with an absence of cognate B cells experience a dramatic reduction in $CXCR5^{+}Bcl6^{+}$ CD4 T cell numbers shortly after initial expansion (Liu et al., 2012). Additionally, B cell-specific genetic deletion of SAP and ICOSL leads to dramatic defects in Tfh cell differentiation *in vivo* (Nurieva et al., 2008), demonstrating a specific role for B cells in Tfh cell development. Studies have observed "monogamous" cognate interactions between pre-Tfh cells and B cells in the interfollicular region, where the conjugates were highly motile and were led by the B cell towards the B cell follicle (Okada et al., 2005; Kerfoot et al., 2011). During this T-B contact, pre-Tfh cells undergo a second round of Bcl6 upregulation and an increase in CXCR5 and PD-1 expression, but no increased ICOS expression (Baumjohann et al., 2011). Interestingly, a small number of $CXCR5^{+}$ pre-Tfh cells could still be found inside the follicle in the absence of cognate B cells, but had dramatically decreased numbers and quickly lost the $ICOS^{+}PD\text{-}1^{+}$ Tfh cell phenotype (Kerfoot et al., 2011). Nevertheless, this indicates that B cells are not absolutely required for initial pre-Tfh cell differentiation and entry into the follicle, but are absolutely necessary for Tfh cell maintenance. Furthermore, a study has shown that B cells do not provide a unique signal to pre-Tfh cells to induce their differentiation into $PD\text{-}1^{+}ICOS^{+}CXCR5^{+}$ Tfh cells per se. Rather, it appears that ongoing antigen presentation is responsible for the process and that B cells are the main source of APCs at that stage of the immune response (Deenick et al., 2010). However, whether the Tfh cells undergo further epigenetic changes, or whether their gene expression profile has simply stabilized is still unclear. Many factors are involved in B cell-mediated Tfh cell differentiation, the majority of which I will elaborate on in the following section. However, as a whole, the molecular mechanisms of Tfh cell differentiation remain poorly understood.

1.4 MEDIATORS OF Tfh CELL DIFFERENTIATION AND FUNCTION

A lack of Tfh cells leads to immunodeficiency, while unchecked Tfh cell development has been linked to many cases of autoimmunity (Yu and Vinuesa, 2010b; Tangye et al., 2013). Not surprisingly, there are several checkpoints and many different mediators of Tfh cell differentiation and function. In this section, I will discuss in greater details some of these key regulators of Tfh cell development such as ICOS, Bcl6, PD-1 and CD28. I will also describe the role of the adaptor protein SAP in T-B interactions, as well as the key cytokines necessary for B cell help IL-21 and IL-4.

1.4.1 ICOS

As ICOS costimulation is a central component of my thesis, I will devote a whole section to ICOS later in this introduction (see S*ection 2 – The Inducible Costimulator*). Therefore, in this section, I will only briefly describe evidence that ICOS is involved in Tfh cell generation in humans and mice, and potential signal transduction mechanisms.

ICOS deficiencies in humans have been reported to cause common variable immunodeficiency (CVID), which is characterized by a severe reduction in class-switched antibodies and an inability to mount specific antibody responses to vaccination or natural infection (Grimbacher et al., 2003; Yong et al., 2009). Consistently, mice with ICOS or ICOSL deficiencies also have severe defects in Tfh cell generation and GC reactions (Dong et al., 2001a; Dong et al., 2001b; McAdam et al., 2001; Tafuri et al., 2001; Mak et al., 2003; Nurieva et al., 2003b; Wong et al., 2003; Akiba et al., 2005; Bossaller et al., 2006). On the other hand, mice with increased surface expression levels of ICOS have increased Tfh cell numbers and are prone to autoimmunity (Yu et al., 2007; Linterman et al., 2009a; Linterman et al., 2009b; Pratama et al., 2013; Vogel et al., 2013). However, the exact mechanisms by which ICOS mediates Tfh cell differentiation remained largely unknown when I first began my research in this field.

One possible mechanism of ICOS-mediated Tfh cell differentiation may be through IL-21 production. Tfh cells express high levels of ICOS and IL-21 (Chtanova et al., 2004) and studies have suggested that ICOS-mediated IL-21 production by T cell plays an important role in mediating Tfh cell differentiation through autocrine secretion (Nurieva et al., 2008; Vogelzang et

al., 2008). ICOS costimulation is directly linked to increased expression of the transcription factor c-Maf (Nurieva et al., 2003a; Bauquet et al., 2009), which regulates the expression of both IL-21 (Vogelzang et al., 2008; Bauquet et al., 2009; Hiramatsu et al., 2010; Kroenke et al., 2012) and IL-4 (Nurieva et al., 2003a). This may explain, at least in part, how ICOS costimulation induces the generation of Tfh cells. A second possibility for the promotion of Tfh cell differentiation through ICOS costimulation may also be its ability to induce IL-21 expression through Ca^{2+}-NFAT, as the IL-21 promoter contains three NFAT binding sites (Kim et al., 2005).

In all, we have limited understanding of the biochemical and mechanistic processes through which ICOS regulates the differentiation and function of Tfh cells. Therefore, our group has focused our experiments to try to better understand the mechanisms by which ICOS costimulation, specifically through PI3K, induces the generation of Tfh cells.

1.4.2 Bcl6

B cell lymphoma 6 protein, or Bcl6, is a transcriptional repressor that was discovered to be essential for GC formation when it was found that mice genetically deficient in Bcl6 were completely unable to form GCs (Dent et al., 1997; Fukuda et al., 1997; Ye et al., 1997). It was later observed to play an essential role in GC B cell development (Dent et al., 1997; Ye et al., 1997). Interest in Bcl6 expression in T cells began when an early genetic transcriptional profile study of human $CXCR5^+$ T cells revealed that Bcl6 was particularly expressed in these cells (Chtanova et al., 2004). More recently, it was observed that Bcl6-transduced activated CD4 T cells preferentially became Tfh cells when transferred back into mice and subjected to immunization and/or infection (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009). This led many to propose that Bcl6 was the “master regulator” of Tfh cells. The studies describing Bcl6 as a key transcriptional mediator for Tfh cell differentiation were only published after submission of our manuscript (Chapter II) for publication. As a result, we were unable to address the role of ICOS on Bcl6 expression in this work. However, since Bcl6 is now a widely accepted central component of Tfh cells and GC B cells, I will describe it here, while the relationship between ICOS and Bcl6 will be discussed later in Chapter IV.

Bcl6 is a member of the BTB-POZ/zinc finger family of proteins. Its C2H2 zinc fingers bind to DNA and its BTB-POZ domain forms an obligatory homodimer. The symmetrical grooves

that are created after homodimerization create a crucial docking site for corepressors, without which Bcl6 is thought to be unable to repress transcription (Dhordain et al., 1997; Huynh and Bardwell, 1998; Huynh et al., 2000). However, Bcl6 also has a middle autonomous repression region often called 'repression domain 2', which may recruit a different set of corepressors (Chang et al., 1996).

Interestingly, Bcl6 seems to regulate a different set of genes in B cells and T cells (Huang et al., 2013). In B cells, Bcl6 is proposed to facilitate the simultaneous rapid proliferation and tolerance against DNA damage that occurs during SHM and CSR by downregulating molecules involved in DNA-damage sensing and cell cycle checkpoints such as ATR and p53 (Phan and Dalla-Favera, 2004; Ranuncolo et al., 2007). This downregulation in B cells is dependent upon the BTB-POZ domain as B cells with a Bcl-6 BTB-POZ loss-of-function mutation have a disruption in GC formation, which is attributed to a defect in B cell survival and proliferation (Huang et al., 2013).

The role of Bcl6 in CD4 T cells is less clear, however. First, CD4 T cells with the same Bcl6 BTB-POZ^{nul} mutation have no defects in Tfh cell formation (Huang et al., 2013), strongly suggesting that Bcl6 acts differently in B and T cells. Second, Bcl6 upregulation has been observed to positively regulate CXCR5 (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009; Kroenke et al., 2012), possibly by inhibiting the expression of miRNA that negatively regulate CXCR5 (Yu et al., 2009), yet this finding has recently been disputed (Baumjohann et al., 2013; Kang et al., 2013). Bcl6 may also increase genes that positively regulate the cell cycle (Kitano et al., 2011). Furthermore, Bcl6$^{-/-}$ CD4 T cells can still upregulate CXCR5 after early DC-engagement (although it is not maintained) (Liu et al., 2012) and some CXCR5^{+} Tfh cells have undetectable levels of Bcl6 (Kitano et al., 2011). Likewise, recently discovered human CXCR5^{+} circulating Tfh-like cells have no detectable Bcl6 expression (Simpson et al., 2010a; Chevalier et al., 2011; Morita et al., 2011). Together, this strongly indicates that Bcl6 is not absolutely necessary for CXCR5 expression. Furthermore, three studies have shown that the transduction of Bcl6 in activated CD4 T cells increased CXCR5 expression only after the cells were transferred back into mice and made to respond to antigen *in vivo*. In other words, Bcl6 transduction was not sufficient to increase CXCR5 expression in CD4 T cells *in vitro* (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009). This conflicts with another study where *in vitro* transduction of Bcl6 in primary human

CD4 T cells was sufficient to increase CXCR5 expression, as well as regulate other migration genes such as CXCR4, CCR7 and EBI2 (Kroenke et al., 2012). Furthermore, the same study also found that Bcl6 also upregulated proteins critical for T-B interactions such as ICOS, PD-1, SAP and CD40L. However, the reason for the discrepancies between these studies is yet to be clarified.

Bcl6 expression has been shown to antagonize B lymphocyte-induced maturation protein 1 (Blimp1) expression and vice versa (Johnston et al., 2009). Blimp1 is a transcription factor that is highly expressed in the Th effector subsets Th1, Th2 and Th17 (Crotty et al., 2010). It was therefore believed that the balance between Bcl6 and Blimp1 levels would dictate whether a differentiating T cell would become a Tfh cell or one of the other effector subsets. However, a recent study has shown that the novel follicular regulatory T (Tfr) cell, a Tfh cell subset that has characteristics of a Treg cell, co-expressed high levels of Bcl6 and Blimp1 (Linterman et al., 2011). Therefore, a simple Bcl6-Blimp1 antagonism does not seem to explain Tfh cell lineage choice over other Th cell subsets.

Finally, it has been observed that mature Tfh cells exhibit a similar gene expression profile to early memory precursor CD8 T cells (Choi et al., 2013b). Interestingly, Bcl6 plays a role in the development of memory CD8 T cells (Ichii et al., 2002; Ichii et al., 2004) and also plays a role in the protection of memory precursor CD4 T cells from apoptosis and may be involved in survival of long-term memory CD4 T cells (Ichii et al., 2007). Furthermore, as the immune reaction progresses, Bcl6 expression in Tfh cells diminishes (Baumjohann et al., 2011; Kitano et al., 2011), correlating with decreased proliferation and increased expression of IL-7Rα (Kitano et al., 2011; Choi et al., 2013b), possibly indicating a memory phenotype (Sallusto et al., 2004). It is therefore speculated that Bcl6 may play a role in the development of memory Tfh cells, although how this is achieved remains unclear.

1.4.3 SLAM-SAP

X-linked lymphoproliferative (XLP) syndrome is a rare genetic disorder that is characterized by an abnormal, and often fatal, response to Epstein-Barr virus (EBV). EBV infects mature B cells and XLP patients fail to clear these infected B cells. It was discovered that the gene associated with most cases of the disease was *SH2D1A*, which encodes SAP (SLAM associated protein), an adaptor protein for the SLAM (signaling lymphocyte activation molecule) family of

cell surface receptors (Latour and Veillette, 2003). SAP-deficient patients who had survived EBV infection, or who were never infected with EBV, still develop lymphoproliferative disorders, usually of B cell origin (Schwartzberg et al., 2009; Cannons et al., 2011).

SLAM family members are part of the greater CD2 superfamily of immunoglobulin receptors. They typically contain an immunoreceptor tyrosine-based switch motif (ITSM) in their intracellular domain and interact with an identical SLAM receptor on their partnering cell (with a few exceptions). They are expressed on a wide variety of hematopoietic cells and have diverse roles including regulating costimulation, T cell cytokine production, NK cell- and CD8 T cell-mediated cytotoxicity, adhesion between hematopoietic cells, T cell reactivation-induced cell death, the development of innate T lymphocytes, as well as functions for neutrophils and macrophages. SAP on the other hand is an intracellular adaptor protein that is composed primarily of a single SH2 domain that binds specifically to SLAM family members (Veillette, 2006; Schwartzberg et al., 2009; Cannons et al., 2011; Qi, 2012).

Relevant to Tfh cells, it was discovered that SAP was crucial for the development of GC B cells and Tfh cells (Qi et al., 2008; Cannons et al., 2010; Yusuf et al., 2010). Importantly, SAP is dispensable for DC-mediated pre-Tfh cell activation, but is crucial for the maintenance of the B cell-mediated Tfh cell phenotype (Cannons et al., 2010). The SLAM family member CD84 and Ly108 are believed to play the role of adhesion molecules in the coupling of T and B cells (Cannons et al., 2010; Yusuf et al., 2010; Cannons et al., 2011). Importantly, SAP-deficient B or T cells had dramatically lower conjugation times and this led to lower levels of IL-21 and IL-4, but not Bcl6 or ICOS (Cannons et al., 2010; Yusuf et al., 2010). On the other hand, SAP can be upregulated by Bcl6 (Kroenke et al., 2012) and its increased expression correlates with Tfh cell differentiation (Yusuf et al., 2010). The working model is that during initial DC engagement of naïve T cells, SLAM-SAP is dispensable for pre-Tfh cell differentiation. However, SAP is required for long term conjugation between pre-Tfh cells and B cells, a lack of which prevents the further differentiation of Tfh cells, IL-21 and IL-4 expression, and leads to the collapse of the GC reaction.

Interestingly, SAP-deficiency is able to abrogate autoimmunity in *sanroque* mice, where uncontrolled ICOS expression is thought to lead to an increased number of Tfh cells and spontaneous GC formation (Linterman et al., 2009b). This suggests that ICOS costimulation requires functional SLAM-SAP interactions for its own function. Therefore, although I do not

directly investigate the role of SLAM-SAP in my thesis, its role in Tfh cell differentiation is still crucial and must be taken into consideration.

1.4.4 PD-1

Programed Death 1, or PD-1, is member of the CD28 superfamily of costimulatory receptors which is expressed on the surface of T cells, B cells, natural killer T (NKT) cells, activated monocytes and DCs (Keir et al., 2008). In T cells, its surface expression mirrors that of ICOS such that it is barely expressed on naïve T cells, but highly expressed on activated T cells. However, in contrast to CD28 and ICOS, PD-1 negatively regulates TCR signals. The intracellular tail of PD-1 contains an immunoreceptor tyrosine-based inhibitor motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM) that recruits SH2-domain containing tyrosine phosphatase (SHP)-1 and SHP-2 (Okazaki et al., 2001; Chemnitz et al., 2004; Parry et al., 2005). However, it is known that the ITSM motif (not ITIM) plays a major role in the downregulation of TCR and BCR signaling.

Importantly, PD-1 is highly expressed on Tfh cells and has become one of the key surface marker of Tfh cells, along with CXCR5 and ICOS (Keir et al., 2007; Yu and Vinuesa, 2010a). It was therefore suggested that PD-1 served to broadly negatively regulate Tfh cells in order to control autoimmunity. By this logic, removing PD-1 would increase Tfh cell numbers and/or function, resulting in more GCs and increased levels of class-switched antibodies with higher affinity. However, three recent studies have cast doubt on this hypothesis.

The first study demonstrated that neither PD-1 nor the PD-1 ligands (PDL-1 and PDL-2) were necessary for Tfh cell or GC development, and no difference was seen during the primary response (Good-Jacobson et al., 2010). However, the authors found that PD-1$^{-/-}$ and PDL1$^{-/-}$ mice had less antigen-specific long-lived PCs after 30 days than WT mice, and less memory B cells in PD-1$^{-/-}$ mice, but not PDL1$^{-/-}$ mice. Interestingly, the remaining PCs had higher antibody affinity than in WT mice. This was explained by showing increased cell death of GC B cells in the PD-1-deficient mice. Additionally, they found a small increase in Tfh cell numbers over time compared to WT mice, but these Tfh cells had consistently lower levels of IL-21 and IL-4. These results proved to be counterintuitive at first as they were the first to suggest that PD-1 played a more complex role in Tfh cell function than previously thought.

A second study showed that the bacterial composition in the gut of PD-$1^{-/-}$ mice was altered compared to WT mice; where WT mice favored a microbiota consisting of "healthy" bacteria, PD-$1^{-/-}$ mice had increased levels of "bad" bacteria (Kawamoto et al., 2012). In conflict with the first study, they showed that the gut IgA antibody affinity was lower in PD-$1^{-/-}$ mice and that there were more Tfh cells in PPs. Nevertheless, they did also confirm increased cell death of plasmablasts in PD-$1^{-/-}$ mice, but no change in proliferation. Furthermore, although they showed higher numbers of class-switched IgA^+ B cells in PP, there was significantly lower numbers of IgA^+ B cells in the lamina propria. Therefore, this study suggests that PD-1 on Tfh cells may act to ensure that only the highest affinity B cells receive survival cues.

Finally, a third paper published by Arlene Sharpe and colleagues partially reconciles inconsistencies surrounding the role of this inhibitory costimulatory receptor by demonstrating that PD-1 has an inhibitory role in the generation of Tfr cells (Sage et al., 2013). In PD-$1^{-/-}$ and PDL$1^{-/-}$ mice, there were increased numbers of Tfr cells in LNs and that these Tfr cells had increased immunosuppressive capabilities. Furthermore, while CD28 and ICOS were necessary for the development of Tfr cells, PD-1 inhibited their development. Therefore, it appears that PD-1 inhibits the function of Tfh cells, but it also negatively regulates the development of Tfr cells. However, more research is required to further understand the role of PD-1 in the humoral response, as it is apparent that its role is more complex than originally believed.

1.4.5 CD28

CD28 stimulation is required for an effective humoral response, as CD28-deficient mice exhibit major defects in GC reactions, antibody production, Tfh cell differentiation and infection clearance (Shahinian et al., 1993; Ferguson et al., 1996; Walker et al., 1999; Okkenhaug et al., 2001; Walker et al., 2003; Linterman et al., 2009a). CD28 is required for the initial DC-mediated priming of T cells and achieves this through multiple mechanisms. However, there are two main mechanisms that stand out. First, CD28 costimulation leads to increased proliferation as a result of increased IL-2 production, paired with higher Bcl2 and Bcl-xL expression (Riley et al., 2002; Parry et al., 2003; Parry et al., 2007). This is in contrast to ICOS, which has a limited costimulatory effect on IL-2 production. Second, CD28 costimulation leads to a sharp increase in ICOS expression (McAdam et al., 2000) and this is crucial for Tfh cell differentiation. However, it is important to note that CD28 deficiencies affect all other Th subsets (Rudd and Schneider, 2003).

In other words, there was previously no evidence that CD28 has any unique function in driving Tfh cell differentiation. Rather, it seems that CD28 promote the initial expansion of T cell clones through IL-2 and Bcl-xL expression.

1.4.6 CYTOKINES

The production of specific cytokines by APCs during initial CD4 T cell priming promotes epigenetic changes in the proliferating Th cell which induce its differentiation into Th1, Th2, Th17, Tregs and Tfh cells (Oestreich and Weinmann, 2012). In turn, this induces the production of a specific set of a cytokines which help in their effector function. Here I will briefly discuss the role of the key cytokines in Tfh cell development and function.

1.4.6.1 IL-21

The cytokine IL-21 was originally described as an inhibitor of NK and Th1 cells (Kasaian et al., 2002; Wurster et al., 2002), but was later found to synergize with IL-15 to induce CD8 T cell proliferation (Zeng et al., 2005). The functional activity of DCs can also be modulated by IL-21 (Wang et al., 2003). Most importantly, IL-21 directly mediates antibody production in B cells (Ozaki et al., 2002) and is highly expressed in Tfh cells (Chtanova et al., 2004). Recent work has identified IL-21 as a possible mediator of Tfh cell differentiation (Nurieva et al., 2008; Vogelzang et al., 2008).

IL-21 was once considered so central to Tfh cell function that early studies referred to all IL-21 producing CD4 T cells as Tfh cells. However, this was later revised when it was discovered that other cell subsets can also produce IL-21, notably Th17 and NK T cells (Korn et al., 2007; Nurieva et al., 2007a; Wei et al., 2007; Suto et al., 2008). Nevertheless, IL-21 is still a major factor that defines Tfh cells and is a crucial component of T cell-mediated B cell help.

IL-21 is part of the common cytokine-receptor γ-chain (γ_c) family of cytokines. It signals through the IL-21R and γ_c and activates JAK-family tyrosine kinases JAK1 and JAK3, where JAK1 binds IL-21R and JAK3 binds γ_c. These kinases induce IL-21-dependent activation and nuclear localization of STAT3, STAT1 and to a lesser extent STAT5A and STAT5B (Leonard and Spolski, 2005). As I have already discussed, IL-6 in combination with IL-21 is an important component of early Tfh cell differentiation (Nurieva et al., 2008; Suto et al., 2008; Vogelzang et al., 2008; Nurieva et al., 2009; Eto et al., 2011; Choi et al., 2013b). However, IL-21 also plays an

important role in B cell help. It was once theorized that IL-4 was the main cytokine involved in CD4 T cell-mediated B cell activation. However, the observation that IL-4- and IL-4R-deficient mice could still undergo GC reactions and produce class-switched antibodies, albeit reduced in amplitude, suggested that there are redundant or overlapping mechanisms that compensate for IL-4 deficiency (Kuhn et al., 1991; Kopf et al., 1993; Noben-Trauth et al., 1997; Mohrs et al., 1999; King and Mohrs, 2009). It was later discovered that IL-21R-deficient mice exhibit dramatically lower levels of IgG, but much higher levels of IgE, in the serum of immunized mice (Ozaki et al., 2002) proposing an explanation for the phenotype observed in IL-4-deficient mice. Interestingly, these mice had higher levels of IgE, but the reasons for this are still unclear. IL-21 deficiency was also later determined to affect B cells more profoundly than CD4 T cells, as IL-21R deficiency in B cells led to the abrogation of GC formation and antibody production (Linterman et al., 2010; Zotos et al., 2010). Therefore, it appears that T cell-mediated IL-21 production plays a role in Tfh cell development, as well as B cell differentiation into antibody producing cells.

Many studies have demonstrated that IL-21 plays a more complicated role in B cell activation than previously thought. First, *in vitro* cultures of freshly isolated B cells have demonstrated that IL-21 has a pro-apoptotic effect on B cells independent of Bcl2 expression, and that apoptosis is markedly enhance when B cells are costimulated with LPS or IgM crosslinking. However, less apoptosis is observed for B cells that have been stimulated with anti-CD40-specific antibodies (Mehta et al., 2003; Jin et al., 2004; Ozaki et al., 2004). Second, *in vivo* studies have shown that IL-21 potently induces increased IgG1 levels and CSR, but curiously is not crucial for affinity maturation, despite the fact that both processes are dependent upon AID (Ettinger et al., 2005; Ettinger et al., 2007; Kuchen et al., 2007). This suggests that IL-21 plays a dual role in B cells, where it induces CSR of B cells in contact with T cells, but promotes cell death of B cells that have not.

Finally, although IL-6 exposure is crucial for increased IL-21 expression in CD4 T cells (Dienz et al., 2009; Eddahri et al., 2009), ICOS costimulation is also a potent inducer of IL-21 production (Vogelzang et al., 2008; Bauquet et al., 2009). Additionally, SAP-deficient T cells fail to produce IL-21 (Yusuf et al., 2010). However, this is likely because SAP-deficient T cells cannot support T-B interactions long enough for other signaling molecules such as ICOS to induce IL-21

production. In all, IL-21 plays an important role in Tfh differentiation, B cell activation and antibody production.

1.4.6.2 IL-4

IL-4 [also known as B cell stimulating factor 1 (BSF1)] has long been known to be crucial for T cell-mediated B cell differentiation into antibody producing PCs (Howard et al., 1982; Paul and Ohara, 1987). IL-4 is mainly produced by activated T cells, but is also produced by mast cells, basophils and eosinophils (Luzina et al., 2012). Like IL-21, IL-4 is a member of the γ_c family of cytokines and its receptor is therefore composed of the γ_c and the IL-4R chain. Upon activation, IL-4 activates JAK3 (γ_c) and JAK1 (IL-4R). Furthermore, tyrosines on the IL-4R chain can recruit and activate STAT6 as well as insulin receptor substrate (IRS)1 and IRS2, which can in turn activate PI3K. It is known that IRS2 is generally hematopoietically expressed and IRS1 is generally nonhematopoietically expressed. In B cells, the presence or absence of CD40 stimulation greatly changes the effect of IL-4R signaling (Siepmann et al., 1996). Additionally, CD40 stimulation can increase IL-4R expression on B cells 100-1000 fold and together, CD40 and IL-4R stimulation leads to B cell activation, CSR and SHM (Nelms et al., 1999).

Functionally, IL-4 is best known for defining the so-called Th2 phenotype of lymphocytes. The finding that Th2 cells, which express high levels of IL-4, are better than IFN-γ-producing Th1 cells at inducing IgG1 and IgE antibody production by B cells *in vitro* has led to the long-held notion that IL-4 production by Th2 cells are the main mediators of humoral immunity (Mosmann et al., 1986). However, three crucial observations have led to confusion over the role of IL-4 in humoral immunity. First, although IL-4- and IL-4R-deficient BALB/c mice have reduced levels of IgG1 and IgE in immunizations and infections, they produce higher levels of IgG2a, IgG2b and IgA (Kopf et al., 1993; Noben-Trauth et al., 1997; Mohrs et al., 1999), suggesting that the humoral response is not completely collapsed in the absence of IL-4. Second, the emerging understanding that IL-21 is also a crucial cytokine in T cell-mediated B cell help (Ozaki et al., 2002), may have given rise to the perception that IL-4 is less relevant that previously believed in T cell-mediated B cell help. Third, the discovery that Tfh cells, not Th2 cells, are the helper T cells that migrate to the B cell zone and GCs to provide cognate help to B cells (Johnston et al., 2009; Nurieva et al., 2009; Reinhardt et al., 2009; Yu et al., 2009) may have also compounded the belief that IL-4 is less important than previously believed. Notably, STAT6-deficient mice, which are unable to

generate any Th2 cells, produce wild-type levels of IgG1 in response to anti-allotypic anti-IgD monoclonal antibody injection (Kaplan et al., 1996). In fact, Th2 cells migrate to the periphery and recruit eosinophils (Reinhardt et al., 2009) and also co-express IL-13, whereas Tfh cells make only IL-4 and not IL-13 (Liang et al., 2011). Together, these findings led to uncertainty over the role of IL-4 in antibody production.

A recent study has reported that Tfh cells did not produce any IL-4 during a Th2 response against keyhole limpet hemocyanin with complete Freud's adjuvant (Nurieva et al., 2008). However, this proved to be somewhat misleading as it has recently been revealed that Tfh cells in the GC, as opposed to those in the B cell follicle, produce much higher levels of IL-4 (Yusuf et al., 2010), so that average IL-4 production of Tfh cells may in fact be low. This study showed an interesting example of the importance of IL-4 during an antibody response. The authors infected mice with LCMV (Yusuf et al., 2010), which is a classical and potent inducer of a IFN-γ-dominated Th1 response, and analyzed Tfh cells in the B cell follicle. What they observed was that compared to Tfh cells outside of the GC, Tfh cells residing within the GC expressed slightly higher levels (~2-3x) of Bcl6, ICOS, PD-1 and IL-21 mRNA. Strikingly however, GC Tfh cells expressed 21x more IL-4 than B cell follicle Tfh cells, despite the fact that they had induced a Th1 response. Interestingly, this increased IL-4 expression did not correlate with an increased expression in the Th2 master regulator GATA-3, confirming that these were not Th2 cells.

Still, recent studies have shown a unique relationship between IL-4 and Tfh cells. However, before discussing Tfh cells, it is important to note one particular study by Richard Locksley and colleagues on IL-4 production by Th2 cells. This group showed that IL-4 protein synthesis did not correlate with IL-4 mRNA levels. When naïve CD4 T cells were primed *in vivo* to become Th2 cells, they expressed high levels of IL-4 mRNA. However, these cells did not actually produce IL-4 protein unless they were stimulated through the TCR (Scheu et al., 2006). In other words, IL-4 transcription and IL-4 translation are uncoupled. Later studies looking at Tfh cells found that during a Th2 response, the only T cells in draining lymphoid organs that produced IL-4 protein were found strictly inside the GC and in direct contact with B cells (King and Mohrs, 2009; Reinhardt et al., 2009; Zaretsky et al., 2009). When analyzed, these IL-4 protein-producing cells had the highest expression of PD-1, ICOS, CXCR5 and IL-21. This demonstrated a striking complexity in the regulation of IL-4 protein by Tfh cells during an antibody response. This ensured

that only Tfh cells that were in direct contact with B cells produced IL-4, presumably to prevent the generation of autoantibodies.

Despite the fact that IL-21 has emerged as an important cytokine in humoral immunity in the past decade, IL-4 is still critically important during T cell-mediated B cell help. Recent studies characterizing the highly complex transcriptional and translational regulation of IL-4 synthesis only further highlight the importance of understanding the mechanisms of its production.

1.4.6.3 IL-2

IL-2 is usually considered an integral part of early T cell activation (Morgan et al., 1976; Boyman and Sprent, 2012). TCR stimulation alone is able to induce IL-2 production, but the unique ability of CD28 costimulation to induce massive amounts of IL-2 has been the subject of intense research. T cell-mediated production of IL-2 feeds back onto the T cell and drives proliferation. However, in the case of Tfh cells, three recently published studies have demonstrated that IL-2 may in fact inhibit Tfh cell differentiation (Ballesteros-Tato et al., 2012; Johnston et al., 2012; Nurieva et al., 2012).

Naïve CD4 T cells that have constitutively active STAT5, which is downstream of IL-2 signaling, are less likely to differentiate into Tfh cells (Johnston et al., 2012; Nurieva et al., 2012) and induce lower antibody titers (Nurieva et al., 2012) in immunized mice. Furthermore, the addition of recombinant IL-2 during influenza infection reduces the number of Tfh cells, GC B cells and influenza-specific antibody titers (Ballesteros-Tato et al., 2012). On the other hand, genetic deletion of STAT5 (Johnston et al., 2012; Nurieva et al., 2012) or IL-2Rα (Ballesteros-Tato et al., 2012) in naïve CD4 T cells significantly increased Tfh cell and GC B cell numbers. Likewise, injection of a blocking anti-IL-2 antibody during immunization also increased Tfh cell number (Nurieva et al., 2012).

This reduced Tfh cell differentiation was not attributed to increased numbers of Treg, which is normally induced by increased IL-2 (Ballesteros-Tato et al., 2012; Johnston et al., 2012). Rather, the IL-2-STAT5 axis upregulated Blimp-1, which antagonizes Bcl6 and inhibited Tfh cell differentiation (Ballesteros-Tato et al., 2012; Johnston et al., 2012; Nurieva et al., 2012). Intriguingly, it has been observed that pre-Tfh cells express lower levels of CD25, the high affinity subunit of the IL-2 receptor (Choi et al., 2011), suggesting that the Tfh cell program actively seeks

to reduce IL-2 responsiveness. However, beyond therapeutic targeting, the significance of the inhibitory effect of IL-2 on Tfh cells remains elusive.

1.4.6.4 IL-12

Three recent studies have observed a crucial role for IL-12 in the development of human, but not mouse, Tfh cells (Ma et al., 2009; Schmitt et al., 2009; Schmitt et al., 2013). Human naïve CD4 T cells that were polarized *in vitro* with IL-12 expressed high levels of Tfh cell-related markers such as CXCR5 and ICOS (Ma et al., 2009) and DC-mediated production of IL-12 led to the differentiation of IL-21-producing Tfh cells in a manner dependent on STAT4 (Schmitt et al., 2009; Schmitt et al., 2013). Importantly, co-culture of IL-12 primed Tfh-like cells induced B cells to produce immunoglobulins in a fashion dependent on IL-21 and ICOS (Schmitt et al., 2009). Finally, human patients lacking functional IL-12Rβ1, a receptor for both IL-12 and IL-23, displayed substantially less circulating memory Tfh cells and memory B cells than healthy subjects (Schmitt et al., 2013). Furthermore, these IL-12Rβ1-deficient patients had impaired GC formation in lymph nodes and substantially lower avidity tetanus-specific serum antibodies in immunized individuals than age-matched control subjects.

These studies demonstrate a major role for IL-12 in the differentiation of human Tfh cells, and highlight a major difference between human and mouse Tfh cells.

1.5 NON-CONVENTIONAL Tfh-LIKE CELLS

While conventional Tfh cells provide B cell help during a TD antibody response, there are recently discovered non-conventional Tfh-like cells that may play a role in the antibody response. Although I do not address these cells in my work, ICOS has been shown to be involved, or might be involved, in their development.

1.5.1 EXTRAFOLLICULAR Th CELLS

In addition to seeding the GC, a subset of B cells that receive help from T cells at the T-B border will upregulate CD138, migrate to the red pulp border and initiate an extrafollicular focus. Extrafollicular Th cells are CD4 T cells that are localized to these extrafollicular foci and initiate the induction of short-lived plasmablasts (Odegard et al., 2008; Vinuesa et al., 2010; Goodnow et al., 2011). These plasmablasts produce low-affinity but class-switched IgG antibodies early (3-4

days) during an antibody response (Goodnow et al., 2011; Chan and Brink, 2012). Importantly, studies in lupus mouse models show that extrafollicular Th express Bcl6 and their function is critically dependent on ICOS, CD40L and IL-21, but not SAP (Odegard et al., 2008; Tangye et al., 2013). Extrafollicular Th cells do not express CXCR5, but rather are attracted to extrafollicular sites by the CXCR4-CXCL12 axis. Finally, extrafollicular Th cells might also give rise to conventional Tfh cells (Bentebibel et al., 2011; Crotty, 2011). Further studies are needed to outline the major differences between extrafollicular Th cells and Tfh cells, beyond their localization and chemokine receptor expression profiles.

1.5.2 FOLLICULAR Treg CELLS

The first report of $Foxp3^+$ Tregs inside the GC was published almost a decade ago (Lim et al., 2004). However, their existence was largely ignored for the next 7-8 years and interest in these cells has only recently re-emerged. Three new studies report that of all Tfh cells, approximately 10-15% exhibit characteristics of both Tfh cells and Tregs, and have thus been given the name of follicular Tregs cells (Tfr) (Chung et al., 2011; Linterman et al., 2011; Wollenberg et al., 2011). Although they exhibit high expression of the Tfh cell markers CXCR5, Bcl6, PD-1 and ICOS, Tfr cells lack the expression of CD40L, IL-4 and IL-21. Paradoxically, they also co-express high levels of Blimp (Linterman et al., 2011), which was previously believed to antagonize Bcl6 expression (Johnston et al., 2009). Importantly, abrogating either Tfr cell development or localization was shown to enhance GC reactions and antibody production (Chung et al., 2011; Wollenberg et al., 2011). These Tfr cells were demonstrated to arise from thymus-derived Tregs and not from inducible Treg or Tfh cells (Linterman et al., 2011). Interestingly, the inhibitory costimulatory receptor PD-1 is more highly expressed on Tfr cells than Tfh cells, and PD-1 deficiency leads to reduced Tfr cell frequency and higher antibody levels (Sage et al., 2013). Thus, PD-1 seems to be crucial for the development of Tfr cells. The role of ICOS in Tfr cell differentiation and function is currently unknown, although ICOS is highly expressed on the surface of Tfr cells (Chung et al., 2011; Linterman et al., 2011; Sage et al., 2013). However, a recent study has identified ICOS as a mediator of Treg homeostasis and function in non-obese diabetic (NOD) mice (Kornete et al., 2012). Therefore, it should be investigated whether ICOS plays a role in the development of Tfr cells.

Finally, it has not yet been tested experimentally if Tfr cells negatively regulate Tfh cell-mediated B cell help through the same mechanisms as Treg cell-mediated repression. Tfr cells do express high levels of IL-10 (Linterman et al., 2011), much like Treg cells, and IL-10 has been reported to inhibit B cell help by Tfh cells (Cai et al., 2012). However, as Tfh cells express equivalent levels of IL-10 compared to Tfr cells, it is unclear how Tfr cells would achieve inhibition solely through IL-10. Clearly, more studies are needed to better understand the role of Tfr cells in humoral immunity.

1.5.3 NKTfh CELLS

Natural killer T (NKT) cells are innate-like T cells expressing a semi-invariant TCR repertoire that recognize lipid and glycolipid antigens presented by the non-polymorphic MHC molecule CD1 (Bendelac et al., 2007). The ability of NKT cells to rapidly expand and produce effector cytokines link the innate and the adaptive immune responses. Recent studies have uncovered a small population of NKT cells that have acquired a Tfh-like phenotype (NKTfh) and can be detected in human tonsils (Chang et al., 2012; King et al., 2012; Tonti et al., 2012). These NKTfh cells produce IL-21, can support the rapid formation of GCs and induce detectable levels of antigen-specific IgG with some evidence of affinity maturation. However, the inability of NKTfh cells to invoke a long-lived memory response against lipid antigen was strikingly different than a conventional Tfh cell response. Furthermore, the magnitude of the antibody response induced by NKTfh cells was also significantly weaker than those induced by Tfh cells (Chang et al., 2012; King et al., 2012; Tonti et al., 2012).

Interestingly, NKTfh cells express high levels of ICOS on their surface (Tonti et al., 2012). Additionally, ICOS has been reported to play a major role in the NKT function and homeostasis in a mouse model of asthma (Akbari et al., 2008; Chung et al., 2008). Therefore, it is possible that ICOS may also play a role in the development and function of NKTfh cells.

1.5.4 γδ Tfh CELLS

The finding that the absence of conventional αβ T cells still leads to some, albeit much weaker, production of antigen-specific antibodies has led to the realization that γδ T cells can provide help to B cells and generate GCs (Vantourout and Hayday, 2013). Much like NKT cells, γδ T cells express a semi-invariant TCR repertoire, but these recognize non-peptidic

phosphoantigens that are derived from microbial metabolites (Vantourout and Hayday, 2013). Two groups have shown that some human γδ T cells express CXCR5 and localize to the GC in response to these antigens (Brandes et al., 2003; Caccamo et al., 2006; Caccamo et al., 2012). Furthermore, it was shown that IL-21 increased their numbers (Caccamo et al., 2012) suggesting that γδ T cells may depend on Tfh cells or IL-21-producing Tfh-like cells for their Th cell abilities. Finally, it is still unclear how exactly these cells help B cells, but there is evidence that it can be through the production IL-4 and IL-10 (Tangye et al., 2013; Vantourout and Hayday, 2013).

1.5.5 HUMAN CIRCULATING Tfh-LIKE CELLS

We have known for over a decade that there exists a pool of circulating $CXCR5^+$ CD4 T cells in human blood (Breitfeld et al., 2000; Schaerli et al., 2000). However, the relationship of these cells with Tfh cells in secondary lymphoid organs is still poorly understood. There are clear difference between circulating $CXCR5^+$ CD4 T cells in the blood and Tfh cells. For example, circulating $CXCR5^+$ CD4 T cells do not express Bcl6, have lower levels of ICOS and PD-1, and by definition, are not found inside B cell follicles (Chevalier et al., 2011; Morita et al., 2011).

Interestingly, these cells do have some unique characteristics. Subsets of circulating $CXCR5^+$ CD4 T cells have been identified by assessing the co-expression of CXCR3 and CCR6, chemokine receptors associated with Th1 and Th17 cells, respectively. This allowed for their classification into Th1-like, Th17-like and Th2-like (the latter being $CXCR3^-CCR6^-$) (Morita et al., 2011). The Th17-like and Th2-like circulating $CXCR5^+$ CD4 T cells expressed higher levels of IL-21 and importantly, were over-represented in human autoimmunity (Morita et al., 2011).

If Tfh cells and these circulating $CXCR5^+$ CD4 T cells are indeed related, the link between these two cell types is still unclear. However, they do produce IL-21, IL-10 and CXCL13, which can be expressed by Tfh cells, and are efficient at inducing antibody production in *in vitro* co-cultures with B cells (Chevalier et al., 2011; Morita et al., 2011). Furthermore, the recent finding that Bcl6 is downregulated overtime to near undetectable levels, but still allows for the expression of CXCR5 and correlates with an increase in markers usually associated with a memory phenotypes (Kitano et al., 2011) does suggest that these cells may have experienced some aspect of a Tfh cell "program".

1.6 Tfh CELLS IN HUMAN DISEASES

Multiple studies have found that the deregulation of Tfh cells leads to autoimmunity or immunodeficiency in mouse models (King et al., 2008; Vinuesa et al., 2009; Crotty, 2011; Linterman et al., 2012; Qi, 2012). Unsurprisingly, there is even greater interest in understanding their role in human diseases. However, due to the nature of their localization in secondary lymphoid organs, they are technically difficult to study in human patients, with a few exceptions. Tonsils from individuals undergoing tonsillectomy are the notable exception (Breitfeld et al., 2000; Schaerli et al., 2000; Bentebibel et al., 2011), but these studies on tonsils rarely target individuals with genetic defects. However, recent studies have shown that quantifying the frequency of circulating $CXCR5^+$ CD4 Tfh-like cells is a reliable predictor of vaccine success and the magnitude of the induced response (Pallikkuth et al., 2011; Pallikkuth et al., 2012) and therefore may provide additional insight into Tfh cell activity in humans.

Notably, lack of Tfh cell activity has been observed in patients with primary immunodeficiencies. For example, mutations in the gene encoding SAP causes XLP, where patients are unable to mount an effective immune response against EBV, and this disease is associated with defects in Tfh cell differentiation (Cannons et al., 2011). Likewise, mutations in ICOS lead to common variable immunodeficiency in humans, which is characterized by a severe defect in antibody production (Grimbacher et al., 2003; Warnatz et al., 2006; Yong et al., 2009). Notably, patients with defects in ICOS, as well as CD40L, have considerably fewer circulating $CXCR5^+$ CD4 T cells as well as defects in GCs (Bossaller et al., 2006). Furthermore, patients with autosomal dominant hyper-IgE syndrome, which is caused by mutations in STAT3, have fewer circulating $CXCR5^+$ CD4 T cells, and their naïve CD4 T cells fail to different into Tfh cell-like cells *in vitro* (Ma et al., 2012). Finally, patients with mutations in the gene that encodes for IL-12Rβ1 have fewer circulating $CXCR5^+$ CD4 T cells and have reduced numbers of memory B cells, abnormal GCs and low-avidity antibody responses in response to tetanus toxin (Schmitt et al., 2013). Taken together, genetic defects in SAP, ICOS, CD40L, STAT3 and IL-12Rβ1, all of which have been proven to play a crucial role in Tfh cell development and function in mouse models (except for IL-12), have also been demonstrated to cause Tfh cell-related immunodeficiencies in humans.

Conversely, increased or uncontrolled Tfh cell differentiation and/or function is associated with multiple autoimmune disorders in humans. Increased frequencies of circulating $CXCR5^{+}ICOS^{hi}$ CD4 T cells have been found in patients with systemic lupus erythematosus, Sjögren's syndrome, rheumatoid arthritis, juvenile dermatomyositis, autoimmune thyroid disease and myasthenia gravis (Tangye et al., 2013). These usually correlate with higher serum levels of autoantibody and IL-21, as well as disease severity.

In some cases, Tfh cells may also give rise to malignant tumors. Peripheral T cell lymphomas (PTCLs) are rare haematological malignancies constituting approximately 5-10% of all non-Hodgkin's lymphomas (Tangye et al., 2013). These included angioimmunoblastic T cell lymphoma (AITL), follicular T cell lymphoma (FTCL) and PTCL-not-otherwise-specified (PTCL-NOS). Some of the cardinal features of AITL and FTCL include B cell activation, follicular hyperplasisa, hypergammaglobulinaemia and autoantibody production. Importantly, a substantial proportion of cases of PTCL show an increase in markers associated with Tfh cells. It is unclear why Tfh cells could give rise to several types of PTCLs, but targeting Tfh cell markers may help treat these diseases.

2. THE INDUCIBLE COSTIMULATOR

The specific recognition of cognate antigen presented by MHC molecules induces TCR signaling, but it is the stimulation of costimulatory and coinhibitory receptors that truly give context in which TCR is engaged. It is costimulation that directs T cell function and determines T cell fate. For example, when APCs recognize pathogens or tissue damage, costimulatory ligands are upregulated to induce T cell expansion and differentiation, and ultimately T cell immunity. On the other hand, lack of costimulation or dominance of coinhibition over costimulation leads to T cell tolerance (Sharpe and Freeman, 2002). The discovery of the prototypical CD28 costimulatory receptor allowed for the incorporation of T cells into the two-signal paradigm for immune cell activation (Bretscher and Cohn, 1970; June et al., 1987; Mueller et al., 1989). Since then, up to 25 costimulatory and coinhibitory receptors have been discovered in T cells (Greenwald et al., 2005; Watts, 2005; Veillette, 2006; Chen and Flies, 2013).

ICOS is a costimulatory receptor of the CD28 family of costimulatory receptors and is found on activated T cells. Costimulation of ICOS enhances many T cell responses including cytokine production and proliferation. It is associated with an effective humoral response and recent findings have shown that this is due to its crucial role in the development of Tfh cells. In this section, I will review what is known about this important costimulatory receptor, beginning with a summary of the biochemical aspects of ICOS, such as structure and signaling pathways, followed by an in-depth review of its known role in T cell immunity. However, as ICOS is such a central aspect of my research, I will begin with a history of its discovery and early characterization.

2.1 A BRIEF HISTORY

ICOS, originally named H4, was first discovered in 1996 by one of the leading figures in fundamental immunological research, Charles Janeway Jr. and his colleagues (Redoglia et al., 1996). By immunizing Armenian hamsters with a mouse Th2 cell clone, they produced a monoclonal antibody that recognized a protein selectively expressed on activated T cells, but not B cells, macrophages or fibroblasts. This protein, named H4, was physically associated with the TCR and co-precipitated with a tyrosine kinase activity. It was therefore suggested that H4 might function as a T cell-specific costimulatory molecule.

Three years later, two studies were published in *Nature* describing, in both humans and mice, a novel costimulatory receptor found selectively on activated T cells (Hutloff et al., 1999; Yoshinaga et al., 1999). As its expression in activated T cells was, by definition, inducible, it was dubbed the Inducible Costimulator, or ICOS. Notably, ICOS enhanced all basic T cell responses to foreign antigens such as proliferation, and secretion of specific cytokines such as IL-4, IFN-γ and IL-10, as well as provided a boost to antibody secretion by B cells. Also important to note is that ICOS was highly expressed in the LZ of GCs (Hutloff et al., 1999), in which Th interact with B cells. ICOS also displayed similarity with the prototypical costimulatory receptor CD28. However, ICOS and its ligand B7RP-1, also described in one of the studies (Yoshinaga et al., 1999), did not interact at all with CD28 or its binding partners. Finally, using the monoclonal antibody C398.4A, first produced by Janeway and colleagues to identify H4, the Dianziani group confirmed that the T cell activation molecule H4 and the CD28-like molecule ICOS were identical (Buonfiglio et al., 1999).

The subsequent two years saw a flurry of papers regarding ICOS. B7RP-1, with varying nomenclature (ie: ICOSL (Aicher et al., 2000), B7h (Swallow et al., 1999), AILIM-L (Tamatani et al., 2000), GL50 (Ling et al., 2000), LICOS (Brodie et al., 2000) and B7-H2 (Wang et al., 2000)) was quickly confirmed to be the sole ligand of ICOS (Yoshinaga et al., 1999). CD28 costimulation was demonstrated to optimize ICOS expression upon TCR engagement (McAdam et al., 2000) and the importance of ICOS in controlling infections (Coyle et al., 2000; Kopf et al., 2000) and cytokine production (Coyle et al., 2000; McAdam et al., 2000; Wang et al., 2000) was further confirmed. One early study showed that ICOS can act as an adhesion molecule (Tamatani et al., 2000). Finally, more rigorous experimentation and characterization of the role of ICOS in T cell activation could be performed after three independent groups generated $ICOS^{-/-}$ mice (Dong et al., 2001a; McAdam et al., 2001; Tafuri et al., 2001). All three strains were reported to have significant defects in antibody responses and GC reactions. Despite the striking phenotype in $ICOS^{-/-}$ mice, the exact mechanisms for the role of ICOS in T cell-mediated antibody responses remained unknown for several years.

In the year following the discovery of ICOS, two studies came out identifying CD4 T cells that expressed CXCR5 and could be found inside B cell follicles of human tonsils; ie: Tfh cells (Breitfeld et al., 2000; Schaerli et al., 2000). The authors of the studies showed that, along with

CXCR5, Tfh cells expressed high levels of CD69, CD40L, HLA-DR and most importantly, ICOS. This supported the earlier finding that ICOS expression could be detected in the LZ of GCs (Hutloff et al., 1999). However, it took another five years to discover that ICOS is crucial in the development of Tfh cells, as $ICOS^{-/-}$ mice did not generate any Tfh cells and in turn, lacked GCs (Akiba et al., 2005). However, it remained unclear until recently as to how ICOS facilitates Tfh cell generation at the molecular and cellular level.

2.2 STRUCTURAL FEATURES AND SIGNALING CAPACITIES OF ICOS

ICOS is a type I transmembrane glycoprotein that consists of a single IgV-like domain, one ~23 amino-acid transmembrane region and a 35 amino-acid cytoplasmic tail (Hutloff et al., 1999). Homology-based modeling of the IgV-like domain alongside targeted mutagenesis mapped the ICOSL-binding region to a "FDPPPF" motif, which is analogous yet distinct from the "MYPPPY" motif of CD28/CTLA-4 (Wang et al., 2002). Two cysteine residues at position 42 and 109 stabilize the IgV-like domain, while the cysteine residue at position 136 is required to form the disulfide bridge between the homodimeric chains (Hutloff et al., 1999) (Figure 2).

ICOS is found on the cell surface as a homodimeric disulfide-linked protein of an apparent relative molecular mass of 55-60 kDa in humans and 47-57 kDa in mice. It is composed of 27 kDa and 29 kDa chains in humans (Hutloff et al., 1999; Beier et al., 2000) and approximately 26 kDa and 29 kDa in mice (Redoglia et al., 1996; Mages et al., 2000). In humans, the ICOS mRNA encodes a 199 amino-acid chain (200 amino-acid chain in mice) with a predicted relative molecular mass of 22.6 kDa (20.3 kDa in mice (Mages et al., 2000)). The discrepancy between the predicted and apparent sizes, as well as the two differently sized chains, is due to post-translational modifications, most notably due to glycosylation (Redoglia et al., 1996; Hutloff et al., 1999).

The amino-acid sequence of ICOS shares 17% and 19% identity with CD28 in humans and mice, respectively (Hutloff et al., 1999; Yoshinaga et al., 1999), whereas sequence similarity (as defined by structural similarity) is approximately 39% (Hutloff et al., 1999). Although it may seem low, this is significant as CD28 shares 26% amino-acid identity with the functionally related co-inhibitory CTLA-4 protein (Brunet et al., 1987). The human and mouse amino-acid sequences of ICOS share approximately 70% identity (Yoshinaga et al., 1999; Mages et al., 2000), which is

almost the same percentage of similarity between human and mouse CD28 (69%) (Yoshinaga et al., 1999).

ICOS is known to potentiate two TCR-mediated signaling pathways: the PI3K pathway (Coyle et al., 2000; Arimura et al., 2002; Parry et al., 2003) and the Ca^{2+} pathway (Parry et al., 2003; Nurieva et al., 2007b). While ICOS binds PI3K directly, the mechanism of Ca^{2+} mobilization by ICOS is less clear. It was assumed, however, that ICOS-mediated Ca^{2+} potentiation was an indirect result of PI3K activity, as PI3K induces Ca^{2+} mobilization in T cells (Hsu et al., 2000).

2.2.1 COMPARISON OF CYTOPLASMIC TAILS OF CD28 FAMILY MEMBERS

ICOS has a cytoplasmic tail of 35 amino acids, which is approximately the same length as the cytoplasmic tails of both CD28 and CTLA-4. Common to all three receptors is a Tyr-Xaa-Xaa-Met (YxxM) PI3K-binding consensus motif: YMNM for CD28, YVKM for CTLA-4 and YMFM for ICOS. However, whereas CD28 and CTLA-4 can bind multiple other adaptor proteins such as Grb2, Gads, Lck, Fyn and Itk for CD28, and PP2A, SHP2 and AP1/2 for CLTA-4, ICOS has only been reported to bind to PI3K upon phosphorylation of the tyrosine residue in the YMFM motif (Coyle et al., 2000; Parry et al., 2003; Rudd and Schneider, 2003; Zang et al., 2006; Nurieva et al., 2007b).

ICOS has no intrinsic kinase activity and therefore cannot auto-phosphorylate the tyrosine in its YMFM motif. This residue is phosphorylated in activated T cells (Coyle et al., 2000) and it is presumed to be the result of a Src family kinase, but this has yet to be determined experimentally. The conformational changes that lead to ICOS signal transduction upon binding to its ligand are not clear as this has not been directly studied. ICOS has a highly conserved and positively charged K^{167}KKY motif in the membrane proximal region, but its role in mediated ICOS-mediating signaling is unknown. Furthermore, ICOS has two evolutionarily conserved lysine residues near the end of its cytoplasmic tail (K^{191}K). The CD3ε chain is known to bury its cytoplasmic tail in the lipid bilayer through positively charged amino acids and release it upon TCR ligand binding (Xu et al., 2008; Gagnon et al., 2012). Therefore, it is possible that the cytoplasmic tail of ICOS is also buried in the lipid bilayer and released upon activation. This could theoretically inhibit enzyme access to the tyrosine in the YMFM motif under non-stimulatory conditions. However, this speculative mechanism is yet to be tested.

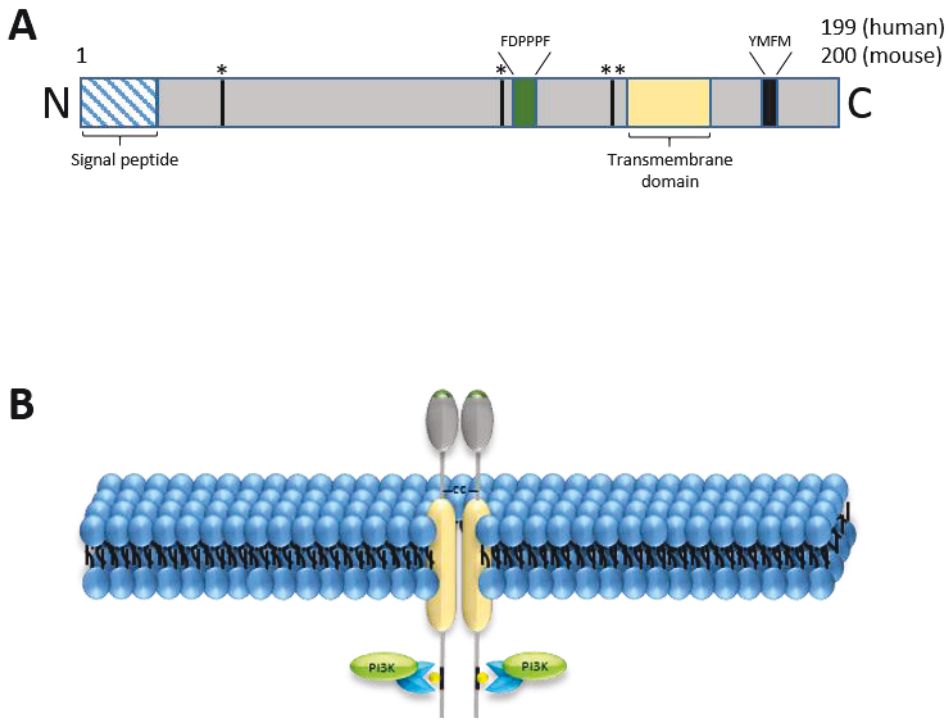

Figure 2. Schematic representations of ICOS. A) A linear representation of ICOS. The signal peptide, two cysteine residues (*) for intramolecular disulfide bond with the immunoglobulin domain, one cysteine residue (**) for homodimerization, ligand binding motif (FDPPPF), transmembrane segment and the cytoplasmic tail with a PI3K-binding motif (YMFM) are indicated. B) Model of ICOS in the lipid bilayer membrane. Also depicted are the regulatory (blue) and catalytic (green) subunits of PI3K, bound to the phosphorylated tyrosine from the YMFM PI3K-binding motif.

Once the tyrosine in the YMFM motif is phosphorylated, it can recruit the SH2 domain-containing PI3K regulatory subunits p85α and p50α (Coyle et al., 2000; Fos et al., 2008). This in turn leads to the activation of PI3K and the induction of PI3K signaling. Interestingly, the ICOS cytoplasmic tail is much more potent than that of CD28 in activating PI3K (Arimura et al., 2002; Parry et al., 2003). This may be due to the binding competition between Grb2 and PI3K for binding to the same region on the CD28 cytoplasmic tail. It is also worth noting that it has been repeatedly shown that CD28-mediated PI3K activation is largely dispensable for most T cell activity *in vivo* (Okkenhaug et al., 2001; Parry et al., 2003; Deane et al., 2007; Gogishvili et al., 2008; Dodson et al., 2009; Pagan et al., 2012).

2.2.2 PI3K ACTIVATION

PI3K is implicated in many facets of T cell activity, which includes but is not limited to development, differentiation, homeostatis, chemotaxis, trafficking and translation (Okkenhaug and Fruman, 2010). As the impact of PI3K activity in T cells is too vast to be covered in this subsection, I will expand on the subject in a section devoted to PI3K in "*Section 3 – The role of PI3K signaling in T cells"*.

2.2.3 CALCIUM SIGNALING

A rise in intracellular Ca^{2+} concentration is crucial for T cell activation and modulation of TCR signal strength. The initial steps of TCR-mediated calcium signaling are well established (Koretzky and Myung, 2001). Upon TCR engagement, Src-family kinases (eg: LCK) are 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 the zeta-chain-associated protein kinase 70 (ZAP70), which phosphorylates the adaptor protein linker for activated T cells (LAT). Tyrosine-phosphorylated LAT then recruits several SH2-containing proteins, including phospholipase C-γ1 (PLC-γ1), to lipid rafts. PLC-γ1 then cleaves phosphoinositide-(4,5)-biphosphate (PI(4,5)P2) at the plasma membrane to generate inositol-(1,4,5)-trisphosphate (IP3) and diacyl glycerol (DAG). IP3 then travels through the cytoplasm and induces the release of Ca^{2+} stored in 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 (Lewis, 2001;

Luik and Lewis, 2007). Importantly, the relationship between PLC-γ1 and ICOS, as well as the role of ICOS-mediated Ca^{2+} flux potentiation have yet to be fully examined.

2.3 REGULATION OF ICOS EXPRESSION

ICOS is expressed at low levels on naïve T cells and is upregulated significantly as early as 24 hours after TCR and CD28 costimulation (Hutloff et al., 1999; Yoshinaga et al., 1999; McAdam et al., 2000). Interestingly, early studies found that Th2 cells expressed much higher levels of ICOS than Th1 cells (McAdam et al., 2000), suggesting that ICOS may play a role in IL-4 production. ICOS expression can be regulated at the transcriptional level after T cell activation by the Src family kinase Fyn and the MAP kinase Erk (Tan et al., 2006). ICOS can also positively regulate its own transcription through the same mechanism (Tan et al., 2006). Fyn activates calcineurin, which in turn dephosphorylates NFATc2 and induces its nuclear localization (Zamoyska et al., 2003). In the nucleus, NFATc2 and Erk bind independently to the *icos* promoter and activate its transcription. Interestingly, the Th1- and Th2-promoting cytokines IL-12 and IL-4 can both lead to upregulation of ICOS expression (Wassink et al., 2004), suggesting that T cells that have lasting contact with DCs increase ICOS expression.

ICOS expression can also be negatively regulated at the posttranscriptional level. The RING-type ubiquitin-ligases Roquin-1 and Roquin-2 mediate ICOS mRNA degradation through a process dependent on miRNA-101, which binds the 3' untranslated region (UTR) of ICOS (Vinuesa et al., 2005; Yu et al., 2007; Pratama et al., 2013; Vogel et al., 2013). Mice deficient in Roquin-1 and its paralog Roquin-2 (Roquin1/2-DKO) have higher surface expression of ICOS, leading to increase number of Tfh cells and aberrant GC formation (Pratama et al., 2013; Vogel et al., 2013). The same phenotype is also found in Roquin-1 *sanroque* mutant mice where mutant Roquin-1 binds ICOS mRNA, but does not degrade it and prevents its degradation by Roquin-2 (Linterman et al., 2009a; Linterman et al., 2009b).

Finally, ICOS is expressed on Tfh cells as well as Th1, Th2, Treg, Th17 and unpolarized activated CD4 T cells (Th0) (Simpson et al., 2010b). Furthermore, ICOS is also highly expressed on effector and memory T cells in mice upon aging (Lohning et al., 2003). These cells can be inflammatory with distinct expression profiles and thus ICOS can be used as a marker for activation. Recent studies have shown that ICOS is regulated differently in different 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 however, NFATc2 also binds to the ICOS promoter, but the master regulator of Th2 cells GATA-3, operates via an *icos* 3'UTR (Tan et al., 2008). This may be highly relevant as Th2 cells express higher levels of ICOS than do Th1 cells (Coyle et al., 2000).

2.4 B7RP-1: THE ICOS LIGAND

B7RP-1 is a member of the B7 family of costimulatory ligands and it is the only known ligand of ICOS. Interestingly, B7RP-1 also binds to CD28 and CTLA-4, albeit with much lower affinity (Yao et al., 2011). However, this only happens in humans, and not mice. There are many differences between B7RP-1 and the prototypical B7 ligands B7-1 and B7-2. For example, while B7-1 and B7-2 are expressed exclusively on hematopoietic cells, B7RP-1 is expressed on both hematopoietic cells and cells of non-hematopoeitic origin, such as fibroblasts and endothelial cells (Greenwald et al., 2005). Of note, B7RP-1 mRNA can be detected in lymphoid and non-lymphoid organs including the kidney, liver, peritoneum, lung and testes. Furthermore, in contrast to the CD28 ligands B7-1 and B7-2, as well as the PD-1 ligand B7-H1, B7-RP1 has no known bi-directional co-signaling (Munn et al., 2004; Butte et al., 2007).

The regulation of B7RP-1 does not occur in the same manner as the prototypical B7 ligands B7.1 and B7.2, which are expressed at low levels during steady state and are upregulated upon TLR stimulus (Greenwald et al., 2005). B7RP-1 is constitutively expressed at steady state and its level of expression does not increase upon crosslinking of CD40 or the BCR (Aicher et al., 2000). Rather, signaling through the BCR and the IL-4R downregulates the expression of B7RP-1 and this can be prevented through CD40 stimulation (Liang et al., 2002). Furthermore, ligation of BCR, PMA stimulation and ICOS binding leads to B7RP-1 shedding and this can be prevented through TLR7/8 and TLR9 stimulation (Logue et al., 2006). Interestingly, ICOS-expressing, but PFA-fixed, Chinese hamster ovary cells (ie: non-functional) are also sufficient to downregulate B7RP-1 expression in B cells (Logue et al., 2006), suggesting that ICOS binding might cause conformational changes in B7RP-1 and making it susceptible to proteases.

It has been suggested that TLR stimulation, most notably TLR7/8 and TLR9 stimulation, may be a necessary third signal during mature B cell activation (Ruprecht and Lanzavecchia,

2006). Therefore, one possible mechanism through which TLR stimulation achieves full B cell activation is by ensuring maximal B7RP-1 expression. In other words, only B cells having received a full range of appropriate stimuli can in turn induce ICOS costimulation. Otherwise, improperly stimulated B cells remove that final stimulatory cue for T cells. Consistent with this observation, T cell-specific overexpression of ICOS in transgenic mice leads to downregulation of B7RP-1 *in vivo* and gives the paradoxical phenotype similar to that of $ICOS^{-/-}$ mice (Watanabe et al., 2008). This would also suggest that ICOS costimulation might in fact be a limiting factor during T-B interaction.

2.5 DOWNSTREAM EFFECTS OF ICOS COSTIMULATION

The costimulation of ICOS has been associated with increased production of IL-4, IL-21, IFN-γ and IL-10, as well as increased migration, proliferation and CD40L expression. Early studies were rigorous in detailing the mechanistic effects of ICOS costimulation at the cellular level and there is some basic understanding of ICOS signaling downstream of PI3K. However, it is important to consider that the vast majority of recent studies on ICOS have examined its role by extrapolating the phenotype of $ICOS^{-/-}$ mice.

2.5.1 REGULATION OF GENE EXPRESSION

ICOS costimulation may be closely linked to expression of IL-4 and IL-21, but their upregulation is dependent upon ICOS-mediated c-Maf induction (Nurieva et al., 2003a; Hiramatsu et al., 2010) which, along with nuclear translocation of NFATc1, binds to the IL-4 and IL-21 promoters (Yoshida et al., 1998; Nurieva et al., 2003a; Nurieva et al., 2007b; Bauquet et al., 2009; Hiramatsu et al., 2010). It should be noted that NFATc1 nuclear translocation is induced through the Ca^{2+} signaling pathway, thus ICOS can induce NFAT-related genes through both pathways. Furthermore, c-Maf is a transcription factor associated with Th2 cells (Hwang et al., 2002; Rengarajan et al., 2002), and this may explain why ICOS-deficient CD4 T cells are more likely to become Th1 cells *in vivo* (Nurieva et al., 2003a).

The mechanism by which ICOS costimulation leads to increased IL-10 and IFN-γ production is still relatively unclear. Transduction of constitutively active AKT into mouse CD4 T cells leads to increased ICOS-mediated IL-10 and IFN-γ production (Arimura et al., 2004). In contrast, pharmacological inhibition of AKT during ICOS costimulation leads to decreased levels

of IL-10 production (Okamoto et al., 2003). This suggests that ICOS-mediated IL-10 and IFN-γ production are a product of the PI3K pathway, but the molecular mechanisms downstream of PI3K are still unclear. Furthermore, ICOS has been reported to maintain high levels of CD40L surface expression on T cells (Kaminski et al., 2009), but the exact mechanisms have not yet been reported. It is also worth mentioning that early studies on ICOS costimulation reported conflicting data on the role of ICOS in induction of IL-2 production (Coyle et al., 2000; McAdam et al., 2000; Yoshinaga et al., 2000). However, later studies using chimeric ICOS-CD28 receptors convincingly demonstrated that ICOS is a poor activator of IL-2 production (Parry et al., 2003). Rather, CD28 is the predominant inducer of IL-2 and occurs through a PI3K-independent mechanism. Likewise, in contrast with CD28, which significantly induces Bcl-xL, ICOS has only a weak effect on transcription of this gene (Parry et al., 2003; Watanabe et al., 2005a). It is therefore still unclear how ICOS costimulation directly promotes survival and proliferation of activated T cells. In all, we have a surprisingly poor understanding of the role of ICOS signaling in the modulation of many of its target genes.

2.5.2 ICOS AS AN ADHESION MOLECULE

One particular effector function of ICOS that is often overlooked is the ability of ICOS to act as an adhesion molecule. An early ICOS study suggested that crosslinking of ICOS led to the aggregation of both human and rat thymoma cell lines (Tamatani et al., 2000). Later studies demonstrated that ICOS costimulation leads to cell elongation, or “spreading”, and actin polymerization in a TCR-independent fashion, which could not be induced upon CD28 costimulation (Okamoto et al., 2004; Nukada et al., 2006; Franko and Levine, 2009). Interestingly, it was shown that ICOS-dependent elongation, but not the formation of thin finger-like filopodia and micro-spikes, was PI3K-depenent (Franko and Levine, 2009). Furthermore, this ICOS-mediated elongation acted through the GTPase RhoA in a PI3K-dependent manner (Nukada et al., 2006; Franko and Levine, 2009). Finally, it was found that ICOS could mediate *in vitro* transendothelial migration of polarized Th1 cells, but not Th2 cells (Okamoto et al., 2004). However, no mechanism explaining this was established and the phenotype was not confirmed *in vivo*.

2.6 THE ROLE OF ICOS IN THE DIFFERENTATION AND FUNCTION OF OTHER T CELL SUBSETS

So far, I have discussed in depth the role of ICOS in the development and function of Tfh cells. However, data suggests that ICOS promotes all CD4 T cell responses and that the particular involvement of ICOS signaling depends on the immunization/infection model (Simpson et al., 2010b). For example, $ICOS^{-/-}$ mice are incapable of effectively controlling viral and worm infections, owing to impaired Th1 and Th2 response respectively (Kopf et al., 2000). Likewise, defects in ICOS signaling have been reported to affect Th17 cell development (Bauquet et al., 2009; Gao et al., 2012) and Treg cells homeostasis (Marks et al., 2007; Kornete et al., 2012; Lischke et al., 2012) as well.

2.6.1 Th1 AND Th2 CELLS

The decreased numbers of Th2 cells in mice with ICOSL-deficient APCs has validated a role for ICOS signaling in the positive regulation of Th2 cell differentiation (Nurieva et al., 2003a; Vieira et al., 2004). Furthermore, Th2 cells express higher levels of ICOS than Th1 cells (Coyle et al., 2000; Arimura et al., 2002). This regulation occurs not only through induction of c-Maf and IL-4, but also by increasing the expression of the IL-4R (Nurieva et al., 2003a; Watanabe et al., 2005b). It should be noted, however, that there are contradicting studies proposing that ICOS signaling does not promote Th2 cell differentiation, but rather promotes the expansion of differentiated Th2 cells (Gonzalo et al., 2001; Tesciuba et al., 2001). Finally, ICOS ablation dramatically reduces the development of Th2-mediated airway inflammation in an autoimmune mouse model (Clay et al., 2009). On the other hand, ICOS does not appear to be necessary in Th1 cell differentiation as these cells can develop in the absence of ICOS signaling (Simpson et al., 2010b). However, ICOS may positively regulate the effector function of Th1 cells by inducing the production of IFN-γ, such that the deletion of ICOS in NOD mice led to decreased levels of IFN-γ and lower levels of insulitis and hyperglycemia (Hawiger et al., 2008)

2.6.2 Th17 CELLS

Studies characterizing the role of ICOS in Th17 cell development are conflicting. We have shown that the anti-chlamydial Th17 response is controlled by ICOS partially through PI3K (Gao et al., 2012). Our *in vitro* experiments indicated that ICOS promotes both initial differentiation and

IL-23-mediated expansion of Th17 cells. However, another study showed that ICOS is required at the stage of IL-23-dependent expansion of Th17 cells, but not their initial differentiation (Bauquet et al., 2009). Likewise, injection of anti-ICOS blocking monoclonal antibodies during the effector phase of experimental autoimmune encephalomyelitis (EAE), which is strongly influenced by Th17 cells, abrogates the disease (Rottman et al., 2001). However, blocking ICOS during Th17 cell priming has no effect. More studies are therefore needed to clarify the role of ICOS in Th17 development and function.

2.6.3 Treg CELLS

In addition to its function in regulating effector T cell subsets, ICOS may also be required to control tolerance through the homeostasis of Treg cells. $ICOS^{-/-}$ mice were found to have a reduction in the number of $CD4^{+}Foxp3^{+}$ Treg cells in varying autoimmune mouse models (Gotsman et al., 2006; Burmeister et al., 2008; Guo et al., 2008; Kornete et al., 2012). While it has been shown that CD28 is crucial for initial Treg development and homeostasis (Bour-Jordan et al., 2004; Tai et al., 2005), data suggests that ICOS plays a more important role in Treg cell homeostasis. This could be a result of poor proliferation and survival as ICOS costimulation expands Treg cell number *in vitro* and promotes their survival (Ito et al., 2008). Studies in $ICOS^{-/-}$ mice have revealed that ICOS costimulation is not necessary for the *de novo* peripheral conversion of Treg cells (Guo et al., 2008). Furthermore, ICOS can promote the secretion of IL-10 by Treg cells in a model of airway inflammation, demonstrating that ICOS also plays a role in the function of Treg cells.

2.6.8 CD8 T CELLS

Although most studies have focused on the role of ICOS in CD4 T cells, ICOS also plays a role in CD8 cytotoxic T lymphocytes (CTLs). Early studies showed that ectopic expression of B7RP-1 in tumor cells increased tumor rejection by CD8 T cells in the absence of CD4 T cells (Liu et al., 2001; Wallin et al., 2001). Interestingly, while ICOS costimulation efficiently increased naïve T cell priming by tumor cells, it was surprisingly more effective at mobilizing a recall response in adoptive transfer of rested CD8 T cells that originated from mice that had previously rejected tumor cells (Wallin et al., 2001). Therefore, ICOS markedly augments the CD8 T cell recall response.

Later studies also revealed a role for ICOS in CTL-mediated response against intracellular bacterium. For example, anti-ICOS blockade is reported to decrease the CTL response against *Listeria monocytogenes* (Mittrucker et al., 2002; Miyahira et al., 2003), while $ICOS^{-/-}$ mice exhibit a dramatically reduced ability to regulate bacterial clearance during *Salmonella enterica* infection, at least in part through a direct impact on CD8 T cells (Vidric et al., 2006). However, this may not be due to initial expansion, as influenza-specific CD8 T cells expanded normally in $ICOS^{-/-}$ mice in response to influenza infection (Vidric et al., 2005). These studies suggest that ICOS is indispensable for CD8 T cell responses, primarily during the effector phase.

We and others have recently shown that ICOS is essential in inducing acute graft-versus-host disease (GVHD) in murine models of allogeneic bone marrow transplantation, which is mediated by both CD4 and CD8 T cells (Shlomchik, 2007). However, we have recently shown that CD8 T cells are able to induce GVHD utilizing a PI3K-independent ICOS signaling mechanism, whereas CD4 T cells did so in a PI3K-dependent ICOS signaling manner (Li et al., 2013). This suggests that ICOS signaling may depend more on Ca^{2+} signaling in CD8 T cells as opposed to CD4 T cells.

3. THE ROLE OF PI3K IN T CELLS

Few enzymes that specialize in signal transduction are involved in such a large array of functions, cell types and species as PI3K. In cells, PI3K signaling can regulate growth, metabolism, activation, proliferation and migration, and its signaling is indispensable for the proper functioning of almost all cell types. Furthermore, PI3K is highly conserved from yeast to humans, further proof of its importance (Foukas and Withers, 2010). The function of PI3K is to phosphorylate the 3' hydroxyl group of the larger inositol group of the phosphatidylinositide (PtdIns) lipid, which has a broad range of repercussion for the cell. There are three classes of PI3K (class I, II and III) and each is composed of a regulatory subunit and a catalytic subunit. Each class is differentiated from the other by their structure, function, ability to bind other regulatory protein, tissue expression or a combination of these (Yuan and Cantley, 2010). However, the most widely studied of these classes of PI3K is by far the class I PI3Ks, of which there are two types: class IA PI3Ks, which bind to phosphotyrosine-based motifs, and class IB PI3Ks, which bind to the βγ subunits of G protein coupled receptors (GPCRs).

PI3K is too large of a topic to be covered in its entirety in this report, and even PI3K signaling is dauntingly complex and difficult to summarize comprehensively. For example, the kinase AKT (also known as PKB), which is just one of the many downstream mediators of PI3K, has over 100 different substrates (Manning and Cantley, 2007). Therefore, in this section I will cover class IA PI3Ks (due to their ability to bind to ICOS) in the context of T cell-mediated immunity with a strong emphasis on the effects of the costimulatory receptors ICOS and CD28 on this signaling cascade.

3.1 PI3K SIGNAL TRANSDUCTION

Mammalian cells are chaotic environments containing approximately 10^{10} individual proteins that are more or less randomly diffused within a 15 μm^3 space (Yuan and Cantley, 2010). The ability to transmit an orderly signal in that chaos is key to providing directionality as well as signals for growth and survival. One of the most valuable enzymes that has the ability to impose such order is PI3K, which is why it is so highly conserved from yeast to mammals.

There are three core properties that allow PI3K to carry out this orderly signal. First, its lipid substrates, phosphoinositide-(4)-phosphate (PI(4)P) and PI(4,5)-P_2, are highly specific but extremely low in abundance. While lipids compose only 4% of the total cell mass, only 5% of total lipids are PtdIns and of those, less than 1% are phosphorylated (Mulgrew-Nesbitt et al., 2006). This ensures that PI3K signaling is deliberate, dynamic, non-promiscuous and exquisitely localized. Second, PI3K generates membrane-anchored products, specifically PI(3,4,5)P_3, that nucleate protein signaling complexes. While cytoplasmic products can diffuse relatively quickly, membrane-bound products diffuse more slowly and this allows for greater directionality of the signal (Gassama-Diagne et al., 2006). Finally, PI3K has the ability to physically associate itself with membrane-bound proteins that can sense extracellular stimuli, specifically phosphotyrosine-based motifs and GPCRs.

PtdIns have polar and non-polar regions, making them amphiphiles that can imbed themselves into the lipid bilayer. They consist of a glycerol backbone, two non-polar fatty acid tails and a phosphate group substituted with an inositol polar head group (Milne et al., 2005). PtdIns can be phosphorylated at position 3', 4' and 5' of the inositol group and all seven variations (three mono-phosphate, three bi-phosphate, one tri-phosphate) have been found in animals (Parry et al., 2007). After activation, PI3K phosphorylates PI(4,5)P_2 at position 3' to produce PI(3,4,5)P_3 at the cell membrane. This triphosphate form will recruit pleckstrin homology (PH) domain-containing proteins, such as phosphoinositide-dependent kinase (PDK)-1 and AKT, to the membrane and the conformational changes that are induced upon docking promote the activation of most of these proteins (Fayard et al., 2010). This triggers a complex network of signaling cascades, which generally tend to positively regulate cellular growth, proliferation and differentiation. The binding of a receptor to its ligand will often lead to the phosphorylation of a tyrosine in its cytoplasmic tail. In the case of class IA PI3Ks, the SH2-containing regulatory subunit will bind to this phosphorylated tyrosine and therefore localize to the plasma membrane, allowing the PI3K catalytic subunit to produce PI(3,4,5)P_3, inducing the signaling cascade.

Finally, a targeted mass spectrometric analysis identified eight different PI(3,4,5)P_3 forms based on their lipid chains (Milne et al., 2005). This has led researchers to speculate about a possible extra layer of modulation based on fatty acid chains, possibly explaining different outcomes of PI3K signaling during stimulation, as is the case with CD28 and ICOS (Parry et al.,

2007). However, this remains to be tested experimentally and more studies are needed to address this.

3.2 SUBUNITS

Class IA PI3Ks are composed of a regulatory subunit (p85α, p85β, p55α, p55γ or p50α) and a catalytic subunit (p110α, p110β or p110δ). The full-length regulatory p85 subunits p85α and p85β consist of a SH3 domain, a Rac-binding domain flanked by two proline-rich domains and two SH2 domains linked by the inter-SH2 domain. The shorter regulatory subunits p55γ and the p85α-splice variants p55α and p50α lack the SH3 domain, one of the proline-rich domains and the Rac-binding domain. On the other hand, the catalytic p110 subunits contain an adapter binding domain, which binds to the iSH2 domain of p85, a Ras-binding domain (RBD), a C2 domain, a helical domain and a kinase domain. Our understanding of the complex interactions between the regulatory subunits and the catalytic subunits is based on multiple biochemical and crystallographic studies (Backer, 2010). In short, the regulatory subunits both stabilize and inhibit the catalytic subunits, where binding of the regulatory subunit to a phosphotyrosine releases its inhibitory activity on the catalytic subunit without actually releasing the catalytic subunit. In fact, binding of both types of subunits is incredibly tight and is irreversible under cellular conditions (Backer, 2010). This ensures that monomers of the catalytic subunits are degraded in the absence of the regulatory subunits, preventing unregulated signaling.

In the context of the immune system, some subunits play essential roles in mediating PI3K signaling. First, it should be noted that one particular catalytic subunit, p110δ, is highly enriched in lymphoid cells and appears to be the main mediator of TCR-induced PI3K signaling (Chantry et al., 1997; Vanhaesebroeck et al., 1997; Okkenhaug et al., 2002). One study showed that mice with a defective p110δ subunit had defects in GC formation (Okkenhaug et al., 2002). However, the authors of the study were unable to distinguish whether this was attributable to defects in B cells, T cells or both. Furthermore, it was recently discovered that ICOS, but not CD28, recruits the regulatory subunit p50α (Fos et al., 2008). p50α is a more potent inducer of PI(3,4,5)P3 than p85α (Inukai et al., 1997; Inukai et al., 2001) and may explain why ICOS induces a stronger PI3K signal than does CD28. Although it is likely that many PI3K subunits are crucial in the immune response, it appears that p110δ and p50α may have a predominant role during a T cell-dependent response.

3.3 AKT SIGNALING

AKT is a PH domain-containing serine/threonine kinase that is one of the best-characterized downstream mediator of PI3K signaling. AKT substrates are involved in a wide range of cellular and physiological processes including cell cycle progression, cell growth, cell differentiation, cell survival, metabolism, angiogenesis and motility (Manning and Cantley, 2007; Fayard et al., 2010). The full activation of AKT requires its phosphorylation at two sites; both of which are dependent upon the binding of AKT to $PI(3,4,5)P_3$ at the plasma membrane via its PH domain. The effects of this recruitment are two-fold. First, the binding of AKT to $PI(3,4,5)P_3$ via the PH domain induces a major conformational change which facilitates AKT phosphorylation (Calleja et al., 2003; Milburn et al., 2003). Second, $PI(3,4,5)P_3$ binding allows for the co-localization of AKT and PDK-1, inducing the phosphorylation of the catalytic domain of AKT by PDK-1 at position T308, one of the key residues for AKT activation (Alessi et al., 1997). However, for AKT to be fully activated, it must be phosphorylated by mammalian target of rapamycin complex 2 (mTORC2) at a second key residue, S473, located in the hydrophobic motif within its regulatory domain (Hresko and Mueckler, 2005; Jacinto et al., 2006; Facchinetti et al., 2008; Ikenoue et al., 2008). Although it is known that this second step is $PI(3,4,5)P_3$-dependent, it is unclear how mTORC2 is recruited to the plasma membrane as it does not have a PH domain (Gan et al., 2011).

There are three AKT isoforms in mammals: AKT1, AKT2 and AKT3, and each is encoded by a separate gene. However, they share more than 80% amino acid sequence identity and have a comparable structural organization (Fayard et al., 2010). While AKT1 is ubiquitously expressed, AKT2 is mainly expressed in insulin-sensitive tissues and AKT3 is only expressed in the brain and testes (Franke, 2008). T cell express both AKT1 and AKT2 (Fayard et al., 2010) and evidence suggests that they compensate eachother.

The effects of AKT activation are broad and are responsible for much of the complexity of PI3K signaling. AKT has approximately 100 non-redundant substrates, of which ~75% have a RxRxx[S/T]B consensus sequence (where x is any amino acid and B is a bulky hydrophobic residue) (Manning and Cantley, 2007). Through both phosphorylation and the varying nature of its substrates, AKT can both activate and inhibit the functions of its targets. However, the net effect is usually a “positive” one and correlates with an activating phenotype. In all, the phosphorylation

of AKT substrates positively regulates cellular metabolism, survival, growth, mobility and proliferation, as well as glucose uptake and angiogenesis (Manning and Cantley, 2007; Fayard et al., 2010). For example, AKT can phosphorylate and inhibit the tuberous sclerosis complex 2 (TSC2), which in itself is a negative regulator of mTORC1. This has the effect of initiating cap-dependent translation, promoting cell growth and proliferation (Powell et al., 2012), as I will discuss in great detail in *Section 4 - Regulation of cap-dependent translation*.

3.4 NEGATIVE REGULATION OF PI3K ACTIVITY BY PTEN AND SHIP

PI3K signaling induces a potent cascade leading to the activation of a large network of downstream pathways. Therefore, cellular levels of $PI(3,4,5)P_3$ must be tightly regulated to prevent unrestrained activation. Importantly, uncontrolled PI3K activity has been observed to induce malignancies and autoimmunity in multiple mouse models (Liang and Slingerland, 2003). At least two lipid phosphatases are reported to dephosphorylate $PI(3,4,5)P_3$ *in vivo*: phosphatase and tensin homologue (PTEN) and SH2-domain-containing inositol polyphosphate 5-phosphatase (SHIP) (Parry et al., 2007).

PTEN dephosphorylates $PI(3,4,5)P_3$ at position 3' to make the inactive form $PI(4,5)P_2$, directly counteracting the effect of PI3K. Furthermore, experiments in Jurkat cells strongly suggest that PTEN is important to keep basal levels of $PI(3,4,5)P_3$ down in response to the low but constitutive PI3K activity (Edmunds et al., 1999). In T cells, PTEN allows for the discrimination between transient and persistent TCR stimulation, as well as TCR stimulation in the presence or absence of costimulation. A noteworthy study showed that in PTEN-deficient mice, TCR stimulation alone is sufficient to induce hyperactivation of the PI3K pathway and enhance IL-2 production (Buckler et al., 2006). In other words, PTEN imposes a threshold for TCR stimulation. On the other hand, the second $PI(3,4,5)P_3$ phosphatase SHIP dephosphorylates $PI(3,4,5)P_3$ at position 5' to make $PI(3,4)P_2$. Interestingly, this form is still biologically active and can recruit AKT as well several other PH-containing proteins (Parry et al., 2007). An acute or persistent activation of PI3K will have a different impact on the level of available $PI(3,4,5)P_3$ available to PTEN. As a result, SHIP will produce diverse amounts of $PI(3,4)P_2$, which will recruit different sets of mediators than $PI(3,4,5)P_3$ and induce a different response. This may explain how different receptors, notably CD28 and ICOS, can induce a rather distinctive set of responses upon

costimulation. Furthermore, unlike PTEN, which binds to the membrane through its C2 domain, SHIP is recruited to the membrane by binding ITIM motifs on the cytoplasmic tail of receptors, and must be phosphorylated to become activated (Edmunds et al., 1999; Leslie and Downes, 2002; Parry et al., 2007). This suggests that SHIP has a particular role in shaping the effect of PI3K activity at the immunological synapse. Finally, CD28 costimulation is known to activate SHIP (Edmunds et al., 1999), which may explain the different effects on PI3K signaling in response to CD28 and ICOS costimulation.

4. REGULATION OF CAP-DEPENDENT TRANSLATION

The notion that an increase in a specific mRNA leads to an equal increase of the respective protein, has been challenged in recent years. Comparative genomics versus proteomics studies have indicated that mRNA levels are a poor indicator of protein levels in eukaryotic cells (Gygi et al., 1999; Ideker et al., 2001). Eukaryotic cells and tissues require that each protein be expressed at the right abundance, time and location, in order to function properly. Some cellular events depend on an immediate increase in protein concentration in response to a specific stimulus. Therefore, to depend solely on transcription would be slow and inefficient. This quick regulation of protein levels is thus largely achieved through the translational activity of ribosomes. The regulation of mRNA translation allows for a more direct and rapid regulation of protein expression. Although the regulation of mRNA levels through transcription (or mRNA degradation) is clearly an important contributor to protein synthesis, the regulation of mRNA translation is at least as important as transcription.

The translation of mRNA is divided into three stages: initiation, elongation and termination. Each stage requires its own set of conditions and factors, and each can be regulated to control levels of protein synthesis. However, the main rate-limiting step in the process of translation is the initiation stage (Sonenberg and Hinnebusch, 2009; Livingstone et al., 2010). This stage is controlled by a large family of eukaryotic translation initiation factors (eIFs) which bind mRNA directly, predominantly through the direct or indirect binding of the 5',7-methyl guanosine (5') cap structure. Upstream of eIFs is mTORC1, which acts as a central node and sensor of multiple intracellular pathway stimuli. Many growth factors or hormone receptors, as well as changes in energy and amino acid levels, will regulate translation through mTORC1.

In this section, I will introduce the subject of the regulation of cap-dependent translation with a strong focus on the initiation step. I will also discuss the known roles of mTOR in the immune system and the importance of ICOS in mediating translation in activated CD4 T cells, an area of research that has as-of-yet not been thoroughly explored.

4.1 mTOR AS A CENTRAL SENSORY NODE OF CAP-DEPENDENT TRANSLATION

mTOR is a serine-threonine kinase belonging to the PI3K-related kinase family. It nucleates at least two distinct multi-protein complexes: mTORC1 and mTORC2. While mTORC2 is believed to play an important role in cytoskeletal rearrangement, mTORC1 is required for autophagy, microtubule organization, mitochondrial metabolism and biogenesis, lipid synthesis and metabolism (Wullschleger et al., 2006). Importantly, mTORC1 is also the main regulator of cap-dependent translation (Laplante and Sabatini, 2009). Multiple signaling pathways converge on mTORC1 to regulate its activity, including cytoplasmic concentrations of glucose and amino acids, WNT signaling, tyrosine kinase receptors and TNF receptors. On the other hand, cellular stress, as well as decreased levels of ATP and oxygen can all inhibit mTORC1 activity. Although each of these pathways are characterized to various extents, they are extremely complex and beyond the scope of my thesis. I will therefore focus on the role of mTOR in mediating cap-dependent translation and concentrate strictly on the pathway activated by the PI3K pathway.

PI3K is known to be potent activator of cap-dependent translation. It achieves this through a major sensory node upstream of mTORC1, tuberous sclerosis complex (TSC). TSC functions as a GTPase-activating protein (GAP) for the small GTPase Rheb (Ras homolog enriched in brain). The active GTP-bound form of Rheb directly interacts with mTORC1 to stimulate its activity (Long et al., 2005; Sancak et al., 2007). TSC negatively regulates mTORC1 activity by converting Rheb into its inactive form. However, the PI3K substrate AKT can phosphorylate and inhibit the activity of TSC (Fayard et al., 2010). Furthermore, the kinase PDK-1, which is also immediately downstream of PI3K, can also directly phosphorylate and activate S6 kinase (S6K), a key mediator of cap-dependent translation (Fenton and Gout, 2011) (Figure 3).

Once active, mTORC1 phosphorylates and activates S6K as well as phosphorylates and inhibits eIF4E binding proteins (4E-BP) 1, 2 and 3. Both 4E-BPs and S6K induce the initiation of cap-dependent translation through different mechanisms (Chung et al., 1992; Jefferies et al., 1994; Lin et al., 1994; Pause et al., 1994) and induce the translation of different sets of mRNA species. Knock-down experiments in mouse embryonic fibroblasts have shown that S6K regulates cell growth but is dispensable for proliferation, while 4E-BPs acts on proliferation but has no effect on cell growth (Dowling et al., 2010).

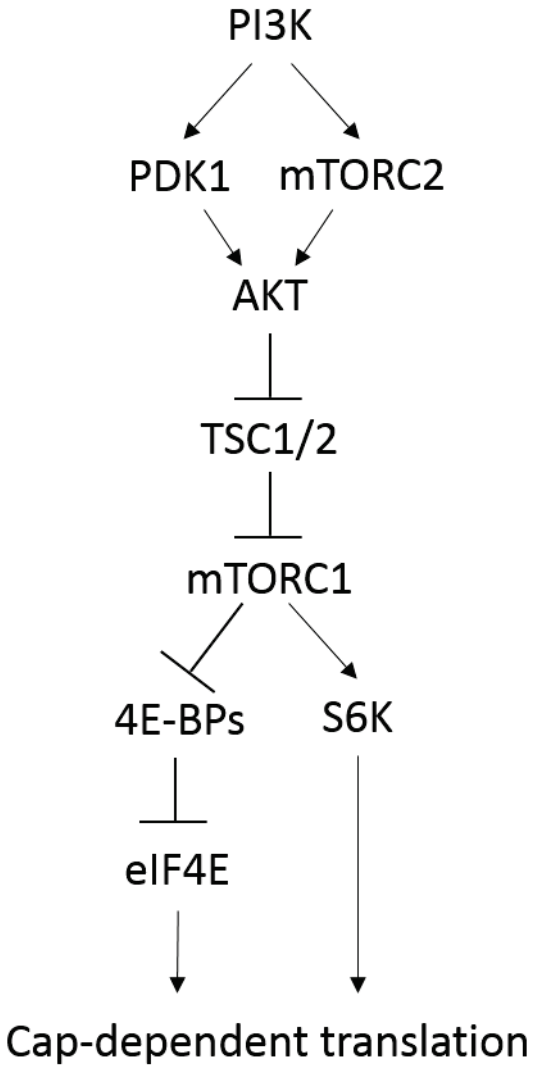

Figure 3. PI3K/AKT/mTORC1 pathway leads to cap-dependent translation. An overview of PI3K downstream pathways that regulate cap-dependent translation.

4.1.1 TRANSLATION INITIATION, eIFs AND 4E-BP

Translation initiation in eukaryotes is a complex and highly regulated process involving at least 12 protein factors composed of 24 distinct polypeptides (Jackson et al., 2010; Hinnebusch, 2011; Aitken and Lorsch, 2012). The ultimate goal of the translation initiation stage is to assemble the large (60S) and the small (40S) ribosomal subunits into an elongation-competent (80S) ribosome with the initiating methionyl(Met)-tRNA over the start codon (AUG) of the mRNA transcript.

Initiation starts with the formation of the ternary complex (TC) of Met-tRNA, eIF2 and GTP, which assembles with the small 40S ribosomal subunit eIFs 1, 1A, 3 and 5 to form the 43S pre-initiation complex (PIC) (Figure 4). Concurrently, eIF4E binds the 5' cap structure and the large scaffolding protein eIF4G recruits the RNA helicase eIF4A to the 5' cap by directly binding eIF4E (Hinnebusch and Lorsch, 2012). Amongst the eIFs, eIF4E is the least abundant and is thought to be limiting for translation initiation (Duncan et al., 1987). Together, eIF 4E, 4G and 4A compose the multi-protein complex known as eIF4F. It should also be noted that 4E-BPs negatively regulates translation initiation at this stage by inhibiting the binding and recruitment of eIF4G to eIF4E, but I will discuss this later. eIF4G also binds the poly-A binding protein (PABP) which as the name implies, binds to the poly-A tail of the mRNA. This induces the "closed-loop" structure necessary for a stable, circular messenger ribonucleoprotein (mRNP). Importantly, the helicase eIF4A unwinds the secondary structure of the 5' UTR to create a single-stranded landing pad for the PIC, which is recruited through eIF4G binding directly to eIF3 (Hinnebusch, 2011; Aitken and Lorsch, 2012; Hinnebusch and Lorsch, 2012).

The PIC scans the mRNA leader for an AUG start codon once it has been recruited to a linearized 5' cap structure. Base pairing of the Met-tRNA to AUG will arrest the PIC at the start codon, where GTP-eIF2 in the TC is converted to its GDP-bound state as a result of the GTPase activity of eIF5. Following the release of GDP-eIF2 and several other eIFs, the joining of the 60S is catalyzed by eIF5B to produce a functional 80S ribosome, which begins the elongation phase of protein synthesis. The reactivation of GDP-eIF2 to its active GTP-eIF2 form by eIF2B is regulated by eIF2α kinase. Cellular stress activates eIF2α kinase, which phosphorylates eIF2 to inhibit its activation by eIF2B, depleting the pool of functional TC for further translation initiation (Gomez et al., 2002).

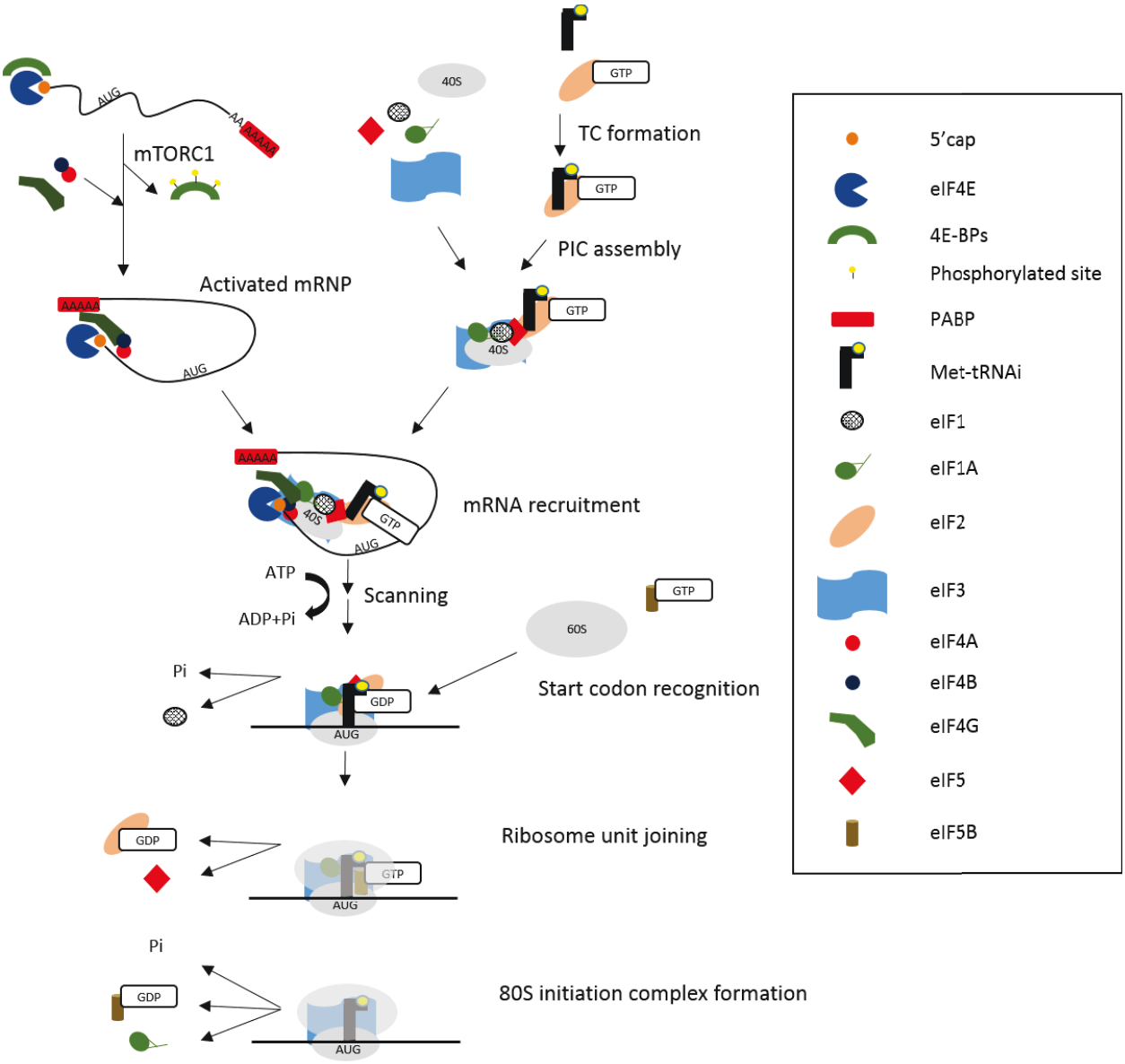

Figure 4. Schematic of translation initiation in eukaryotes. Translation begins with the formation of the ternary complex (TC), which is recruited to the 40S ribosomal subunit with the help of eIFs 1, A1, 3 and 5 to form the pre-initiation complex (PIC). Meanwhile, phosphorylation of 4E-BPs by mTORC1 allows for the recruitment of eIFs 4A, 4B and 4G to eIF4E (together making eIF4F). PABP is then recruited to eIF4G, allowing for a stable circular mRNP to form. This induces the recruitment of the PIC to the mRNP and the helicase eIF4A linearizes secondary structures in the 5' UTR. The PIC then begins scanning for the start codon (AUG). Start codon recognition triggers the release of eIF1 and the conversion of eIF2-GTP to eIF2-GDP, arresting the scanning process. eIF2-GDP and eIF5 are then release, allowing for the recruitment of the 60S ribosomal subunit, mediated by eIF5B. GTP hydrolysis by eIF5B induces the joining of the 40S and 60S subunit to create the 80S initiation complex. (Adapted from Aitkin *et al*. 2012)

Although there exist many ways to control the initiation of cap-dependent translation, one of the main mechanisms is through 4E-BP phosphorylation. When hypophosphorylated, 4E-BPs binds eIF4E with high affinity and prevents the recruitment of eIF4G. However, 4E-BPs can be directly hyperphophorylated by mTORC1, which prevents 4E-BPs from binding eIF4E, allowing for the recruitment of eIF4G and the PIC to the 5' cap structure.

To summarize, under non-stimulatory conditions, eIF4E binds the 5' cap of mRNA, but the additional binding of 4E-BPs prevents any further activity. However, under stimulatory conditions, mTORC1 hyperphosphorylates 4E-BPs and it is thus released from eIF4E. This allows the scaffolding protein eIF4G to be recruited to the 5' cap, and by virtue of its binding sites for PABP, eIF4A and eIF5, allow for the circulization and stabilization of the mRNP, the linearization of the mRNA 5' UTR, and recruitment of the PIC to the 5' cap structure, precipitating PIC scanning and the full 80S ribosome assembly at the start codon.

4.1.2 S6K

S6K is a serine/threonine kinase whose name is derived from its ability to phosphorylate the ribosomal protein S6 (rpS6). The inducible phosphorylation of rpS6, a component of the 40S ribosome subunit, is a universal response to mitogens in eukaryotes, and is often associated with mRNA translation. S6K can also modulate the activity of a host of other proteins which in turn can regulate cytoskeletal rearrangement, proliferation, splicing, and cell survival, as well as its own regulation through negative feedback (Fenton and Gout, 2011).

The activity of S6K is controlled by its phosphorylation state, where increased phosphorylation correlates with increased activity. In activating conditions, S6K is directly phosphorylated by PDK-1 at position T229 in its activation loop, and this is only possible after its C-terminal regulatory domain has been phosphorylated. The kinase responsible for this phosphorylation remains unknown, but several proline-directed kinases, including ERK1/2, JNK1/2 and CDK1, have been proposed (Mukhopadhyay et al., 1992; Fenton and Gout, 2011). S6K activation is, however, associated with the phosphorylation of T389, which is directly mediated by mTORC1 (Weng et al., 1998).

Most of what we know regarding S6K comes from genetic studies in *C. elegans*, *Drosophila* and mice. Genetic deletion of *Drosophila* S6K results in death in most flies at the larval stage, and the ones that survive are much smaller than wild type flies. Interestingly, this was attributed to a reduction in the size of individual cells rather than a reduction in the number of cells (Montagne et al., 1999). Genetic studies in mice have also produced the same small-size phenotype (Kawasome et al., 1998; Shima et al., 1998). However, ablation of *dPI3K* and *dAKT* in flies also produces a small-size phenotype, accompanied by a reduction in total cell numbers (Weinkove and Leevers, 2000). This suggested that the bifurcation between cell growth and proliferation was downstream of PI3K-AKT. Finally, in a study published by Nahum Sonenberg and colleagues, knockdown experiments in mouse embryonic fibroblasts (MEFs) showed that 4E-BPs controlled proliferation and that S6K controlled cell growth (Dowling et al., 2010). Therefore, it appears that in higher organisms, 4E-BPs and S6K have non-overlapping functions.

Mechanistically, S6K controls translation by phosphorylating the five C-terminal serine residues of rpS6 in a sequential manner (Ferrari et al., 1991). S6K can also regulate translation initiation by phosphorylating and activating the cap-binding complex component eIF4B (Raught et al., 2004) and by phosphorylating the negative regulator of eIF4A, Pdcd4, leading it to its degradation (Dorrello et al., 2006). Furthermore, S6K can also regulate translation elongation by phosphorylating and inhibiting eEF2K (Wang et al., 2001). However, despite a good deal of knowledge on the mechanisms, it is still unclear exactly how S6K regulates a set of mRNA species that specifically regulate cell growth.

4.2 mTORC1-SPECIFIC TRANSLATOME

Despite a wealth of studies detailing the molecular mechanisms of mTORC1-mediated cap-dependent translation, knowledge of the actual overall translational program is surprisingly poorly defined. mTOR was discovered after an effort by pharmaceutical companies to find new antibiotics. The search yielded the compound called rapamycin, which was extracted from the *Streptomyces hygroscopicus* bacterium from the soils of the island of Rapa Nui (Vezina et al., 1975). It proved to be a poor antibiotic, but it was discovered to suppress the growth of yeast and had immunosuppressive and antitumor properties (Sabatini, 2006). mTOR was subsequently shown to be the inhibitory allosteric target of rapamycin and was named accordingly (Schmelzle and Hall, 2000). Importantly, many of the inhibitory studies regarding mTORC1-sensitive

translation targets from the past 30 years have been performed using rapamycin. However, recent studies have shown that rapamycin only partially inhibits mTORC1 activity (Thoreen et al., 2009; Hsieh et al., 2012), confounding much of the findings thus far. Furthermore, one could predict that eliminating 4E-BP1/2 would lead to unregulated protein synthesis and embryonic lethality. However, 4E-BP1/2-DKO mice (4E-BP2 has been shown to compensate for lack of 4E-BP1 (Sonenberg and Hinnebusch, 2009)) are not visibly different from wild type mice, although they have been shown to have increased sensitivity to diet-induced obesity (Le Bacquer et al., 2007), increased resistance to vesicular stomatitis virus (VSV) infection (Colina et al., 2008) and *Leishmania* infection (Jaramillo et al., 2011), and a higher tendency to develop autistic behavior (Gkogkas et al., 2013). This suggests that only a selected few mRNA transcripts are controlled through mTORC1 and 4E-BP1/2.

The newly discovered small molecule ATP-competitive inhibitor of mTOR Torin1 was discovered to be a better inhibitor of both mTORC1 and mTORC2 (Thoreen et al., 2009). In a study where MEFs were cultured with Torin1 and translational profiling was assessed by a new technique called ribosome footprinting (RF), a deep-sequencing-based assay of ribosome-protected mRNA transcripts which ultimately measures translational activity, global translational activity was only partially affected. However, around 200 mRNA transcripts were particularly sensitive to mTOR inhibition in MEFs and of these, the vast majority were highly enriched for pyrimidines near the 5' cap, or contained a 5' terminal oligopyrimidine (TOP) motif, a sequence of 4-14 uninterrupted pyrimidines starting immediately after the 5' cap (Thoreen et al., 2012). TOP mRNAs tend to code for proteins associated with translation (Meyuhas, 2000; Iadevaia et al., 2008). The study also showed that these mRNAs, whose translation was repressed when mTORC1 was inactive, were being repressed in a 4E-BP-dependent manner.

Another study, also using RF analysis, showed that two other ATP-competitive inhibitors of mTOR, PP242 and INK128, could selectively repress approximately 150 target mRNA in PC3 human prostate cancer cells, where mTOR is constitutively hyperactive (Hsieh et al., 2012). Importantly, 90% of mTOR-responsive mRNAs possess TOP motifs and/or pyrimidine-rich translational elements (PRTEs), which consist of an invariant uridine at position 6 after the 5' cap flanked by pyrimidines. Notably, knockdown experiments showed that PRTE-mediated translation

repression was 4E-BP-dependent. In PC3 cells, the mTORC1 target mRNAs encoded for pro-invasion proteins associated with metastasis.

Finally, it had previously been proposed that the length of the 5' UTR played an important regulatory role in translation initiation (Hay and Sonenberg, 2004; Livingstone et al., 2010). mRNAs encoding for the proteins cyclinD1, ODC, VEGF, IRF7 and IκBα have all been demonstrated to possess an unusually long 5'UTR which folds into a highly stable secondary structure that prevents translation initiation (Colina et al., 2008; Livingstone et al., 2010; Herdy et al., 2012). Elegant biochemical experiments have shown that the unwinding of the secondary structure and subsequent translation initiation was dependent on the recruitment of the RNA helicase eIF4A in a 4E-BP1-dependent manner (Livingstone et al., 2010). However, both of the previously mentioned mTOR inhibitor studies have revealed through global deep-sequencing RF analysis that the length of the 5' UTR does not correlate in any way with mTOR mRNA targets (Hsieh et al., 2012; Thoreen et al., 2012). Therefore, there appears to exist two broad mechanisms to negatively regulate mRNA translation of mTOR target mRNAs: 1) pyrimidine-rich sequences at or near the 5' cap structure and 2) having a long 5'UTR whose secondary structure has high thermal stability.

4.2.1 MECHANISMS OF TOP/PRTE mRNA TRANSLATION

Although the existence of mTOR target mRNAs with "polypyrimidine" sequences near the 5' cap structure has been known for nearly two decades (Jefferies et al., 1994), the mechanisms by which they are regulated are still poorly understood. It was initially reported that the translation of these mRNAs, later termed TOP mRNAs, was regulated through S6K (Jefferies et al., 1997). However, this was later proven to be inaccurate, as S6K activity and TOP mRNA translation initiation were merely correlative (Tang et al., 2001; Barth-Baus et al., 2002; Meyuhas and Dreazen, 2009). As discussed earlier, the translation of TOP mRNAs was demonstrated to be dependent upon 4E-BPs (Hsieh et al., 2012; Thoreen et al., 2012). Furthermore, other independent chemical inhibitor and knockdown experiments have shown that TOP mRNA translation initiation is decidedly downstream of PI3K and TSC activation (Tang et al., 2001; Patursky-Polischuk et al., 2009). Unfortunately, there is no current mechanistic model for the induction of translation initiation of TOP/PRTE mRNAs by mTOR/4E-BP1. More detailed experiments will be required elucidate this mechanism.

4.3 CAP-DEPENDENT VERSUS CAP-INDEPENDENT TRANSLATION

When discussing mRNA translation in the context of the immune system, it is necessary to first understand that the translation of some mRNA species is dependent upon neither mTOR nor the 5' cap structure. Many of these mRNA transcripts contain an internal ribosome entry site (IRES) element, which was first identified in poliovirus and encephalomyocarditis viruses (both RNA viruses) in the late 1980s (Jang et al., 1988; Pelletier and Sonenberg, 1988). Briefly, IRES elements are a non-coding sequence 5' of the open reading frame (ORF) with secondary and tertiary structures allow ribosomes to assemble and begin protein synthesis independently of the 5' cap structure (Balvay et al., 2007). It should be noted that although IRES-mediated translation is cap-independent and can largely bypass the need for eIFs, it still requires some non-canonical IRES *trans*-activating factors for efficient translation (Sonenberg and Hinnebusch, 2009).

IRES-containing mRNAs are common among viruses, but they have also been reported in eukaryotes, mammals, mice and humans (Mohr and Sonenberg, 2012). In the case of viruses, many have evolved strategies to repress host translation for a variety of reasons, usually to protect themselves against the antiviral response (Mohr and Sonenberg, 2012). However, all viruses still depend upon their host for protein synthesis and therefore, cap-independent translation allows for efficient protein synthesis in the presence of inhibited host initiation factors. In contrast, eukaryotic IRES mRNAs are usually involved in the stress response. A good of example of a mammalian IRES-containing mRNA is the one that encodes for binding immunoglobulin protein (BiP; or heat shock 70 kDa protein 5A (HSP5A)), an ER chaperone protein. ER over-burdening induces a stress response that inhibits translation through eIF2 phosphorylation (Gomez et al., 2002). However, cap-independent translation of BiP through its IRES element increases BiP protein levels, which facilitates protein folding in the ER and therefore alleviates ER burden (Johannes and Sarnow, 1998).

It is important to take into consideration cap-independent translation when discussing translation mechanisms, especially in the case of the immune system. However, my work focuses almost exclusively on cap-dependent translation, I will not further discuss cap-independent translation.

4.4 REGULATING THE IMMUNE RESPONSE THROUGH mTOR

Together with other studies using genetic deletion of components associated specifically with mTORC1 and mTORC2, research on rapamycin has allowed us to further understand the many roles of mTOR in the immune system. Immunosuppression was one of the first observed effects of rapamycin after its discovery (Sabatini, 2006). Initial models suggested that this was due to the inhibitory effect of rapamycin on T cell proliferation, which mTOR was later found to control through the degradation of the cell cycle inhibitor p27 and increased expression of cyclin D3 (Sengupta et al., 2010). Other early observations showed that rapamycin induced T cell anergy and at the time, researchers believed that this was due to the block in proliferation (Schwartz, 2003). However, subsequent experiments illustrated that cell cycle arrest in G1 in the absence of mTOR inhibition did not induce anergy and that inducing proliferation in the presence of rapamycin did not rescue anergy (Colombetti et al., 2006). There was therefore a dissociation between the ability of rapamycin to inhibit proliferation and to induce anergy. In other words, rapamycin induced anergy not by inhibiting proliferation, but by inhibiting mTOR. These studies provided the initial insight in terms of the ability of mTOR to regulate T cell fate. In this section, I will briefly review of the role of mTOR in the immune system with an emphasis on T cells.

mTOR has been reported to play a crucial role in CD4 and CD8 T cells, B cells, macrophages, DCs, monocytes and other cell types, as well as in general innate immunity (Chi, 2012; Mohr and Sonenberg, 2012; Powell et al., 2012). As I have already discussed, genetic deletion of 4E-BP1/2 in mice, a main mediator of mTOR activity, makes them less prone to VSV infection due to an increase in IRF7 translation, which leads to increased levels of IFN-α (Colina et al., 2008). Likewise, plasmacytoid DC produce significantly less IFN-α and IFN-β when treated with LPS in the presence of rapamycin (Weichhart et al., 2008), possibly because they produce less IRF7. Furthermore, mature DCs and monocytes treated with LPS in the presence of rapamycin produce less IL-12, a pro-inflammatory cytokine, while deletion of TSC2 (leading to hyperactive mTORC1) leads to increased production of IL-10, an anti-inflammatory cytokine (Ohtani et al., 2008). In B cells, mTOR plays a crucial role in mediating CD40 signaling, which may explain why mTOR hypomorphic mice have impaired B cell development (Zhang et al., 2011). On the other hand, B cell specific deletion of TSC1, which leads to hyperactive mTORC1, also leads to defects in B cell maturation (Lazorchak et al., 2010). Although the mechanisms behind these

phenotypes are unknown, they suggest that levels of mTORC1 are crucial in regulating many cells of the immune system.

CD4 T cells encounter a multitude of signals during initial T cell activation produced both by other cells and their environment (Chi, 2012). *In vivo*, many of these signals are subtle and often with opposing effect. Likewise, the lineage-specific transcription factors T-bet, GATA3, RORγt and Bcl6 (for Th1, Th2, Th17 and Tfh respectively) can all be upregulated during initial naïve T cell activation (Murphy and Stockinger, 2010). Therefore, T cells must integrate a plurality of signals to derive instruction for effector lineage commitment. Given that mTOR does exactly that, it serves as an elegant mechanism in integrating cues from the immune microenvironment to instruct helper cell differentiation. Interestingly, priming mTOR-deficient T cells leads to normal activation marker expression and IL-2 production, but these cells are unable to differentiate into Th1, Th2 or Th17 cells (Chi, 2012). mTOR is reported to activate many transcription factors associated with T cell differentiation including T-bet, STAT3, STAT4 and STAT6 (Powell et al., 2012). On the other hand, mTOR-deficient T cells spontaneously upregulate Foxp3, even in the absence of exogenous cytokines, promoting Treg differentiation (Delgoffe et al., 2009). The list of roles that mTOR plays in the immune is simply too extensive to be summarized here, but most of the known mechanisms do not actually relate to translation. Only a few studies have looked directly at the role of specific mRNA translation in the immune system and even fewer have done so in CD4 T cells specifically. As translation regulation is such an important layer of the proper functioning of a cell, much more research must be done in the field to further understand the mechanisms of CD4 T cell-mediated immune functions.

4.5 A CASE FOR IL-4

The production of IL-4 by Tfh cells is a critical process in regulating an efficient humoral response (King and Mohrs, 2009). On the other hand, IL-4 protein production is tightly regulated and uncontrolled production of IL-4 can lead to autoimmunity (Ruger et al., 2000; Chan et al., 2001). Interestingly, recent studies have demonstrated that IL-4 protein synthesis is regulated at the level of translation, as well as transcription (Scheu et al., 2006; King and Mohrs, 2009; Reinhardt et al., 2009; Zaretsky et al., 2009; Liang et al., 2011).

Two seminal studies published by the Markus Mohrs group and the Richard Locksley group found that when naïve CD4 T cells were differentiated into Th2 cells *in vivo*, they expressed high levels of IL-4 mRNA, but unexpectedly did not produce any IL-4 protein. However, when the activated T cells were restimulated through the TCR receptor, IL-4 protein synthesis was initiated and correlated with IL-4 mRNA loading onto polysomes (Mohrs et al., 2005; Scheu et al., 2006). In other words, the production of IL-4 mRNA and IL-4 protein were uncoupled. It was also observed that T cell activation induced an integrated stress response (ISR) and therefore repressed translation was attributed to increased eIF2α phosphorylation (Scheu et al., 2006). However, no other component of the translation initiation machinery was assessed.

Most studies addressing the relationship between T cells expressing IL-4 mRNA but not IL-4 protein (IL-4-competent T cells) and T cells actually producing the IL-4 protein (IL-4-producing T cells) were done using IL-4 4get/KN2 dual reporter mice. In these 4get/KN2 mice, one IL-4 allele codes for enhanced green fluorescent protein (eGFP) downstream of an IRES element inserted at the 3' end of *Il4* [IL-4/GFP-enhanced transcript (4get)], allowing for the identification of cells with an actively transcribed IL-4 locus. The other IL-4 allele has been replaced by human CD2 (huCD2) cDNA introduced at the *Il4* start site (KN2), allowing for the identification of IL-4-producing T cells using anti-CD2 antibodies (Riviere et al., 1998; Hu-Li et al., 2001; Mohrs et al., 2001; Mohrs et al., 2005).

In mice infected with the enteric helminth *Heligmosomoides polygyrus (Hp)*, a potent inducer of a Th2 response, the highest concentration of IL-4-competent Th2 cells are found in the liver, peritoneal exudate cells and bronchoalveolar lavage, but the relative number of IL-4-producing cells is strikingly low (Mohrs et al., 2005). However, roughly half of all CD4 T cells found in the mesenteric LNs (mesLNs), PP, intraepithelial lymphocytes and one fifth of CD4 T cells in the lamina propria (LP) are IL-4-producing cells (Mohrs et al., 2005; King and Mohrs, 2009). It should be noted that mesLNs, PPs and even the LP are locations of B cell differentiation into APCs (Kawamoto et al., 2012; Victora and Nussenzweig, 2012). Importantly, immunohistological analyses have shown that among the IL-4-competent CD4 T cells in the mesLNs, all the IL-4-producing cells are found exclusively inside the B cell zones and GCs (King and Mohrs, 2009). Furthermore, these cells also express higher levels of PD-1, ICOS and IL-21, all characteristics associated with Tfh cells.

Other studies using *Leishmania major (L. major)*, serum egg antigen or OVA infection/immunization models similarly found that IL-4-producing CD4 T cells were found exclusively in B cell zones and GCs, and co-expressed Tfh cell markers such as ICOS and PD-1 (Reinhardt et al., 2009; Zaretsky et al., 2009). In one of these studies, the authors infected 4get/KN2 mice with *L. major* and collected the draining LNs, elegantly demonstrating that some of the IL-4-producing CD4 T cells were physically bound to B cells as doublets during FACS sorting. When analyzed by semi-quantitative PCR, these doublets expressed transcripts for IgG1 and AID (Reinhardt et al., 2009). This suggests that 1) B cells are potentially responsible for turning IL-4-competent T cells into IL-4-producing T cells, and that 2) IL-4-producing T cells are functionally capable of inducing B cell differentiation *in vivo*. Of note, blocking ICOS during the course of the immune response inhibited the generation of any IL-4-producing cells, although this may have also prevented the differentiation of IL-4-competent Tfh cells (Reinhardt et al., 2009).

RATIONALE AND OBJECTIVES

The expression of the CD28 homologue ICOS on CD4 T cells is important for the generation of Tfh cells in both humans and mice. However, the molecular mechanisms that lead to the differentiation of Tfh cells upon ICOS costimulation are unclear. A previous study has shown that mice with a defective p110δ PI3K catalytic subunit, which is highly enriched in lymphoid cells, have severe defects in GC formation. However, the authors of the study were unable to determine if this effect was a result of a defect in T cells, B cells or both. While CD28 is able to signal through PI3K, it also signals through Grb2, Gab, Lck and Itk. On the other hand, ICOS is reported to signal exclusively through PI3K. Furthermore, ICOS induces stronger PI3K signaling that CD28. Consistently, CD28-mediated PI3K activation has repeatedly shown to be dispensable for most T cell function *in vivo*. *Therefore, we hypothesize that ICOS-mediated PI3K activation is crucial for the generation of Tfh cells.*

Tfh cells produce high levels of IL-4, a crucial cytokine for B cell growth and differentiation. Previous work by others has shown that the production of IL-4 protein is repressed in activated CD4 T cells, despite high levels of IL-4 mRNA, but can be induced upon TCR stimulation. In other words, IL-4 mRNA transcription and translation are uncoupled. Furthermore, in an *in vivo* response against helminths, the production of IL-4 protein is restricted to Tfh cells inside the B cell follicle. One of the main mediators of cap-dependent translation is mTORC1, which is potently activated upon PI3K signaling. *Since B cells express ICOSL in the context of cognate T-B interaction and ICOS is a potent activator of PI3K signaling, we hypothesize that ICOS-mediated PI3K activation enhances TCR-mediated IL-4 translation through mTORC1 and that this is essential for T cell-mediated B cell help.*

Accordingly, our objectives were to investigate the molecular events of ICOS-mediated PI3K activation in the context of Tfh cell differentiation and function. This could be further subdivided into two specific objectives, the first of which was to:

1. *Determine whether ICOS-mediated PI3K signaling is crucial for Tfh cell differentiation*
    a. Develop a strain of mutant mice which is unable to induce PI3K signaling upon ICOS costimulation (ICOS-YF);
    b. Evaluate the ability of ICOS-YF mice to generate Tfh cells, as well as GC induction;
    c. Determine whether CD28 and ICOS play differential roles in Tfh cell differentiation and function.

Following the discovery that ICOS-mediated PI3K activation was indeed crucial for Tfh cell differentiation, we sought to test the role of ICOS-mediated PI3K activation in the function of Tfh cells. Therefore, our second objective was to:

2. *Determine whether the ICOS-PI3K axis enhanced IL-4 translation, as well as T cell-mediated B cell help*
    a. Confirm that ICOS-mediated PI3K signaling activated the translation machinery at the molecular level;
    b. Determine whether the ICOS-PI3K axis increases IL-4 mRNA translation;
    c. Observe the role of ICOS in T cell-mediated B cell help.

REFERENCES

Aicher, A., Hayden-Ledbetter, M., Brady, W.A., Pezzutto, A., Richter, G., Magaletti, D., Buckwalter, S., Ledbetter, J.A., and Clark, E.A. (2000). Characterization of human inducible costimulator ligand expression and function. J Immunol 164, 4689-4696.

Aitken, C.E., and Lorsch, J.R. (2012). A mechanistic overview of translation initiation in eukaryotes. Nature structural & molecular biology 19, 568-576.

Akbari, O., Stock, P., Meyer, E.H., Freeman, G.J., Sharpe, A.H., Umetsu, D.T., and DeKruyff, R.H. (2008). ICOS/ICOSL interaction is required for CD4+ invariant NKT cell function and homeostatic survival. J Immunol 180, 5448-5456.

Akiba, H., Takeda, K., Kojima, Y., Usui, Y., Harada, N., Yamazaki, T., Ma, J., Tezuka, K., Yagita, H., and Okumura, K. (2005). The role of ICOS in the CXCR5+ follicular B helper T cell maintenance in vivo. J Immunol 175, 2340-2348.

Alessi, D.R., James, S.R., Downes, C.P., Holmes, A.B., Gaffney, P.R., Reese, C.B., and Cohen, P. (1997). Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. Current biology : CB 7, 261-269.

Allen, C.D., Ansel, K.M., Low, C., Lesley, R., Tamamura, H., Fujii, N., and Cyster, J.G. (2004). Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. Nature immunology 5, 943-952.

Ansel, K.M., McHeyzer-Williams, L.J., Ngo, V.N., McHeyzer-Williams, M.G., and Cyster, J.G. (1999). In vivo-activated CD4 T cells upregulate CXC chemokine receptor 5 and reprogram their response to lymphoid chemokines. The Journal of experimental medicine 190, 1123-1134.

Arimura, Y., Kato, H., Dianzani, U., Okamoto, T., Kamekura, S., Buonfiglio, D., Miyoshi-Akiyama, T., Uchiyama, T., and Yagi, J. (2002). A co-stimulatory molecule on activated T cells, H4/ICOS, delivers specific signals in T(h) cells and regulates their responses. International immunology 14, 555-566.

Arimura, Y., Shiroki, F., Kuwahara, S., Kato, H., Dianzani, U., Uchiyama, T., and Yagi, J. (2004). Akt is a neutral amplifier for Th cell differentiation. The Journal of biological chemistry 279, 11408-11416.

Backer, J.M. (2010). The regulation of class IA PI 3-kinases by inter-subunit interactions. Current topics in microbiology and immunology 346, 87-114.

Ballesteros-Tato, A., Leon, B., Graf, B.A., Moquin, A., Adams, P.S., Lund, F.E., and Randall, T.D. (2012). Interleukin-2 Inhibits Germinal Center Formation by Limiting T Follicular Helper Cell Differentiation. Immunity.

Balvay, L., Lopez Lastra, M., Sargueil, B., Darlix, J.L., and Ohlmann, T. (2007). Translational control of retroviruses. Nature reviews. Microbiology 5, 128-140.

Barth-Baus, D., Stratton, C.A., Parrott, L., Myerson, H., Meyuhas, O., Templeton, D.J., Landreth, G.E., and Hensold, J.O. (2002). S6 phosphorylation-independent pathways regulate translation of 5'-terminal oligopyrimidine tract-containing mRNAs in differentiating hematopoietic cells. Nucleic acids research 30, 1919-1928.

Batista, F.D., and Harwood, N.E. (2009). The who, how and where of antigen presentation to B cells. Nature reviews 9, 15-27.

Baumjohann, D., Kageyama, R., Clingan, J.M., Morar, M.M., Patel, S., de Kouchkovsky, D., Bannard, O., Bluestone, J.A., Matloubian, M., Ansel, K.M., and Jeker, L.T. (2013). The microRNA cluster miR-17 approximately 92 promotes TFH cell differentiation and represses subset-inappropriate gene expression. Nature immunology 14, 840-848.

Baumjohann, D., Okada, T., and Ansel, K.M. (2011). Cutting Edge: Distinct waves of BCL6 expression during T follicular helper cell development. J Immunol 187, 2089-2092.

Bauquet, A.T., Jin, H., Paterson, A.M., Mitsdoerffer, M., Ho, I.C., Sharpe, A.H., and Kuchroo, V.K. (2009). The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells. Nature immunology 10, 167-175.

Beier, K.C., Hutloff, A., Dittrich, A.M., Heuck, C., Rauch, A., Buchner, K., Ludewig, B., Ochs, H.D., Mages, H.W., and Kroczek, R.A. (2000). Induction, binding specificity and function of human ICOS. European journal of immunology 30, 3707-3717.

Bendelac, A., Savage, P.B., and Teyton, L. (2007). The biology of NKT cells. Annual review of immunology 25, 297-336.

Bentebibel, S.E., Schmitt, N., Banchereau, J., and Ueno, H. (2011). Human tonsil B-cell lymphoma 6 (BCL6)-expressing CD4+ T-cell subset specialized for B-cell help outside germinal centers. Proceedings of the National Academy of Sciences of the United States of America 108, E488-497.

Bossaller, L., Burger, J., Draeger, R., Grimbacher, B., Knoth, R., Plebani, A., Durandy, A., Baumann, U., Schlesier, M., Welcher, A.A*., et al.* (2006). ICOS deficiency is associated with a severe reduction of CXCR5+CD4 germinal center Th cells. J Immunol 177, 4927-4932.

Bour-Jordan, H., Salomon, B.L., Thompson, H.L., Szot, G.L., Bernhard, M.R., and Bluestone, J.A. (2004). Costimulation controls diabetes by altering the balance of pathogenic and regulatory T cells. The Journal of clinical investigation 114, 979-987.

Boyman, O., and Sprent, J. (2012). The role of interleukin-2 during homeostasis and activation of the immune system. Nature reviews 12, 180-190.

Brandes, M., Willimann, K., Lang, A.B., Nam, K.H., Jin, C., Brenner, M.B., Morita, C.T., and Moser, B. (2003). Flexible migration program regulates gamma delta T-cell involvement in humoral immunity. Blood 102, 3693-3701.

Breitfeld, D., Ohl, L., Kremmer, E., Ellwart, J., Sallusto, F., Lipp, M., and Forster, R. (2000). Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. The Journal of experimental medicine 192, 1545-1552.

Bretscher, P., and Cohn, M. (1970). A theory of self-nonself discrimination. Science (New York, N.Y 169, 1042-1049.

Brodie, D., Collins, A.V., Iaboni, A., Fennelly, J.A., Sparks, L.M., Xu, X.N., van der Merwe, P.A., and Davis, S.J. (2000). LICOS, a primordial costimulatory ligand? Current biology : CB 10, 333-336.

Brunet, J.F., Denizot, F., Luciani, M.F., Roux-Dosseto, M., Suzan, M., Mattei, M.G., and Golstein, P. (1987). A new member of the immunoglobulin superfamily--CTLA-4. Nature 328, 267-270.

Buckler, J.L., Walsh, P.T., Porrett, P.M., Choi, Y., and Turka, L.A. (2006). Cutting edge: T cell requirement for CD28 costimulation is due to negative regulation of TCR signals by PTEN. J Immunol 177, 4262-4266.

Buonfiglio, D., Bragardo, M., Bonissoni, S., Redoglia, V., Cauda, R., Zupo, S., Burgio, V.L., Wolff, H., Franssila, K., Gaidano, G*., et al.* (1999). Characterization of a novel human surface molecule selectively expressed by mature thymocytes, activated T cells and subsets of T cell lymphomas. European journal of immunology 29, 2863-2874.

Burmeister, Y., Lischke, T., Dahler, A.C., Mages, H.W., Lam, K.P., Coyle, A.J., Kroczek, R.A., and Hutloff, A. (2008). ICOS controls the pool size of effector-memory and regulatory T cells. J Immunol 180, 774-782.

Butte, M.J., Keir, M.E., Phamduy, T.B., Sharpe, A.H., and Freeman, G.J. (2007). Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses. Immunity 27, 111-122.

Caccamo, N., Battistini, L., Bonneville, M., Poccia, F., Fournie, J.J., Meraviglia, S., Borsellino, G., Kroczek, R.A., La Mendola, C., Scotet, E*., et al.* (2006). CXCR5 identifies a subset of Vgamma9Vdelta2 T cells which secrete IL-4 and IL-10 and help B cells for antibody production. J Immunol 177, 5290-5295.

Caccamo, N., Todaro, M., La Manna, M.P., Sireci, G., Stassi, G., and Dieli, F. (2012). IL-21 regulates the differentiation of a human gammadelta T cell subset equipped with B cell helper activity. PloS one 7, e41940.

Cai, G., Nie, X., Zhang, W., Wu, B., Lin, J., Wang, H., Jiang, C., and Shen, Q. (2012). A regulatory role for IL-10 receptor signaling in development and B cell help of T follicular helper cells in mice. J Immunol 189, 1294-1302.

Calleja, V., Ameer-Beg, S.M., Vojnovic, B., Woscholski, R., Downward, J., and Larijani, B. (2003). Monitoring conformational changes of proteins in cells by fluorescence lifetime imaging microscopy. The Biochemical journal 372, 33-40.

Cannons, J.L., Qi, H., Lu, K.T., Dutta, M., Gomez-Rodriguez, J., Cheng, J., Wakeland, E.K., Germain, R.N., and Schwartzberg, P.L. (2010). Optimal Germinal Center Responses Require A Multistage T Cell:B Cell Adhesion Process Involving Integrins, SLAM-Associated Protein, and CD84. Immunity.

Cannons, J.L., Tangye, S.G., and Schwartzberg, P.L. (2011). SLAM family receptors and SAP adaptors in immunity. Annual review of immunology 29, 665-705.

Chan, L.S., Robinson, N., and Xu, L. (2001). Expression of interleukin-4 in the epidermis of transgenic mice results in a pruritic inflammatory skin disease: an experimental animal model to study atopic dermatitis. The Journal of investigative dermatology 117, 977-983.

Chan, T.D., and Brink, R. (2012). Affinity-based selection and the germinal center response. Immunological reviews 247, 11-23.

Chang, C.C., Ye, B.H., Chaganti, R.S., and Dalla-Favera, R. (1996). BCL-6, a POZ/zinc-finger protein, is a sequence-specific transcriptional repressor. Proceedings of the National Academy of Sciences of the United States of America 93, 6947-6952.

Chang, P.P., Barral, P., Fitch, J., Pratama, A., Ma, C.S., Kallies, A., Hogan, J.J., Cerundolo, V., Tangye, S.G., Bittman, R.*, et al.* (2012). Identification of Bcl-6-dependent follicular helper NKT cells that provide cognate help for B cell responses. Nature immunology 13, 35-43.

Chantry, D., Vojtek, A., Kashishian, A., Holtzman, D.A., Wood, C., Gray, P.W., Cooper, J.A., and Hoekstra, M.F. (1997). p110delta, a novel phosphatidylinositol 3-kinase catalytic subunit that associates with p85 and is expressed predominantly in leukocytes. The Journal of biological chemistry 272, 19236-19241.

Chemnitz, J.M., Parry, R.V., Nichols, K.E., June, C.H., and Riley, J.L. (2004). SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation. J Immunol 173, 945-954.

Chen, L., and Flies, D.B. (2013). Molecular mechanisms of T cell co-stimulation and co-inhibition. Nature reviews 13, 227-242.

Chevalier, N., Jarrossay, D., Ho, E., Avery, D.T., Ma, C.S., Yu, D., Sallusto, F., Tangye, S.G., and Mackay, C.R. (2011). CXCR5 expressing human central memory CD4 T cells and their relevance for humoral immune responses. J Immunol 186, 5556-5568.

Chi, H. (2012). Regulation and function of mTOR signalling in T cell fate decisions. Nature reviews 12, 325-338.

Choi, Y.S., Eto, D., Yang, J.A., Lao, C., and Crotty, S. (2013a). Cutting edge: STAT1 is required for IL-6-mediated Bcl6 induction for early follicular helper cell differentiation. J Immunol 190, 3049-3053.

Choi, Y.S., Kageyama, R., Eto, D., Escobar, T.C., Johnston, R.J., Monticelli, L., Lao, C., and Crotty, S. (2011). ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. Immunity 34, 932-946.

Choi, Y.S., Yang, J.A., Yusuf, I., Johnston, R.J., Greenbaum, J., Peters, B., and Crotty, S. (2013b). Bcl6 expressing follicular helper CD4 T cells are fate committed early and have the capacity to form memory. J Immunol 190, 4014-4026.

Chtanova, T., Tangye, S.G., Newton, R., Frank, N., Hodge, M.R., Rolph, M.S., and Mackay, C.R. (2004). T follicular helper cells express a distinctive transcriptional profile, reflecting their role as non-Th1/Th2 effector cells that provide help for B cells. J Immunol 173, 68-78.

Chung, J., Kuo, C.J., Crabtree, G.R., and Blenis, J. (1992). Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the 70 kd S6 protein kinases. Cell 69, 1227-1236.

Chung, Y., Nurieva, R., Esashi, E., Wang, Y.H., Zhou, D., Gapin, L., and Dong, C. (2008). A critical role of costimulation during intrathymic development of invariant NK T cells. J Immunol 180, 2276-2283.

Chung, Y., Tanaka, S., Chu, F., Nurieva, R.I., Martinez, G.J., Rawal, S., Wang, Y.H., Lim, H., Reynolds, J.M., Zhou, X.H.*, et al.* (2011). Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. Nature medicine 17, 983-988.

Clay, B.S., Shilling, R.A., Bandukwala, H.S., Moore, T.V., Cannon, J.L., Welcher, A.A., Weinstock, J.V., and Sperling, A.I. (2009). Inducible costimulator expression regulates the magnitude of Th2-mediated airway inflammation by regulating the number of Th2 cells. PloS one 4, e7525.

Colina, R., Costa-Mattioli, M., Dowling, R.J., Jaramillo, M., Tai, L.H., Breitbach, C.J., Martineau, Y., Larsson, O., Rong, L., Svitkin, Y.V.*, et al.* (2008). Translational control of the innate immune response through IRF-7. Nature 452, 323-328.

Colombetti, S., Basso, V., Mueller, D.L., and Mondino, A. (2006). Prolonged TCR/CD28 engagement drives IL-2-independent T cell clonal expansion through signaling mediated by the mammalian target of rapamycin. J Immunol 176, 2730-2738.

Coyle, A.J., Lehar, S., Lloyd, C., Tian, J., Delaney, T., Manning, S., Nguyen, T., Burwell, T., Schneider, H., Gonzalo, J.A.*, et al.* (2000). The CD28-related molecule ICOS is required for effective T cell-dependent immune responses. Immunity 13, 95-105.

Crotty, S. (2011). Follicular Helper CD4 T Cells (T(FH)). Annual review of immunology.

Crotty, S., Johnston, R.J., and Schoenberger, S.P. (2010). Effectors and memories: Bcl-6 and Blimp-1 in T and B lymphocyte differentiation. Nature immunology 11, 114-120.

Damdinsuren, B., Zhang, Y., Khalil, A., Wood, W.H., 3rd, Becker, K.G., Shlomchik, M.J., and Sen, R. (2010). Single Round of Antigen Receptor Signaling Programs Naive B Cells to Receive T Cell Help. Immunity.

de Vinuesa, C.G., Cook, M.C., Ball, J., Drew, M., Sunners, Y., Cascalho, M., Wabl, M., Klaus, G.G., and MacLennan, I.C. (2000). Germinal centers without T cells. The Journal of experimental medicine 191, 485-494.

Deane, J.A., Kharas, M.G., Oak, J.S., Stiles, L.N., Luo, J., Moore, T.I., Ji, H., Rommel, C., Cantley, L.C., Lane, T.E., and Fruman, D.A. (2007). T-cell function is partially maintained in the absence of class IA phosphoinositide 3-kinase signaling. Blood 109, 2894-2902.

Deenick, E.K., Chan, A., Ma, C.S., Gatto, D., Schwartzberg, P.L., Brink, R., and Tangye, S.G. (2010). Follicular helper T cell differentiation requires continuous antigen presentation that is independent of unique B cell signaling. Immunity 33, 241-253.

Delgoffe, G.M., Kole, T.P., Zheng, Y., Zarek, P.E., Matthews, K.L., Xiao, B., Worley, P.F., Kozma, S.C., and Powell, J.D. (2009). The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment. Immunity 30, 832-844.

Dent, A.L., Shaffer, A.L., Yu, X., Allman, D., and Staudt, L.M. (1997). Control of inflammation, cytokine expression, and germinal center formation by BCL-6. Science (New York, N.Y 276, 589-592.

Dhordain, P., Albagli, O., Lin, R.J., Ansieau, S., Quief, S., Leutz, A., Kerckaert, J.P., Evans, R.M., and Leprince, D. (1997). Corepressor SMRT binds the BTB/POZ repressing domain of the LAZ3/BCL6 oncoprotein. Proceedings of the National Academy of Sciences of the United States of America 94, 10762-10767.

Dienz, O., Eaton, S.M., Bond, J.P., Neveu, W., Moquin, D., Noubade, R., Briso, E.M., Charland, C., Leonard, W.J., Ciliberto, G.*, et al.* (2009). The induction of antibody production by IL-6 is indirectly mediated by IL-21 produced by CD4+ T cells. The Journal of experimental medicine 206, 69-78.

Dodson, L.F., Boomer, J.S., Deppong, C.M., Shah, D.D., Sim, J., Bricker, T.L., Russell, J.H., and Green, J.M. (2009). Targeted knock-in mice expressing mutations of CD28 reveal an essential pathway for costimulation. Molecular and cellular biology 29, 3710-3721.

Dong, C., Juedes, A.E., Temann, U.A., Shresta, S., Allison, J.P., Ruddle, N.H., and Flavell, R.A. (2001a). ICOS co-stimulatory receptor is essential for T-cell activation and function. Nature 409, 97-101.

Dong, C., Temann, U.A., and Flavell, R.A. (2001b). Cutting edge: critical role of inducible costimulator in germinal center reactions. J Immunol 166, 3659-3662.

Dorrello, N.V., Peschiaroli, A., Guardavaccaro, D., Colburn, N.H., Sherman, N.E., and Pagano, M. (2006). S6K1- and betaTRCP-mediated degradation of PDCD4 promotes protein translation and cell growth. Science (New York, N.Y 314, 467-471.

Dowling, R.J., Topisirovic, I., Alain, T., Bidinosti, M., Fonseca, B.D., Petroulakis, E., Wang, X., Larsson, O., Selvaraj, A., Liu, Y.*, et al.* (2010). mTORC1-mediated cell proliferation, but not cell growth, controlled by the 4E-BPs. Science (New York, N.Y 328, 1172-1176.

Duncan, R., Milburn, S.C., and Hershey, J.W. (1987). Regulated phosphorylation and low abundance of HeLa cell initiation factor eIF-4F suggest a role in translational control. Heat shock effects on eIF-4F. The Journal of biological chemistry 262, 380-388.

Eddahri, F., Denanglaire, S., Bureau, F., Spolski, R., Leonard, W.J., Leo, O., and Andris, F. (2009). Interleukin-6/STAT3 signaling regulates the ability of naive T cells to acquire B-cell help capacities. Blood 113, 2426-2433.

Edmunds, C., Parry, R.V., Burgess, S.J., Reaves, B., and Ward, S.G. (1999). CD28 stimulates tyrosine phosphorylation, cellular redistribution and catalytic activity of the inositol lipid 5-phosphatase SHIP. European journal of immunology 29, 3507-3515.

Eto, D., Lao, C., Ditoro, D., Barnett, B., Escobar, T.C., Kageyama, R., Yusuf, I., and Crotty, S. (2011). IL-21 and IL-6 Are Critical for Different Aspects of B Cell Immunity and Redundantly Induce Optimal Follicular Helper CD4 T Cell (Tfh) Differentiation. PloS one 6, e17739.

Ettinger, R., Sims, G.P., Fairhurst, A.M., Robbins, R., da Silva, Y.S., Spolski, R., Leonard, W.J., and Lipsky, P.E. (2005). IL-21 induces differentiation of human naive and memory B cells into antibody-secreting plasma cells. J Immunol 175, 7867-7879.

Ettinger, R., Sims, G.P., Robbins, R., Withers, D., Fischer, R.T., Grammer, A.C., Kuchen, S., and Lipsky, P.E. (2007). IL-21 and BAFF/BLyS synergize in stimulating plasma cell differentiation from a unique population of human splenic memory B cells. J Immunol 178, 2872-2882.

Facchinetti, V., Ouyang, W., Wei, H., Soto, N., Lazorchak, A., Gould, C., Lowry, C., Newton, A.C., Mao, Y., Miao, R.Q.*, et al.* (2008). The mammalian target of rapamycin complex 2 controls folding and stability of Akt and protein kinase C. The EMBO journal 27, 1932-1943.

Fairfax, K.A., Kallies, A., Nutt, S.L., and Tarlinton, D.M. (2008). Plasma cell development: from B-cell subsets to long-term survival niches. Seminars in immunology 20, 49-58.

Fayard, E., Xue, G., Parcellier, A., Bozulic, L., and Hemmings, B.A. (2010). Protein kinase B (PKB/Akt), a key mediator of the PI3K signaling pathway. Current topics in microbiology and immunology 346, 31-56.

Fazilleau, N., Mark, L., McHeyzer-Williams, L.J., and McHeyzer-Williams, M.G. (2009a). Follicular helper T cells: lineage and location. Immunity 30, 324-335.

Fazilleau, N., McHeyzer-Williams, L.J., Rosen, H., and McHeyzer-Williams, M.G. (2009b). The function of follicular helper T cells is regulated by the strength of T cell antigen receptor binding. Nature immunology 10, 375-384.

Fenton, T.R., and Gout, I.T. (2011). Functions and regulation of the 70kDa ribosomal S6 kinases. The international journal of biochemistry & cell biology 43, 47-59.

Ferguson, S.E., Han, S., Kelsoe, G., and Thompson, C.B. (1996). CD28 is required for germinal center formation. J Immunol 156, 4576-4581.

Ferrari, S., Bandi, H.R., Hofsteenge, J., Bussian, B.M., and Thomas, G. (1991). Mitogen-activated 70K S6 kinase. Identification of in vitro 40 S ribosomal S6 phosphorylation sites. The Journal of biological chemistry 266, 22770-22775.

Fos, C., Salles, A., Lang, V., Carrette, F., Audebert, S., Pastor, S., Ghiotto, M., Olive, D., Bismuth, G., and Nunes, J.A. (2008). ICOS ligation recruits the p50alpha PI3K regulatory subunit to the immunological synapse. J Immunol 181, 1969-1977.

Foukas, L.C., and Withers, D.J. (2010). Phosphoinositide signalling pathways in metabolic regulation. Current topics in microbiology and immunology 346, 115-141.

Franke, T.F. (2008). PI3K/Akt: getting it right matters. Oncogene 27, 6473-6488.

Franko, J.L., and Levine, A.D. (2009). Antigen-independent adhesion and cell spreading by inducible costimulator engagement inhibits T cell migration in a PI-3K-dependent manner. Journal of leukocyte biology 85, 526-538.

Fukuda, T., Yoshida, T., Okada, S., Hatano, M., Miki, T., Ishibashi, K., Okabe, S., Koseki, H., Hirosawa, S., Taniguchi, M.*, et al.* (1997). Disruption of the Bcl6 gene results in an impaired germinal center formation. The Journal of experimental medicine 186, 439-448.

Gagnon, E., Schubert, D.A., Gordo, S., Chu, H.H., and Wucherpfennig, K.W. (2012). Local changes in lipid environment of TCR microclusters regulate membrane binding by the CD3epsilon cytoplasmic domain. The Journal of experimental medicine 209, 2423-2439.

Gan, X., Wang, J., Su, B., and Wu, D. (2011). Evidence for direct activation of mTORC2 kinase activity by phosphatidylinositol 3,4,5-trisphosphate. The Journal of biological chemistry.

Gao, X., Gigoux, M., Yang, J., Leconte, J., Yang, X., and Suh, W.K. (2012). Anti-Chlamydial Th17 Responses Are Controlled by the Inducible Costimulator Partially through Phosphoinositide 3-Kinase Signaling. PloS one 7, e52657.

Garside, P., Ingulli, E., Merica, R.R., Johnson, J.G., Noelle, R.J., and Jenkins, M.K. (1998). Visualization of specific B and T lymphocyte interactions in the lymph node. Science (New York, N.Y 281, 96-99.

Gaspal, F.M., McConnell, F.M., Kim, M.Y., Gray, D., Kosco-Vilbois, M.H., Raykundalia, C.R., Botto, M., and Lane, P.J. (2006). The generation of thymus-independent germinal centers depends on CD40 but not on CD154, the T cell-derived CD40-ligand. European journal of immunology 36, 1665-1673.

Gassama-Diagne, A., Yu, W., ter Beest, M., Martin-Belmonte, F., Kierbel, A., Engel, J., and Mostov, K. (2006). Phosphatidylinositol-3,4,5-trisphosphate regulates the formation of the basolateral plasma membrane in epithelial cells. Nature cell biology 8, 963-970.

Gkogkas, C.G., Khoutorsky, A., Ran, I., Rampakakis, E., Nevarko, T., Weatherill, D.B., Vasuta, C., Yee, S., Truitt, M., Dallaire, P.*, et al.* (2013). Autism-related deficits via dysregulated eIF4E-dependent translational control. Nature 493, 371-377.

Gogishvili, T., Elias, F., Emery, J.L., McPherson, K., Okkenhaug, K., Hunig, T., and Dennehy, K.M. (2008). Proliferative signals mediated by CD28 superagonists require the exchange factor Vav1 but not phosphoinositide 3-kinase in primary peripheral T cells. European journal of immunology 38, 2528-2533.

Gomez, E., Mohammad, S.S., and Pavitt, G.D. (2002). Characterization of the minimal catalytic domain within eIF2B: the guanine-nucleotide exchange factor for translation initiation. The EMBO journal 21, 5292-5301.

Gonzalo, J.A., Tian, J., Delaney, T., Corcoran, J., Rottman, J.B., Lora, J., Al-garawi, A., Kroczek, R., Gutierrez-Ramos, J.C., and Coyle, A.J. (2001). ICOS is critical for T helper cell-mediated lung mucosal inflammatory responses. Nature immunology 2, 597-604.

Good-Jacobson, K.L., Szumilas, C.G., Chen, L., Sharpe, A.H., Tomayko, M.M., and Shlomchik, M.J. (2010). PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cells. Nature immunology.

Goodnow, C.C., Vinuesa, C.G., Randall, K.L., Mackay, F., and Brink, R. (2011). Control systems and decision making for antibody production. Nature immunology 11, 681-688.

Gotsman, I., Grabie, N., Gupta, R., Dacosta, R., MacConmara, M., Lederer, J., Sukhova, G., Witztum, J.L., Sharpe, A.H., and Lichtman, A.H. (2006). Impaired regulatory T-cell response and enhanced atherosclerosis in the absence of inducible costimulatory molecule. Circulation 114, 2047-2055.

Greenwald, R.J., Freeman, G.J., and Sharpe, A.H. (2005). The B7 family revisited. Annual review of immunology 23, 515-548.

Grimbacher, B., Hutloff, A., Schlesier, M., Glocker, E., Warnatz, K., Drager, R., Eibel, H., Fischer, B., Schaffer, A.A., Mages, H.W*., et al.* (2003). Homozygous loss of ICOS is associated with adult-onset common variable immunodeficiency. Nature immunology 4, 261-268.

Guo, F., Iclozan, C., Suh, W.K., Anasetti, C., and Yu, X.Z. (2008). CD28 controls differentiation of regulatory T cells from naive CD4 T cells. J Immunol 181, 2285-2291.

Gygi, S.P., Rochon, Y., Franza, B.R., and Aebersold, R. (1999). Correlation between protein and mRNA abundance in yeast. Molecular and cellular biology 19, 1720-1730.

Hawiger, D., Tran, E., Du, W., Booth, C.J., Wen, L., Dong, C., and Flavell, R.A. (2008). ICOS mediates the development of insulin-dependent diabetes mellitus in nonobese diabetic mice. J Immunol 180, 3140-3147.

Hay, N., and Sonenberg, N. (2004). Upstream and downstream of mTOR. Genes & development 18, 1926-1945.

Herdy, B., Jaramillo, M., Svitkin, Y.V., Rosenfeld, A.B., Kobayashi, M., Walsh, D., Alain, T., Sean, P., Robichaud, N., Topisirovic, I*., et al.* (2012). Translational control of the activation of transcription factor NF-kappaB and production of type I interferon by phosphorylation of the translation factor eIF4E. Nature immunology.

Hinnebusch, A.G. (2011). Molecular mechanism of scanning and start codon selection in eukaryotes. Microbiology and molecular biology reviews : MMBR 75, 434-467, first page of table of contents.

Hinnebusch, A.G., and Lorsch, J.R. (2012). The mechanism of eukaryotic translation initiation: new insights and challenges. Cold Spring Harbor perspectives in biology 4.

Hiramatsu, Y., Suto, A., Kashiwakuma, D., Kanari, H., Kagami, S., Ikeda, K., Hirose, K., Watanabe, N., Grusby, M.J., Iwamoto, I., and Nakajima, H. (2010). c-Maf activates the promoter and enhancer of the IL-21 gene, and TGF-beta inhibits c-Maf-induced IL-21 production in CD4+ T cells. Journal of leukocyte biology 87, 703-712.

Howard, M., Farrar, J., Hilfiker, M., Johnson, B., Takatsu, K., Hamaoka, T., and Paul, W.E. (1982). Identification of a T cell-derived b cell growth factor distinct from interleukin 2. The Journal of experimental medicine 155, 914-923.

Hresko, R.C., and Mueckler, M. (2005). mTOR.RICTOR is the Ser473 kinase for Akt/protein kinase B in 3T3-L1 adipocytes. The Journal of biological chemistry 280, 40406-40416.

Hsieh, A.C., Liu, Y., Edlind, M.P., Ingolia, N.T., Janes, M.R., Sher, A., Shi, E.Y., Stumpf, C.R., Christensen, C., Bonham, M.J*., et al.* (2012). The translational landscape of mTOR signalling steers cancer initiation and metastasis. Nature 485, 55-61.

Hsu, A.L., Ching, T.T., Sen, G., Wang, D.S., Bondada, S., Authi, K.S., and Chen, C.S. (2000). Novel function of phosphoinositide 3-kinase in T cell Ca2+ signaling. A phosphatidylinositol 3,4,5-trisphosphate-mediated Ca2+ entry mechanism. The Journal of biological chemistry 275, 16242-16250.

Hu-Li, J., Pannetier, C., Guo, L., Lohning, M., Gu, H., Watson, C., Assenmacher, M., Radbruch, A., and Paul, W.E. (2001). Regulation of expression of IL-4 alleles: analysis using a chimeric GFP/IL-4 gene. Immunity 14, 1-11.

Huang, C., Hatzi, K., and Melnick, A. (2013). Lineage-specific functions of Bcl-6 in immunity and inflammation are mediated by distinct biochemical mechanisms. Nature immunology.

Hutloff, A., Dittrich, A.M., Beier, K.C., Eljaschewitsch, B., Kraft, R., Anagnostopoulos, I., and Kroczek, R.A. (1999). ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. Nature 397, 263-266.

Huynh, K.D., and Bardwell, V.J. (1998). The BCL-6 POZ domain and other POZ domains interact with the co-repressors N-CoR and SMRT. Oncogene 17, 2473-2484.

Huynh, K.D., Fischle, W., Verdin, E., and Bardwell, V.J. (2000). BCoR, a novel corepressor involved in BCL-6 repression. Genes & development 14, 1810-1823.

Hwang, E.S., White, I.A., and Ho, I.C. (2002). An IL-4-independent and CD25-mediated function of c-maf in promoting the production of Th2 cytokines. Proceedings of the National Academy of Sciences of the United States of America 99, 13026-13030.

Iadevaia, V., Caldarola, S., Tino, E., Amaldi, F., and Loreni, F. (2008). All translation elongation factors and the e, f, and h subunits of translation initiation factor 3 are encoded by 5'-terminal oligopyrimidine (TOP) mRNAs. Rna 14, 1730-1736.

Ichii, H., Sakamoto, A., Arima, M., Hatano, M., Kuroda, Y., and Tokuhisa, T. (2007). Bcl6 is essential for the generation of long-term memory CD4+ T cells. International immunology 19, 427-433.

Ichii, H., Sakamoto, A., Hatano, M., Okada, S., Toyama, H., Taki, S., Arima, M., Kuroda, Y., and Tokuhisa, T. (2002). Role for Bcl-6 in the generation and maintenance of memory CD8+ T cells. Nature immunology 3, 558-563.

Ichii, H., Sakamoto, A., Kuroda, Y., and Tokuhisa, T. (2004). Bcl6 acts as an amplifier for the generation and proliferative capacity of central memory CD8+ T cells. J Immunol 173, 883-891.

Ideker, T., Thorsson, V., Ranish, J.A., Christmas, R., Buhler, J., Eng, J.K., Bumgarner, R., Goodlett, D.R., Aebersold, R., and Hood, L. (2001). Integrated genomic and proteomic analyses of a systematically perturbed metabolic network. Science (New York, N.Y 292, 929-934.

Ikenoue, T., Inoki, K., Yang, Q., Zhou, X., and Guan, K.L. (2008). Essential function of TORC2 in PKC and Akt turn motif phosphorylation, maturation and signalling. The EMBO journal 27, 1919-1931.

Inukai, K., Funaki, M., Anai, M., Ogihara, T., Katagiri, H., Fukushima, Y., Sakoda, H., Onishi, Y., Ono, H., Fujishiro, M.*, et al.* (2001). Five isoforms of the phosphatidylinositol 3-kinase regulatory subunit exhibit different associations with receptor tyrosine kinases and their tyrosine phosphorylations. FEBS letters 490, 32-38.

Inukai, K., Funaki, M., Ogihara, T., Katagiri, H., Kanda, A., Anai, M., Fukushima, Y., Hosaka, T., Suzuki, M., Shin, B.C.*, et al.* (1997). p85alpha gene generates three isoforms of regulatory subunit for phosphatidylinositol 3-kinase (PI 3-Kinase), p50alpha, p55alpha, and p85alpha, with different PI 3-kinase activity elevating responses to insulin. The Journal of biological chemistry 272, 7873-7882.

Ito, T., Hanabuchi, S., Wang, Y.H., Park, W.R., Arima, K., Bover, L., Qin, F.X., Gilliet, M., and Liu, Y.J. (2008). Two functional subsets of FOXP3+ regulatory T cells in human thymus and periphery. Immunity 28, 870-880.

Jacinto, E., Facchinetti, V., Liu, D., Soto, N., Wei, S., Jung, S.Y., Huang, Q., Qin, J., and Su, B. (2006). SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. Cell 127, 125-137.

Jackson, R.J., Hellen, C.U., and Pestova, T.V. (2010). The mechanism of eukaryotic translation initiation and principles of its regulation. Nature reviews. Molecular cell biology 11, 113-127.

Jang, S.K., Krausslich, H.G., Nicklin, M.J., Duke, G.M., Palmenberg, A.C., and Wimmer, E. (1988). A segment of the 5' nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. Journal of virology 62, 2636-2643.

Jaramillo, M., Gomez, M.A., Larsson, O., Shio, M.T., Topisirovic, I., Contreras, I., Luxenburg, R., Rosenfeld, A., Colina, R., McMaster, R.W*., et al.* (2011). Leishmania repression of host translation through mTOR cleavage is required for parasite survival and infection. Cell host & microbe 9, 331-341.

Jefferies, H.B., Fumagalli, S., Dennis, P.B., Reinhard, C., Pearson, R.B., and Thomas, G. (1997). Rapamycin suppresses 5'TOP mRNA translation through inhibition of p70s6k. The EMBO journal 16, 3693-3704.

Jefferies, H.B., Reinhard, C., Kozma, S.C., and Thomas, G. (1994). Rapamycin selectively represses translation of the "polypyrimidine tract" mRNA family. Proceedings of the National Academy of Sciences of the United States of America 91, 4441-4445.

Jin, H., Carrio, R., Yu, A., and Malek, T.R. (2004). Distinct activation signals determine whether IL-21 induces B cell costimulation, growth arrest, or Bim-dependent apoptosis. J Immunol 173, 657-665.

Johannes, G., and Sarnow, P. (1998). Cap-independent polysomal association of natural mRNAs encoding c-myc, BiP, and eIF4G conferred by internal ribosome entry sites. Rna 4, 1500-1513.

Johnston, R.J., Choi, Y.S., Diamond, J.A., Yang, J.A., and Crotty, S. (2012). STAT5 is a potent negative regulator of TFH cell differentiation. The Journal of experimental medicine 209, 243-250.

Johnston, R.J., Poholek, A.C., DiToro, D., Yusuf, I., Eto, D., Barnett, B., Dent, A.L., Craft, J., and Crotty, S. (2009). Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. Science (New York, N.Y 325, 1006-1010.

June, C.H., Ledbetter, J.A., Gillespie, M.M., Lindsten, T., and Thompson, C.B. (1987). T-cell proliferation involving the CD28 pathway is associated with cyclosporine-resistant interleukin 2 gene expression. Molecular and cellular biology 7, 4472-4481.

Kaminski, D.A., Lee, B.O., Eaton, S.M., Haynes, L., and Randall, T.D. (2009). CD28 and inducible costimulator (ICOS) signalling can sustain CD154 expression on activated T cells. Immunology 127, 373-385.

Kang, S.G., Liu, W.H., Lu, P., Jin, H.Y., Lim, H.W., Shepherd, J., Fremgen, D., Verdin, E., Oldstone, M.B., Qi, H*., et al.* (2013). MicroRNAs of the miR-17 approximately 92 family are critical regulators of T differentiation. Nature immunology.

Kaplan, M.H., Schindler, U., Smiley, S.T., and Grusby, M.J. (1996). Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. Immunity 4, 313-319.

Kasaian, M.T., Whitters, M.J., Carter, L.L., Lowe, L.D., Jussif, J.M., Deng, B., Johnson, K.A., Witek, J.S., Senices, M., Konz, R.F*., et al.* (2002). IL-21 limits NK cell responses and promotes antigen-specific T cell activation: a mediator of the transition from innate to adaptive immunity. Immunity 16, 559-569.

Kawamoto, S., Tran, T.H., Maruya, M., Suzuki, K., Doi, Y., Tsutsui, Y., Kato, L.M., and Fagarasan, S. (2012). The inhibitory receptor PD-1 regulates IgA selection and bacterial composition in the gut. Science (New York, N.Y 336, 485-489.

Kawasome, H., Papst, P., Webb, S., Keller, G.M., Johnson, G.L., Gelfand, E.W., and Terada, N. (1998). Targeted disruption of p70(s6k) defines its role in protein synthesis and rapamycin sensitivity. Proceedings of the National Academy of Sciences of the United States of America 95, 5033-5038.

Keir, M.E., Butte, M.J., Freeman, G.J., and Sharpe, A.H. (2008). PD-1 and its ligands in tolerance and immunity. Annual review of immunology 26, 677-704.

Keir, M.E., Francisco, L.M., and Sharpe, A.H. (2007). PD-1 and its ligands in T-cell immunity. Current opinion in immunology 19, 309-314.

Kerfoot, S.M., Yaari, G., Patel, J.R., Johnson, K.L., Gonzalez, D.G., Kleinstein, S.H., and Haberman, A.M. (2011). Germinal center B cell and T follicular helper cell development initiates in the interfollicular zone. Immunity 34, 947-960.

Kim, H.P., Korn, L.L., Gamero, A.M., and Leonard, W.J. (2005). Calcium-dependent activation of interleukin-21 gene expression in T cells. The Journal of biological chemistry 280, 25291-25297.

King, C., Tangye, S.G., and Mackay, C.R. (2008). T follicular helper (TFH) cells in normal and dysregulated immune responses. Annual review of immunology 26, 741-766.

King, I.L., Fortier, A., Tighe, M., Dibble, J., Watts, G.F., Veerapen, N., Haberman, A.M., Besra, G.S., Mohrs, M., Brenner, M.B., and Leadbetter, E.A. (2012). Invariant natural killer T cells direct B cell responses to cognate lipid antigen in an IL-21-dependent manner. Nature immunology 13, 44-50.

King, I.L., and Mohrs, M. (2009). IL-4-producing CD4+ T cells in reactive lymph nodes during helminth infection are T follicular helper cells. The Journal of experimental medicine 206, 1001-1007.

Kishimoto, T. (2005). Interleukin-6: from basic science to medicine--40 years in immunology. Annual review of immunology 23, 1-21.

Kitano, M., Moriyama, S., Ando, Y., Hikida, M., Mori, Y., Kurosaki, T., and Okada, T. (2011). Bcl6 protein expression shapes pre-germinal center B cell dynamics and follicular helper T cell heterogeneity. Immunity 34, 961-972.

Kopf, M., Coyle, A.J., Schmitz, N., Barner, M., Oxenius, A., Gallimore, A., Gutierrez-Ramos, J.C., and Bachmann, M.F. (2000). Inducible costimulator protein (ICOS) controls T helper cell subset polarization after virus and parasite infection. The Journal of experimental medicine 192, 53-61.

Kopf, M., Le Gros, G., Bachmann, M., Lamers, M.C., Bluethmann, H., and Kohler, G. (1993). Disruption of the murine IL-4 gene blocks Th2 cytokine responses. Nature 362, 245-248.

Koretzky, G.A., and Myung, P.S. (2001). Positive and negative regulation of T-cell activation by adaptor proteins. Nature reviews 1, 95-107.

Korn, T., Bettelli, E., Gao, W., Awasthi, A., Jager, A., Strom, T.B., Oukka, M., and Kuchroo, V.K. (2007). IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. Nature 448, 484-487.

Kornete, M., Sgouroudis, E., and Piccirillo, C.A. (2012). ICOS-dependent homeostasis and function of Foxp3+ regulatory T cells in islets of nonobese diabetic mice. J Immunol 188, 1064-1074.

Kroenke, M.A., Eto, D., Locci, M., Cho, M., Davidson, T., Haddad, E.K., and Crotty, S. (2012). Bcl6 and Maf Cooperate To Instruct Human Follicular Helper CD4 T Cell Differentiation. J Immunol.

Kuchen, S., Robbins, R., Sims, G.P., Sheng, C., Phillips, T.M., Lipsky, P.E., and Ettinger, R. (2007). Essential role of IL-21 in B cell activation, expansion, and plasma cell generation during CD4+ T cell-B cell collaboration. J Immunol 179, 5886-5896.

Kuhn, R., Rajewsky, K., and Muller, W. (1991). Generation and analysis of interleukin-4 deficient mice. Science (New York, N.Y 254, 707-710.

Laplante, M., and Sabatini, D.M. (2009). mTOR signaling at a glance. Journal of cell science 122, 3589-3594.

Latour, S., and Veillette, A. (2003). Molecular and immunological basis of X-linked lymphoproliferative disease. Immunological reviews 192, 212-224.

Lazorchak, A.S., Liu, D., Facchinetti, V., Di Lorenzo, A., Sessa, W.C., Schatz, D.G., and Su, B. (2010). Sin1-mTORC2 suppresses rag and il7r gene expression through Akt2 in B cells. Molecular cell 39, 433-443.

Le Bacquer, O., Petroulakis, E., Paglialunga, S., Poulin, F., Richard, D., Cianflone, K., and Sonenberg, N. (2007). Elevated sensitivity to diet-induced obesity and insulin resistance in mice lacking 4E-BP1 and 4E-BP2. The Journal of clinical investigation 117, 387-396.

Lentz, V.M., and Manser, T. (2001). Cutting edge: germinal centers can be induced in the absence of T cells. J Immunol 167, 15-20.

Leonard, W.J., and Spolski, R. (2005). Interleukin-21: a modulator of lymphoid proliferation, apoptosis and differentiation. Nature reviews 5, 688-698.

Leslie, N.R., and Downes, C.P. (2002). PTEN: The down side of PI 3-kinase signalling. Cellular signalling 14, 285-295.

Lewis, R.S. (2001). Calcium signaling mechanisms in T lymphocytes. Annual review of immunology 19, 497-521.

Li, J., Heinrichs, J., Leconte, J., Haarberg, K., Semple, K., Liu, C., Gigoux, M., Kornete, M., Piccirillo, C.A., Suh, W.K., and Yu, X.Z. (2013). Phosphatidylinositol 3-Kinase-Independent Signaling Pathways Contribute to ICOS-Mediated T Cell Costimulation in Acute Graft-Versus-Host Disease in Mice. J Immunol.

Liang, H.E., Reinhardt, R.L., Bando, J.K., Sullivan, B.M., Ho, I.C., and Locksley, R.M. (2011). Divergent expression patterns of IL-4 and IL-13 define unique functions in allergic immunity. Nature immunology.

Liang, J., and Slingerland, J.M. (2003). Multiple roles of the PI3K/PKB (Akt) pathway in cell cycle progression. Cell Cycle 2, 339-345.

Liang, L., Porter, E.M., and Sha, W.C. (2002). Constitutive expression of the B7h ligand for inducible costimulator on naive B cells is extinguished after activation by distinct B cell receptor and interleukin 4 receptor-mediated pathways and can be rescued by CD40 signaling. The Journal of experimental medicine 196, 97-108.

Lim, H.W., Hillsamer, P., and Kim, C.H. (2004). Regulatory T cells can migrate to follicles upon T cell activation and suppress GC-Th cells and GC-Th cell-driven B cell responses. The Journal of clinical investigation 114, 1640-1649.

Lin, T.A., Kong, X., Haystead, T.A., Pause, A., Belsham, G., Sonenberg, N., and Lawrence, J.C., Jr. (1994). PHAS-I as a link between mitogen-activated protein kinase and translation initiation. Science (New York, N.Y 266, 653-656.

Ling, V., Wu, P.W., Finnerty, H.F., Bean, K.M., Spaulding, V., Fouser, L.A., Leonard, J.P., Hunter, S.E., Zollner, R., Thomas, J.L.*, et al.* (2000). Cutting edge: identification of GL50, a novel B7-like protein that functionally binds to ICOS receptor. J Immunol 164, 1653-1657.

Linterman, M.A., Beaton, L., Yu, D., Ramiscal, R.R., Srivastava, M., Hogan, J.J., Verma, N.K., Smyth, M.J., Rigby, R.J., and Vinuesa, C.G. (2010). IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses. The Journal of experimental medicine 207, 353-363.

Linterman, M.A., Liston, A., and Vinuesa, C.G. (2012). T-follicular helper cell differentiation and the co-option of this pathway by non-helper cells. Immunological reviews 247, 143-159.

Linterman, M.A., Pierson, W., Lee, S.K., Kallies, A., Kawamoto, S., Rayner, T.F., Srivastava, M., Divekar, D.P., Beaton, L., Hogan, J.J.*, et al.* (2011). Foxp3+ follicular regulatory T cells control the germinal center response. Nature medicine 17, 975-982.

Linterman, M.A., Rigby, R.J., Wong, R., Silva, D., Withers, D., Anderson, G., Verma, N.K., Brink, R., Hutloff, A., Goodnow, C.C., and Vinuesa, C.G. (2009a). Roquin differentiates the specialized functions of duplicated T cell costimulatory receptor genes CD28 and ICOS. Immunity 30, 228-241.

Linterman, M.A., Rigby, R.J., Wong, R.K., Yu, D., Brink, R., Cannons, J.L., Schwartzberg, P.L., Cook, M.C., Walters, G.D., and Vinuesa, C.G. (2009b). Follicular helper T cells are required for systemic autoimmunity. The Journal of experimental medicine 206, 561-576.

Lischke, T., Hegemann, A., Gurka, S., Vu Van, D., Burmeister, Y., Lam, K.P., Kershaw, O., Mollenkopf, H.J., Mages, H.W., Hutloff, A., and Kroczek, R.A. (2012). Comprehensive analysis of CD4+ T cells in the decision between tolerance and immunity in vivo reveals a pivotal role for ICOS. J Immunol 189, 234-244.

Liu, X., Bai, X.F., Wen, J., Gao, J.X., Liu, J., Lu, P., Wang, Y., Zheng, P., and Liu, Y. (2001). B7H costimulates clonal expansion of, and cognate destruction of tumor cells by, CD8(+) T lymphocytes in vivo. The Journal of experimental medicine 194, 1339-1348.

Liu, X., Yan, X., Zhong, B., Nurieva, R.I., Wang, A., Wang, X., Martin-Orozco, N., Wang, Y., Chang, S.H., Esplugues, E.*, et al.* (2012). Bcl6 expression specifies the T follicular helper cell program in vivo. The Journal of experimental medicine 209, 1841-1852.

Livingstone, M., Atas, E., Meller, A., and Sonenberg, N. (2010). Mechanisms governing the control of mRNA translation. Physical biology 7, 021001.

Logue, E.C., Bakkour, S., Murphy, M.M., Nolla, H., and Sha, W.C. (2006). ICOS-induced B7h shedding on B cells is inhibited by TLR7/8 and TLR9. J Immunol 177, 2356-2364.

Lohning, M., Hutloff, A., Kallinich, T., Mages, H.W., Bonhagen, K., Radbruch, A., Hamelmann, E., and Kroczek, R.A. (2003). Expression of ICOS in vivo defines CD4+ effector T cells with high inflammatory potential and a strong bias for secretion of interleukin 10. The Journal of experimental medicine 197, 181-193.

Long, X., Lin, Y., Ortiz-Vega, S., Yonezawa, K., and Avruch, J. (2005). Rheb binds and regulates the mTOR kinase. Current biology : CB 15, 702-713.

Luik, R.M., and Lewis, R.S. (2007). New insights into the molecular mechanisms of store-operated Ca2+ signaling in T cells. Trends in molecular medicine 13, 103-107.

Luzina, I.G., Keegan, A.D., Heller, N.M., Rook, G.A., Shea-Donohue, T., and Atamas, S.P. (2012). Regulation of inflammation by interleukin-4: a review of "alternatives". Journal of leukocyte biology 92, 753-764.

Ma, C.S., Avery, D.T., Chan, A., Batten, M., Bustamante, J., Boisson-Dupuis, S., Arkwright, P.D., Kreins, A.Y., Averbuch, D., Engelhard, D.*, et al.* (2012). Functional STAT3 deficiency compromises the generation of human T follicular helper cells. Blood 119, 3997-4008.

Ma, C.S., Suryani, S., Avery, D.T., Chan, A., Nanan, R., Santner-Nanan, B., Deenick, E.K., and Tangye, S.G. (2009). Early commitment of naive human CD4(+) T cells to the T follicular helper (T(FH)) cell lineage is induced by IL-12. Immunology and cell biology 87, 590-600.

MacLennan, I.C. (1994). Germinal centers. Annual review of immunology 12, 117-139.

Mages, H.W., Hutloff, A., Heuck, C., Buchner, K., Himmelbauer, H., Oliveri, F., and Kroczek, R.A. (2000). Molecular cloning and characterization of murine ICOS and identification of B7h as ICOS ligand. European journal of immunology 30, 1040-1047.

Mak, T.W., Shahinian, A., Yoshinaga, S.K., Wakeham, A., Boucher, L.M., Pintilie, M., Duncan, G., Gajewska, B.U., Gronski, M., Eriksson, U.*, et al.* (2003). Costimulation through the inducible costimulator ligand is essential for both T helper and B cell functions in T cell-dependent B cell responses. Nature immunology 4, 765-772.

Manning, B.D., and Cantley, L.C. (2007). AKT/PKB signaling: navigating downstream. Cell 129, 1261-1274.

Marks, E., Verolin, M., Stensson, A., and Lycke, N. (2007). Differential CD28 and inducible costimulatory molecule signaling requirements for protective CD4+ T-cell-mediated immunity against genital tract Chlamydia trachomatis infection. Infection and immunity 75, 4638-4647.

McAdam, A.J., Chang, T.T., Lumelsky, A.E., Greenfield, E.A., Boussiotis, V.A., Duke-Cohan, J.S., Chernova, T., Malenkovich, N., Jabs, C., Kuchroo, V.K.*, et al.* (2000). Mouse inducible costimulatory molecule (ICOS) expression is enhanced by CD28 costimulation and regulates differentiation of CD4+ T cells. J Immunol 165, 5035-5040.

McAdam, A.J., Greenwald, R.J., Levin, M.A., Chernova, T., Malenkovich, N., Ling, V., Freeman, G.J., and Sharpe, A.H. (2001). ICOS is critical for CD40-mediated antibody class switching. Nature 409, 102-105.

McHeyzer-Williams, L.J., Pelletier, N., Mark, L., Fazilleau, N., and McHeyzer-Williams, M.G. (2009). Follicular helper T cells as cognate regulators of B cell immunity. Current opinion in immunology 21, 266-273.

McSorley, S.J., and Jenkins, M.K. (2000). Antibody is required for protection against virulent but not attenuated Salmonella enterica serovar typhimurium. Infection and immunity 68, 3344-3348.

Mehta, D.S., Wurster, A.L., Whitters, M.J., Young, D.A., Collins, M., and Grusby, M.J. (2003). IL-21 induces the apoptosis of resting and activated primary B cells. J Immunol 170, 4111-4118.

Meyuhas, O. (2000). Synthesis of the translational apparatus is regulated at the translational level. European journal of biochemistry / FEBS 267, 6321-6330.

Meyuhas, O., and Dreazen, A. (2009). Ribosomal protein S6 kinase from TOP mRNAs to cell size. Progress in molecular biology and translational science 90, 109-153.

Milburn, C.C., Deak, M., Kelly, S.M., Price, N.C., Alessi, D.R., and Van Aalten, D.M. (2003). Binding of phosphatidylinositol 3,4,5-trisphosphate to the pleckstrin homology domain of protein kinase B induces a conformational change. The Biochemical journal 375, 531-538.

Miller, J.F., De Burgh, P.M., and Grant, G.A. (1965). Thymus and the production of antibody-plaque-forming cells. Nature 208, 1332-1334.

Milne, S.B., Ivanova, P.T., DeCamp, D., Hsueh, R.C., and Brown, H.A. (2005). A targeted mass spectrometric analysis of phosphatidylinositol phosphate species. Journal of lipid research 46, 1796-1802.

Mittrucker, H.W., Kohler, A., Mak, T.W., and Kaufmann, S.H. (1999). Critical role of CD28 in protective immunity against Salmonella typhimurium. J Immunol 163, 6769-6776.

Mittrucker, H.W., Kursar, M., Kohler, A., Yanagihara, D., Yoshinaga, S.K., and Kaufmann, S.H. (2002). Inducible costimulator protein controls the protective T cell response against Listeria monocytogenes. J Immunol 169, 5813-5817.

Miyahira, Y., Akiba, H., Ogawa, S.H., Ishi, T., Watanabe, S., Kobayashi, S., Takeuchi, T., Aoki, T., Tezuka, K., Abe, R*., et al.* (2003). Involvement of ICOS-B7RP-1 costimulatory pathway in the regulation of immune responses to Leishmania major and Nippostrongylus brasiliensis infections. Immunology letters 89, 193-199.

Mohr, I., and Sonenberg, N. (2012). Host translation at the nexus of infection and immunity. Cell host & microbe 12, 470-483.

Mohrs, K., Wakil, A.E., Killeen, N., Locksley, R.M., and Mohrs, M. (2005). A two-step process for cytokine production revealed by IL-4 dual-reporter mice. Immunity 23, 419-429.

Mohrs, M., Blankespoor, C.M., Wang, Z.E., Loots, G.G., Afzal, V., Hadeiba, H., Shinkai, K., Rubin, E.M., and Locksley, R.M. (2001). Deletion of a coordinate regulator of type 2 cytokine expression in mice. Nature immunology 2, 842-847.

Mohrs, M., Ledermann, B., Kohler, G., Dorfmuller, A., Gessner, A., and Brombacher, F. (1999). Differences between IL-4- and IL-4 receptor alpha-deficient mice in chronic leishmaniasis reveal a protective role for IL-13 receptor signaling. J Immunol 162, 7302-7308.

Montagne, J., Stewart, M.J., Stocker, H., Hafen, E., Kozma, S.C., and Thomas, G. (1999). Drosophila S6 kinase: a regulator of cell size. Science (New York, N.Y 285, 2126-2129.

Morgan, D.A., Ruscetti, F.W., and Gallo, R. (1976). Selective in vitro growth of T lymphocytes from normal human bone marrows. Science (New York, N.Y 193, 1007-1008.

Morita, R., Schmitt, N., Bentebibel, S.E., Ranganathan, R., Bourdery, L., Zurawski, G., Foucat, E., Dullaers, M., Oh, S., Sabzghabaei, N*., et al.* (2011). Human Blood CXCR5(+)CD4(+) T Cells Are Counterparts of T Follicular Cells and Contain Specific Subsets that Differentially Support Antibody Secretion. Immunity.

Mosmann, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A., and Coffman, R.L. (1986). Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol 136, 2348-2357.

Mueller, D.L., Jenkins, M.K., and Schwartz, R.H. (1989). Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy. Annual review of immunology 7, 445-480.

Mukhopadhyay, N.K., Price, D.J., Kyriakis, J.M., Pelech, S., Sanghera, J., and Avruch, J. (1992). An array of insulin-activated, proline-directed serine/threonine protein kinases phosphorylate the p70 S6 kinase. The Journal of biological chemistry 267, 3325-3335.

Mulgrew-Nesbitt, A., Diraviyam, K., Wang, J., Singh, S., Murray, P., Li, Z., Rogers, L., Mirkovic, N., and Murray, D. (2006). The role of electrostatics in protein-membrane interactions. Biochimica et biophysica acta 1761, 812-826.

Munn, D.H., Sharma, M.D., and Mellor, A.L. (2004). Ligation of B7-1/B7-2 by human CD4+ T cells triggers indoleamine 2,3-dioxygenase activity in dendritic cells. J Immunol 172, 4100-4110.

Murphy, K.M., and Stockinger, B. (2010). Effector T cell plasticity: flexibility in the face of changing circumstances. Nature immunology 11, 674-680.

Nelms, K., Keegan, A.D., Zamorano, J., Ryan, J.J., and Paul, W.E. (1999). The IL-4 receptor: signaling mechanisms and biologic functions. Annual review of immunology 17, 701-738.

Noben-Trauth, N., Shultz, L.D., Brombacher, F., Urban, J.F., Jr., Gu, H., and Paul, W.E. (1997). An interleukin 4 (IL-4)-independent pathway for CD4+ T cell IL-4 production is revealed in IL-4 receptor-deficient mice. Proceedings of the National Academy of Sciences of the United States of America 94, 10838-10843.

Nukada, Y., Okamoto, N., Konakahara, S., Tezuka, K., Ohashi, K., Mizuno, K., and Tsuji, T. (2006). AILIM/ICOS-mediated elongation of activated T cells is regulated by both the PI3-kinase/Akt and Rho family cascade. International immunology 18, 1815-1824.

Nurieva, R., Yang, X.O., Martinez, G., Zhang, Y., Panopoulos, A.D., Ma, L., Schluns, K., Tian, Q., Watowich, S.S., Jetten, A.M., and Dong, C. (2007a). Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. Nature 448, 480-483.

Nurieva, R.I., Chung, Y., Hwang, D., Yang, X.O., Kang, H.S., Ma, L., Wang, Y.H., Watowich, S.S., Jetten, A.M., Tian, Q., and Dong, C. (2008). Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. Immunity 29, 138-149.

Nurieva, R.I., Chung, Y., Martinez, G.J., Yang, X.O., Tanaka, S., Matskevitch, T.D., Wang, Y.H., and Dong, C. (2009). Bcl6 mediates the development of T follicular helper cells. Science (New York, N.Y 325, 1001-1005.

Nurieva, R.I., Chuvpilo, S., Wieder, E.D., Elkon, K.B., Locksley, R., Serfling, E., and Dong, C. (2007b). A costimulation-initiated signaling pathway regulates NFATc1 transcription in T lymphocytes. J Immunol 179, 1096-1103.

Nurieva, R.I., Duong, J., Kishikawa, H., Dianzani, U., Rojo, J.M., Ho, I., Flavell, R.A., and Dong, C. (2003a). Transcriptional regulation of th2 differentiation by inducible costimulator. Immunity 18, 801-811.

Nurieva, R.I., Mai, X.M., Forbush, K., Bevan, M.J., and Dong, C. (2003b). B7h is required for T cell activation, differentiation, and effector function. Proceedings of the National Academy of Sciences of the United States of America 100, 14163-14168.

Nurieva, R.I., Podd, A., Chen, Y., Alekseev, A.M., Yu, M., Qi, X., Huang, H., Wen, R., Wang, J., Li, H.S*., et al.* (2012). STAT5 protein negatively regulates T follicular helper (Tfh) cell generation and function. The Journal of biological chemistry 287, 11234-11239.

Odegard, J.M., Marks, B.R., DiPlacido, L.D., Poholek, A.C., Kono, D.H., Dong, C., Flavell, R.A., and Craft, J. (2008). ICOS-dependent extrafollicular helper T cells elicit IgG production via IL-21 in systemic autoimmunity. The Journal of experimental medicine 205, 2873-2886.

Oestreich, K.J., and Weinmann, A.S. (2012). Master regulators or lineage-specifying? Changing views on CD4+ T cell transcription factors. Nature reviews 12, 799-804.

Ohtani, M., Nagai, S., Kondo, S., Mizuno, S., Nakamura, K., Tanabe, M., Takeuchi, T., Matsuda, S., and Koyasu, S. (2008). Mammalian target of rapamycin and glycogen synthase kinase 3 differentially regulate lipopolysaccharide-induced interleukin-12 production in dendritic cells. Blood 112, 635-643.

Okada, T., Miller, M.J., Parker, I., Krummel, M.F., Neighbors, M., Hartley, S.B., O'Garra, A., Cahalan, M.D., and Cyster, J.G. (2005). Antigen-engaged B cells undergo chemotaxis toward the T zone and form motile conjugates with helper T cells. PLoS biology 3, e150.

Okamoto, N., Nukada, Y., Tezuka, K., Ohashi, K., Mizuno, K., and Tsuji, T. (2004). AILIM/ICOS signaling induces T-cell migration/polarization of memory/effector T-cells. International immunology 16, 1515-1522.

Okamoto, N., Tezuka, K., Kato, M., Abe, R., and Tsuji, T. (2003). PI3-kinase and MAP-kinase signaling cascades in AILIM/ICOS- and CD28-costimulated T-cells have distinct functions between cell proliferation and IL-10 production. Biochemical and biophysical research communications 310, 691-702.

Okazaki, T., Maeda, A., Nishimura, H., Kurosaki, T., and Honjo, T. (2001). PD-1 immunoreceptor inhibits B cell receptor-mediated signaling by recruiting src homology 2-domain-containing tyrosine phosphatase 2 to phosphotyrosine. Proceedings of the National Academy of Sciences of the United States of America 98, 13866-13871.

Okkenhaug, K., Bilancio, A., Farjot, G., Priddle, H., Sancho, S., Peskett, E., Pearce, W., Meek, S.E., Salpekar, A., Waterfield, M.D*., et al.* (2002). Impaired B and T cell antigen receptor signaling in p110delta PI 3-kinase mutant mice. Science (New York, N.Y 297, 1031-1034.

Okkenhaug, K., and Fruman, D.A. (2010). PI3Ks in lymphocyte signaling and development. Current topics in microbiology and immunology 346, 57-85.

Okkenhaug, K., Wu, L., Garza, K.M., La Rose, J., Khoo, W., Odermatt, B., Mak, T.W., Ohashi, P.S., and Rottapel, R. (2001). A point mutation in CD28 distinguishes proliferative signals from survival signals. Nature immunology 2, 325-332.

Ozaki, K., Spolski, R., Ettinger, R., Kim, H.P., Wang, G., Qi, C.F., Hwu, P., Shaffer, D.J., Akilesh, S., Roopenian, D.C*., et al.* (2004). Regulation of B cell differentiation and plasma cell generation by IL-21, a novel inducer of Blimp-1 and Bcl-6. J Immunol 173, 5361-5371.

Ozaki, K., Spolski, R., Feng, C.G., Qi, C.F., Cheng, J., Sher, A., Morse, H.C., 3rd, Liu, C., Schwartzberg, P.L., and Leonard, W.J. (2002). A critical role for IL-21 in regulating immunoglobulin production. Science (New York, N.Y 298, 1630-1634.

Pagan, A.J., Pepper, M., Chu, H.H., Green, J.M., and Jenkins, M.K. (2012). CD28 promotes CD4+ T cell clonal expansion during infection independently of its YMNM and PYAP motifs. J Immunol 189, 2909-2917.

Pallikkuth, S., Parmigiani, A., Silva, S.Y., George, V.K., Fischl, M., Pahwa, R., and Pahwa, S. (2012). Impaired peripheral blood T-follicular helper cell function in HIV-infected nonresponders to the 2009 H1N1/09 vaccine. Blood 120, 985-993.

Pallikkuth, S., Pilakka Kanthikeel, S., Silva, S.Y., Fischl, M., Pahwa, R., and Pahwa, S. (2011). Upregulation of IL-21 receptor on B cells and IL-21 secretion distinguishes novel 2009 H1N1 vaccine responders from nonresponders among HIV-infected persons on combination antiretroviral therapy. J Immunol 186, 6173-6181.

Parry, R.V., Chemnitz, J.M., Frauwirth, K.A., Lanfranco, A.R., Braunstein, I., Kobayashi, S.V., Linsley, P.S., Thompson, C.B., and Riley, J.L. (2005). CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. Molecular and cellular biology 25, 9543-9553.

Parry, R.V., Riley, J.L., and Ward, S.G. (2007). Signalling to suit function: tailoring phosphoinositide 3-kinase during T-cell activation. Trends in immunology 28, 161-168.

Parry, R.V., Rumbley, C.A., Vandenberghe, L.H., June, C.H., and Riley, J.L. (2003). CD28 and inducible costimulatory protein Src homology 2 binding domains show distinct regulation of phosphatidylinositol 3-kinase, Bcl-xL, and IL-2 expression in primary human CD4 T lymphocytes. J Immunol 171, 166-174.

Patursky-Polischuk, I., Stolovich-Rain, M., Hausner-Hanochi, M., Kasir, J., Cybulski, N., Avruch, J., Ruegg, M.A., Hall, M.N., and Meyuhas, O. (2009). The TSC-mTOR pathway mediates translational activation of TOP mRNAs by insulin largely in a raptor- or rictor-independent manner. Molecular and cellular biology 29, 640-649.

Paul, W.E., and Ohara, J. (1987). B-cell stimulatory factor-1/interleukin 4. Annual review of immunology 5, 429-459.

Pause, A., Belsham, G.J., Gingras, A.C., Donze, O., Lin, T.A., Lawrence, J.C., Jr., and Sonenberg, N. (1994). Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5'-cap function. Nature 371, 762-767.

Pelletier, J., and Sonenberg, N. (1988). Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. Nature 334, 320-325.

Phan, R.T., and Dalla-Favera, R. (2004). The BCL6 proto-oncogene suppresses p53 expression in germinal-centre B cells. Nature 432, 635-639.

Powell, J.D., Pollizzi, K.N., Heikamp, E.B., and Horton, M.R. (2012). Regulation of immune responses by mTOR. Annual review of immunology 30, 39-68.

Pratama, A., Ramiscal, R.R., Silva, D.G., Das, S.K., Athanasopoulos, V., Fitch, J., Botelho, N.K., Chang, P.P., Hu, X., Hogan, J.J*., et al.* (2013). Roquin-2 Shares Functions with Its Paralog Roquin-1 in the Repression of mRNAs Controlling T Follicular Helper Cells and Systemic Inflammation. Immunity.

Qi, H. (2012). From SAP-less T cells to helpless B cells and back: dynamic T-B cell interactions underlie germinal center development and function. Immunological reviews 247, 24-35.

Qi, H., Cannons, J.L., Klauschen, F., Schwartzberg, P.L., and Germain, R.N. (2008). SAP-controlled T-B cell interactions underlie germinal centre formation. Nature 455, 764-769.

Ranuncolo, S.M., Polo, J.M., Dierov, J., Singer, M., Kuo, T., Greally, J., Green, R., Carroll, M., and Melnick, A. (2007). Bcl-6 mediates the germinal center B cell phenotype and lymphomagenesis through transcriptional repression of the DNA-damage sensor ATR. Nature immunology 8, 705-714.

Raught, B., Peiretti, F., Gingras, A.C., Livingstone, M., Shahbazian, D., Mayeur, G.L., Polakiewicz, R.D., Sonenberg, N., and Hershey, J.W. (2004). Phosphorylation of eucaryotic translation initiation factor 4B Ser422 is modulated by S6 kinases. The EMBO journal 23, 1761-1769.

Redoglia, V., Dianzani, U., Rojo, J.M., Portoles, P., Bragardo, M., Wolff, H., Buonfiglio, D., Bonissoni, S., and Janeway, C.A., Jr. (1996). Characterization of H4: a mouse T lymphocyte activation molecule functionally associated with the CD3/T cell receptor. European journal of immunology 26, 2781-2789.

Reinhardt, R.L., Liang, H.E., and Locksley, R.M. (2009). Cytokine-secreting follicular T cells shape the antibody repertoire. Nature immunology 10, 385-393.

Rengarajan, J., Mowen, K.A., McBride, K.D., Smith, E.D., Singh, H., and Glimcher, L.H. (2002). Interferon regulatory factor 4 (IRF4) interacts with NFATc2 to modulate interleukin 4 gene expression. The Journal of experimental medicine 195, 1003-1012.

Riley, J.L., Mao, M., Kobayashi, S., Biery, M., Burchard, J., Cavet, G., Gregson, B.P., June, C.H., and Linsley, P.S. (2002). Modulation of TCR-induced transcriptional profiles by ligation of CD28, ICOS, and CTLA-4 receptors. Proceedings of the National Academy of Sciences of the United States of America 99, 11790-11795.

Riviere, I., Sunshine, M.J., and Littman, D.R. (1998). Regulation of IL-4 expression by activation of individual alleles. Immunity 9, 217-228.

Rottman, J.B., Smith, T., Tonra, J.R., Ganley, K., Bloom, T., Silva, R., Pierce, B., Gutierrez-Ramos, J.C., Ozkaynak, E., and Coyle, A.J. (2001). The costimulatory molecule ICOS plays an important role in the immunopathogenesis of EAE. Nature immunology 2, 605-611.

Rudd, C.E., and Schneider, H. (2003). Unifying concepts in CD28, ICOS and CTLA4 co-receptor signalling. Nature reviews 3, 544-556.

Ruger, B.M., Erb, K.J., He, Y., Lane, J.M., Davis, P.F., and Hasan, Q. (2000). Interleukin-4 transgenic mice develop glomerulosclerosis independent of immunoglobulin deposition. European journal of immunology 30, 2698-2703.

Ruprecht, C.R., and Lanzavecchia, A. (2006). Toll-like receptor stimulation as a third signal required for activation of human naive B cells. European journal of immunology 36, 810-816.

Rush, J.S., Liu, M., Odegard, V.H., Unniraman, S., and Schatz, D.G. (2005). Expression of activation-induced cytidine deaminase is regulated by cell division, providing a mechanistic basis for division-linked class switch recombination. Proceedings of the National Academy of Sciences of the United States of America 102, 13242-13247.

Sabatini, D.M. (2006). mTOR and cancer: insights into a complex relationship. Nature reviews. Cancer 6, 729-734.

Sage, P.T., Francisco, L.M., Carman, C.V., and Sharpe, A.H. (2013). The receptor PD-1 controls follicular regulatory T cells in the lymph nodes and blood. Nature immunology 14, 152-161.

Saito, M., Novak, U., Piovan, E., Basso, K., Sumazin, P., Schneider, C., Crespo, M., Shen, Q., Bhagat, G., Califano, A*., et al.* (2009). BCL6 suppression of BCL2 via Miz1 and its disruption in diffuse large B cell lymphoma. Proceedings of the National Academy of Sciences of the United States of America 106, 11294-11299.

Sallusto, F., Geginat, J., and Lanzavecchia, A. (2004). Central memory and effector memory T cell subsets: function, generation, and maintenance. Annual review of immunology 22, 745-763.

Sancak, Y., Thoreen, C.C., Peterson, T.R., Lindquist, R.A., Kang, S.A., Spooner, E., Carr, S.A., and Sabatini, D.M. (2007). PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase. Molecular cell 25, 903-915.

Schaerli, P., Willimann, K., Lang, A.B., Lipp, M., Loetscher, P., and Moser, B. (2000). CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. The Journal of experimental medicine 192, 1553-1562.

Scheu, S., Stetson, D.B., Reinhardt, R.L., Leber, J.H., Mohrs, M., and Locksley, R.M. (2006). Activation of the integrated stress response during T helper cell differentiation. Nature immunology 7, 644-651.

Schmelzle, T., and Hall, M.N. (2000). TOR, a central controller of cell growth. Cell 103, 253-262.

Schmitt, N., Bustamante, J., Bourdery, L., Bentebibel, S.E., Boisson-Dupuis, S., Hamlin, F., Tran, M.V., Blankenship, D., Pascual, V., Savino, D.A*., et al.* (2013). IL-12 receptor beta1 deficiency alters in vivo T follicular helper cell response in humans. Blood 121, 3375-3385.

Schmitt, N., Morita, R., Bourdery, L., Bentebibel, S.E., Zurawski, S.M., Banchereau, J., and Ueno, H. (2009). Human dendritic cells induce the differentiation of interleukin-21-producing T follicular helper-like cells through interleukin-12. Immunity 31, 158-169.

Schwartz, R.H. (2003). T cell anergy. Annual review of immunology 21, 305-334.

Schwartzberg, P.L., Mueller, K.L., Qi, H., and Cannons, J.L. (2009). SLAM receptors and SAP influence lymphocyte interactions, development and function. Nature reviews 9, 39-46.

Schwickert, T.A., Victora, G.D., Fooksman, D.R., Kamphorst, A.O., Mugnier, M.R., Gitlin, A.D., Dustin, M.L., and Nussenzweig, M.C. (2011). A dynamic T cell-limited checkpoint regulates affinity-dependent B cell entry into the germinal center. The Journal of experimental medicine 208, 1243-1252.

Sengupta, S., Peterson, T.R., and Sabatini, D.M. (2010). Regulation of the mTOR complex 1 pathway by nutrients, growth factors, and stress. Molecular cell 40, 310-322.

Shahinian, A., Pfeffer, K., Lee, K.P., Kundig, T.M., Kishihara, K., Wakeham, A., Kawai, K., Ohashi, P.S., Thompson, C.B., and Mak, T.W. (1993). Differential T cell costimulatory requirements in CD28-deficient mice. Science (New York, N.Y 261, 609-612.

Sharpe, A.H., and Freeman, G.J. (2002). The B7-CD28 superfamily. Nature reviews 2, 116-126.

Shima, H., Pende, M., Chen, Y., Fumagalli, S., Thomas, G., and Kozma, S.C. (1998). Disruption of the p70(s6k)/p85(s6k) gene reveals a small mouse phenotype and a new functional S6 kinase. The EMBO journal 17, 6649-6659.

Shlomchik, M.J., and Weisel, F. (2012). Germinal centers. Immunological reviews 247, 5-10.

Shlomchik, W.D. (2007). Graft-versus-host disease. Nature reviews 7, 340-352.

Siepmann, K., Wohlleben, G., and Gray, D. (1996). CD40-mediated regulation of interleukin-4 signaling pathways in B lymphocytes. European journal of immunology 26, 1544-1552.

Simpson, N., Gatenby, P.A., Wilson, A., Malik, S., Fulcher, D.A., Tangye, S.G., Manku, H., Vyse, T.J., Roncador, G., Huttley, G.A*., et al.* (2010a). Expansion of circulating T cells resembling follicular helper T cells is a fixed phenotype that identifies a subset of severe systemic lupus erythematosus. Arthritis and rheumatism 62, 234-244.

Simpson, T.R., Quezada, S.A., and Allison, J.P. (2010b). Regulation of CD4 T cell activation and effector function by inducible costimulator (ICOS). Current opinion in immunology.

Sonenberg, N., and Hinnebusch, A.G. (2009). Regulation of translation initiation in eukaryotes: mechanisms and biological targets. Cell 136, 731-745.

Suto, A., Kashiwakuma, D., Kagami, S., Hirose, K., Watanabe, N., Yokote, K., Saito, Y., Nakayama, T., Grusby, M.J., Iwamoto, I., and Nakajima, H. (2008). Development and characterization of IL-21-producing CD4+ T cells. The Journal of experimental medicine 205, 1369-1379.

Swallow, M.M., Wallin, J.J., and Sha, W.C. (1999). B7h, a novel costimulatory homolog of B7.1 and B7.2, is induced by TNFalpha. Immunity 11, 423-432.

Tafuri, A., Shahinian, A., Bladt, F., Yoshinaga, S.K., Jordana, M., Wakeham, A., Boucher, L.M., Bouchard, D., Chan, V.S., Duncan, G*., et al.* (2001). ICOS is essential for effective T-helper-cell responses. Nature 409, 105-109.

Tai, X., Cowan, M., Feigenbaum, L., and Singer, A. (2005). CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2. Nature immunology 6, 152-162.

Tamatani, T., Tezuka, K., and Hanzawa-Higuchi, N. (2000). AILIM/ICOS: a novel lymphocyte adhesion molecule. International immunology 12, 51-55.

Tan, A.H., Goh, S.Y., Wong, S.C., and Lam, K.P. (2008). T helper cell-specific regulation of inducible costimulator expression via distinct mechanisms mediated by T-bet and GATA-3. The Journal of biological chemistry 283, 128-136.

Tan, A.H., Wong, S.C., and Lam, K.P. (2006). Regulation of mouse inducible costimulator (ICOS) expression by Fyn-NFATc2 and ERK signaling in T cells. The Journal of biological chemistry 281, 28666-28678.

Tang, H., Hornstein, E., Stolovich, M., Levy, G., Livingstone, M., Templeton, D., Avruch, J., and Meyuhas, O. (2001). Amino acid-induced translation of TOP mRNAs is fully dependent on phosphatidylinositol 3-kinase-mediated signaling, is partially inhibited by rapamycin, and is independent of S6K1 and rpS6 phosphorylation. Molecular and cellular biology 21, 8671-8683.

Tangye, S.G., Ma, C.S., Brink, R., and Deenick, E.K. (2013). The good, the bad and the ugly - TFH cells in human health and disease. Nature reviews 13, 412-426.

Tesciuba, A.G., Subudhi, S., Rother, R.P., Faas, S.J., Frantz, A.M., Elliot, D., Weinstock, J., Matis, L.A., Bluestone, J.A., and Sperling, A.I. (2001). Inducible costimulator regulates Th2-mediated inflammation, but not Th2 differentiation, in a model of allergic airway disease. J Immunol 167, 1996-2003.

Thoreen, C.C., Chantranupong, L., Keys, H.R., Wang, T., Gray, N.S., and Sabatini, D.M. (2012). A unifying model for mTORC1-mediated regulation of mRNA translation. Nature 485, 109-113.

Thoreen, C.C., Kang, S.A., Chang, J.W., Liu, Q., Zhang, J., Gao, Y., Reichling, L.J., Sim, T., Sabatini, D.M., and Gray, N.S. (2009). An ATP-competitive mammalian target of rapamycin inhibitor reveals rapamycin-resistant functions of mTORC1. The Journal of biological chemistry 284, 8023-8032.

Tonti, E., Fedeli, M., Napolitano, A., Iannacone, M., von Andrian, U.H., Guidotti, L.G., Abrignani, S., Casorati, G., and Dellabona, P. (2012). Follicular helper NKT cells induce limited B cell responses and germinal center formation in the absence of CD4(+) T cell help. J Immunol 188, 3217-3222.

Tubo, N.J., Pagan, A.J., Taylor, J.J., Nelson, R.W., Linehan, J.L., Ertelt, J.M., Huseby, E.S., Way, S.S., and Jenkins, M.K. (2013). Single naive CD4+ T cells from a diverse repertoire produce different effector cell types during infection. Cell 153, 785-796.

Vanhaesebroeck, B., Welham, M.J., Kotani, K., Stein, R., Warne, P.H., Zvelebil, M.J., Higashi, K., Volinia, S., Downward, J., and Waterfield, M.D. (1997). P110delta, a novel phosphoinositide 3-kinase in leukocytes. Proceedings of the National Academy of Sciences of the United States of America 94, 4330-4335.

Vantourout, P., and Hayday, A. (2013). Six-of-the-best: unique contributions of gammadelta T cells to immunology. Nature reviews 13, 88-100.

Veillette, A. (2006). Immune regulation by SLAM family receptors and SAP-related adaptors. Nature reviews 6, 56-66.

Vezina, C., Kudelski, A., and Sehgal, S.N. (1975). Rapamycin (AY-22,989), a new antifungal antibiotic. I. Taxonomy of the producing streptomycete and isolation of the active principle. The Journal of antibiotics 28, 721-726.

Victora, G.D., and Nussenzweig, M.C. (2012). Germinal centers. Annual review of immunology 30, 429-457.

Victora, G.D., Schwickert, T.A., Fooksman, D.R., Kamphorst, A.O., Meyer-Hermann, M., Dustin, M.L., and Nussenzweig, M.C. (2010). Germinal center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter. Cell 143, 592-605.

Vidric, M., Bladt, A.T., Dianzani, U., and Watts, T.H. (2006). Role for inducible costimulator in control of Salmonella enterica serovar Typhimurium infection in mice. Infection and immunity 74, 1050-1061.

Vidric, M., Suh, W.K., Dianzani, U., Mak, T.W., and Watts, T.H. (2005). Cooperation between 4-1BB and ICOS in the immune response to influenza virus revealed by studies of CD28/ICOS-deficient mice. J Immunol 175, 7288-7296.

Vieira, P.L., Wassink, L., Smith, L.M., Nam, S., Kingsbury, G.A., Gutierrez-Ramos, J.C., Coyle, A.J., Kapsenberg, M.L., and Wierenga, E.A. (2004). ICOS-mediated signaling regulates cytokine production by human T cells and provides a unique signal to selectively control the clonal expansion of Th2 helper cells. European journal of immunology 34, 1282-1290.

Vinuesa, C.G., Cook, M.C., Angelucci, C., Athanasopoulos, V., Rui, L., Hill, K.M., Yu, D., Domaschenz, H., Whittle, B., Lambe, T.*, et al.* (2005). A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity. Nature 435, 452-458.

Vinuesa, C.G., Linterman, M.A., Goodnow, C.C., and Randall, K.L. (2010). T cells and follicular dendritic cells in germinal center B-cell formation and selection. Immunological reviews 237, 72-89.

Vinuesa, C.G., Sanz, I., and Cook, M.C. (2009). Dysregulation of germinal centres in autoimmune disease. Nature reviews 9, 845-857.

Vogel, K.U., Edelmann, S.L., Jeltsch, K.M., Bertossi, A., Heger, K., Heinz, G.A., Zoller, J., Warth, S.C., Hoefig, K.P., Lohs, C.*, et al.* (2013). Roquin Paralogs 1 and 2 Redundantly Repress the Icos and Ox40 Costimulator mRNAs and Control Follicular Helper T Cell Differentiation. Immunity.

Vogelzang, A., McGuire, H.M., Yu, D., Sprent, J., Mackay, C.R., and King, C. (2008). A fundamental role for interleukin-21 in the generation of T follicular helper cells. Immunity 29, 127-137.

Walker, L.S., Gulbranson-Judge, A., Flynn, S., Brocker, T., Raykundalia, C., Goodall, M., Forster, R., Lipp, M., and Lane, P. (1999). Compromised OX40 function in CD28-deficient mice is linked with failure to develop CXC chemokine receptor 5-positive CD4 cells and germinal centers. The Journal of experimental medicine 190, 1115-1122.

Walker, L.S., Wiggett, H.E., Gaspal, F.M., Raykundalia, C.R., Goodall, M.D., Toellner, K.M., and Lane, P.J. (2003). Established T cell-driven germinal center B cell proliferation is independent of CD28 signaling but is tightly regulated through CTLA-4. J Immunol 170, 91-98.

Wallin, J.J., Liang, L., Bakardjiev, A., and Sha, W.C. (2001). Enhancement of CD8+ T cell responses by ICOS/B7h costimulation. J Immunol 167, 132-139.

Wang, G., Tschoi, M., Spolski, R., Lou, Y., Ozaki, K., Feng, C., Kim, G., Leonard, W.J., and Hwu, P. (2003). In vivo antitumor activity of interleukin 21 mediated by natural killer cells. Cancer research 63, 9016-9022.

Wang, S., Zhu, G., Chapoval, A.I., Dong, H., Tamada, K., Ni, J., and Chen, L. (2000). Costimulation of T cells by B7-H2, a B7-like molecule that binds ICOS. Blood 96, 2808-2813.

Wang, S., Zhu, G., Tamada, K., Chen, L., and Bajorath, J. (2002). Ligand binding sites of inducible costimulator and high avidity mutants with improved function. The Journal of experimental medicine 195, 1033-1041.

Wang, X., Li, W., Williams, M., Terada, N., Alessi, D.R., and Proud, C.G. (2001). Regulation of elongation factor 2 kinase by p90(RSK1) and p70 S6 kinase. The EMBO journal 20, 4370-4379.

Warnatz, K., Bossaller, L., Salzer, U., Skrabl-Baumgartner, A., Schwinger, W., van der Burg, M., van Dongen, J.J., Orlowska-Volk, M., Knoth, R., Durandy, A.*, et al.* (2006). Human ICOS deficiency abrogates the germinal center reaction and provides a monogenic model for common variable immunodeficiency. Blood 107, 3045-3052.

Wassink, L., Vieira, P.L., Smits, H.H., Kingsbury, G.A., Coyle, A.J., Kapsenberg, M.L., and Wierenga, E.A. (2004). ICOS expression by activated human Th cells is enhanced by IL-12 and IL-23: increased ICOS expression enhances the effector function of both Th1 and Th2 cells. J Immunol 173, 1779-1786.

Watanabe, M., Hara, Y., Tanabe, K., Toma, H., and Abe, R. (2005a). A distinct role for ICOS-mediated co-stimulatory signaling in CD4+ and CD8+ T cell subsets. International immunology 17, 269-278.

Watanabe, M., Takagi, Y., Kotani, M., Hara, Y., Inamine, A., Hayashi, K., Ogawa, S., Takeda, K., Tanabe, K., and Abe, R. (2008). Down-regulation of ICOS ligand by interaction with ICOS functions as a regulatory mechanism for immune responses. J Immunol 180, 5222-5234.

Watanabe, M., Watanabe, S., Hara, Y., Harada, Y., Kubo, M., Tanabe, K., Toma, H., and Abe, R. (2005b). ICOS-mediated costimulation on Th2 differentiation is achieved by the enhancement of IL-4 receptor-mediated signaling. J Immunol 174, 1989-1996.

Watts, T.H. (2005). TNF/TNFR family members in costimulation of T cell responses. Annual review of immunology 23, 23-68.

Wei, L., Laurence, A., Elias, K.M., and O'Shea, J.J. (2007). IL-21 is produced by Th17 cells and drives IL-17 production in a STAT3-dependent manner. The Journal of biological chemistry 282, 34605-34610.

Weichhart, T., Costantino, G., Poglitsch, M., Rosner, M., Zeyda, M., Stuhlmeier, K.M., Kolbe, T., Stulnig, T.M., Horl, W.H., Hengstschlager, M.*, et al.* (2008). The TSC-mTOR signaling pathway regulates the innate inflammatory response. Immunity 29, 565-577.

Weinkove, D., and Leevers, S.J. (2000). The genetic control of organ growth: insights from Drosophila. Current opinion in genetics & development 10, 75-80.

Weng, Q.P., Kozlowski, M., Belham, C., Zhang, A., Comb, M.J., and Avruch, J. (1998). Regulation of the p70 S6 kinase by phosphorylation in vivo. Analysis using site-specific anti-phosphopeptide antibodies. The Journal of biological chemistry 273, 16621-16629.

Wollenberg, I., Agua-Doce, A., Hernandez, A., Almeida, C., Oliveira, V.G., Faro, J., and Graca, L. (2011). Regulation of the germinal center reaction by Foxp3+ follicular regulatory T cells. J Immunol 187, 4553-4560.

Wong, S.C., Oh, E., Ng, C.H., and Lam, K.P. (2003). Impaired germinal center formation and recall T-cell-dependent immune responses in mice lacking the costimulatory ligand B7-H2. Blood 102, 1381-1388.

Wullschleger, S., Loewith, R., and Hall, M.N. (2006). TOR signaling in growth and metabolism. Cell 124, 471-484.

Wurster, A.L., Rodgers, V.L., Satoskar, A.R., Whitters, M.J., Young, D.A., Collins, M., and Grusby, M.J. (2002). Interleukin 21 is a T helper (Th) cell 2 cytokine that specifically inhibits the differentiation of naive Th cells into interferon gamma-producing Th1 cells. The Journal of experimental medicine 196, 969-977.

Xu, C., Gagnon, E., Call, M.E., Schnell, J.R., Schwieters, C.D., Carman, C.V., Chou, J.J., and Wucherpfennig, K.W. (2008). Regulation of T cell receptor activation by dynamic membrane binding of the CD3epsilon cytoplasmic tyrosine-based motif. Cell 135, 702-713.

Yao, S., Zhu, Y., Zhu, G., Augustine, M., Zheng, L., Goode, D.J., Broadwater, M., Ruff, W., Flies, S., Xu, H*., et al.* (2011). B7-h2 is a costimulatory ligand for CD28 in human. Immunity 34, 729-740.

Ye, B.H., Cattoretti, G., Shen, Q., Zhang, J., Hawe, N., de Waard, R., Leung, C., Nouri-Shirazi, M., Orazi, A., Chaganti, R.S*., et al.* (1997). The BCL-6 proto-oncogene controls germinal-centre formation and Th2-type inflammation. Nature genetics 16, 161-170.

Yong, P.F., Salzer, U., and Grimbacher, B. (2009). The role of costimulation in antibody deficiencies: ICOS and common variable immunodeficiency. Immunological reviews 229, 101-113.

Yoshida, H., Nishina, H., Takimoto, H., Marengere, L.E., Wakeham, A.C., Bouchard, D., Kong, Y.Y., Ohteki, T., Shahinian, A., Bachmann, M*., et al.* (1998). The transcription factor NF-ATc1 regulates lymphocyte proliferation and Th2 cytokine production. Immunity 8, 115-124.

Yoshinaga, S.K., Whoriskey, J.S., Khare, S.D., Sarmiento, U., Guo, J., Horan, T., Shih, G., Zhang, M., Coccia, M.A., Kohno, T*., et al.* (1999). T-cell co-stimulation through B7RP-1 and ICOS. Nature 402, 827-832.

Yoshinaga, S.K., Zhang, M., Pistillo, J., Horan, T., Khare, S.D., Miner, K., Sonnenberg, M., Boone, T., Brankow, D., Dai, T*., et al.* (2000). Characterization of a new human B7-related protein: B7RP-1 is the ligand to the co-stimulatory protein ICOS. International immunology 12, 1439-1447.

Yu, D., Rao, S., Tsai, L.M., Lee, S.K., He, Y., Sutcliffe, E.L., Srivastava, M., Linterman, M., Zheng, L., Simpson, N*., et al.* (2009). The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. Immunity 31, 457-468.

Yu, D., Tan, A.H., Hu, X., Athanasopoulos, V., Simpson, N., Silva, D.G., Hutloff, A., Giles, K.M., Leedman, P.J., Lam, K.P*., et al.* (2007). Roquin represses autoimmunity by limiting inducible T-cell co-stimulator messenger RNA. Nature 450, 299-303.

Yu, D., and Vinuesa, C.G. (2010a). The elusive identity of T follicular helper cells. Trends in immunology.

Yu, D., and Vinuesa, C.G. (2010b). Multiple checkpoints keep follicular helper T cells under control to prevent autoimmunity. Cellular & molecular immunology.

Yuan, T.L., and Cantley, L.C. (2010). Introduction. Current topics in microbiology and immunology 346, 1-7.

Yusuf, I., Kageyama, R., Monticelli, L., Johnston, R.J., Ditoro, D., Hansen, K., Barnett, B., and Crotty, S. (2010). Germinal Center T Follicular Helper Cell IL-4 Production Is Dependent on Signaling Lymphocytic Activation Molecule Receptor (CD150). J Immunol.

Zamoyska, R., Basson, A., Filby, A., Legname, G., Lovatt, M., and Seddon, B. (2003). The influence of the src-family kinases, Lck and Fyn, on T cell differentiation, survival and activation. Immunological reviews 191, 107-118.

Zang, X., Loke, P., Kim, J., Wojnoonski, K., Kusdra, L., and Allison, J.P. (2006). A genetic library screen for signaling proteins that interact with phosphorylated T cell costimulatory receptors. Genomics 88, 841-845.

Zaretsky, A.G., Taylor, J.J., King, I.L., Marshall, F.A., Mohrs, M., and Pearce, E.J. (2009). T follicular helper cells differentiate from Th2 cells in response to helminth antigens. The Journal of experimental medicine 206, 991-999.

Zeng, R., Spolski, R., Finkelstein, S.E., Oh, S., Kovanen, P.E., Hinrichs, C.S., Pise-Masison, C.A., Radonovich, M.F., Brady, J.N., Restifo, N.P.*, et al.* (2005). Synergy of IL-21 and IL-15 in regulating CD8+ T cell expansion and function. The Journal of experimental medicine 201, 139-148.

Zhang, S., Readinger, J.A., DuBois, W., Janka-Junttila, M., Robinson, R., Pruitt, M., Bliskovsky, V., Wu, J.Z., Sakakibara, K., Patel, J.*, et al.* (2011). Constitutive reductions in mTOR alter cell size, immune cell development, and antibody production. Blood 117, 1228-1238.

Zhu, J., Yamane, H., and Paul, W.E. (2010). Differentiation of effector CD4 T cell populations (*). Annual review of immunology 28, 445-489.

Zotos, D., Coquet, J.M., Zhang, Y., Light, A., D'Costa, K., Kallies, A., Corcoran, L.M., Godfrey, D.I., Toellner, K.M., Smyth, M.J.*, et al.* (2010). IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism. The Journal of experimental medicine 207, 365-378.

Chapter II

Inducible costimulator promotes helper T cell differentiation through phosphoinositide 3-kinase

The costimulatory receptor ICOS is important for the differentiation of Tfh cells and for the formation of GCs. However, how ICOS costimulation induces Tfh cell differentiation is currently unknown. In this manuscript, we identify the ICOS-PI3K axis as a crucial mediator of Tfh cell differentiation and GC formation. We also present evidence which suggests that ICOS and CD28 play differential roles in the multistep process of Tfh cell differentiation.

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Inducible costimulator promotes helper T cell differentiation through phosphoinositide 3-kinase

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1. ABSTRACT

The T cell costimulatory receptors, CD28 and the inducible costimulator (ICOS), are required for the generation of follicular B helper T cells (T_{FH}) and germinal center (GC) reaction. A common signal transducer utilized by CD28 and ICOS is the phosphoinositide 3-kinase (PI3K). Although it is known that CD28-mediated PI3K activation is dispensable for GC reaction, the role of ICOS-driven PI3K signaling has not been defined. We show here that knock-in mice that selectively lost the ability to activate PI3K through ICOS had severe defects in T_{FH} generation, GC reaction, antibody class switch, and antibody affinity maturation. In pre-activated $CD4^+$ T cells, ICOS delivered a potent PI3K signal which was critical for the induction of the key T_{FH} cytokines, IL-21 and IL-4. Under the same settings, CD28 was unable to activate PI3K but supported a robust secondary expansion of T cells. Thus, our results demonstrate a non-redundant function of ICOS-PI3K pathway in the generation of T_{FH} and suggest that CD28 and ICOS play differential roles during a multi-step process of T_{FH} differentiation.

2. INTRODUCTION

Follicular B helper T cells (T_{FH}) are a subset of $CD4^+$ T cells that facilitates germinal center (GC) reaction, B cell proliferation, and B cell differentiation (Vinuesa et al., 2005b). T_{FH} cells have an ability to migrate into B cell area using chemokine receptor CXCR5 and they abundantly express costimulatory molecules such as ICOS, PD-1 and CD40L. T_{FH} cells can arise in the absence of factors that mediate Th1, Th2, or Th17 differentiation but depend on Bcl-6 (Nurieva et al., 2008; Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009). T_{FH} cells express a high level of IL-21 which provides a robust stimulus for proliferation and differentiation of B cells (Bryant et al., 2007; Dullaers et al., 2009). IL-21 also plays an indispensible role in the generation of T_{FH} cells, probably by enhancing Bcl-6 expression (Nurieva et al., 2009). Recent studies also revealed an exquisite regulation of IL-4 transcription and translation that allows highly targeted secretion of IL-4 by T_{FH} cells while they form conjugates with cognate B cells (Reinhardt et al., 2009). Thus, IL-21 and IL-4 appear to be crucial for differentiation and/or function of T_{FH} cells.

ICOS is a CD28 family costimulatory receptor that is expressed in recently activated or antigen-experienced T cells (Hutloff et al., 1999; Greenwald et al., 2005). By binding to ICOS ligand (ICOS-L) expressed on antigen presenting cells (APCs), ICOS delivers costimulatory signals that augment T cell proliferation and expression of an array of cytokines including IL-4, IL-10, and IL-21 (Lohning et al., 2003; Greenwald et al., 2005; Vogelzang et al., 2008). Both in mice and humans, interruption of ICOS-ICOS-L interaction leads to impaired GC reaction, Ab class switch and affinity maturation (Dong et al., 2001; McAdam et al., 2001; Tafuri et al., 2001; Grimbacher et al., 2003). Recent findings suggested that these defects in humoral immune responses in ICOS-deficiency is due to the lack of T_{FH} cells (Akiba et al., 2005; Bossaller et al., 2006; Hu et al., 2009). Conversely, dysregulated overexpression of ICOS in *sanroque* mice causes a lupus-like autoimmune disease that is associated with an increased number of T_{FH} cells, spontaneous GC reaction, and augmented IL-21 production (Vinuesa et al., 2005a; Yu et al., 2007; Linterman et al., 2009b).

The prototype T cell costimulator CD28 is also required for GC reaction, humoral immunity, and generation of T_{FH} cells (Ferguson et al., 1996; Linterman et al., 2009a). It is puzzling that the generation of T_{FH} requires both CD28 and ICOS although the two costimulators have a seemingly redundant function in activating PI3K (Arimura et al., 2002; Parry et al., 2003). Whether CD28-mediated PI3K pathway plays significant roles in T cell proliferation, cytokine production, and survival has been a matter of hot debate (Parry et al., 2007). Recent data from knockin mice showed that CD28-mediated PI3K pathways do not have any obvious non-redundant role in T cell functions and humoral immune responses (Dodson et al., 2009).

In order to address the role of ICOS-mediated PI3K signal transduction pathways in the context of the overall ICOS function, we generated a knockin mouse strain in which the cytoplasmic tail of ICOS cannot recruit PI3K. Here we show that the generation of T_{FH} cells critically depends on the PI3K signaling initiated by ICOS. Consequently, GC reaction, Ab class switch and affinity maturation are drastically diminished in the knockin mice. We find evidence that in pre-activated $CD4^+$ T cells, expression of IL-21 and IL-4 is heavily dependent on PI3K and that the dominant activator of PI3K in this context is ICOS, not CD28.

3. RESULTS

3.1 Normal inducible expression pattern of ICOS-YF with altered signaling capacities

We generated knock-in mice, termed ICOS-YF hereafter, possessing a tyrosine-to-phenylalanine mutation at amino acid residue 181 in the cytoplasmic tail of ICOS, a mutation known to abrogate ICOS-mediated PI3K recruitment (Nurieva et al., 2007) (Details in SI Method and Fig. S1). We compared littermates of ICOS-WT (+/+) and ICOS-YF (*yf/yf*) mice along with non-littermate ICOS-KO (-/-) mice that have less than 2 weeks of age difference. All these mice have been backcrossed five generations into C57BL/6.

Since tyrosine residues in the cytoplasmic tails of membrane proteins are often involved in protein trafficking and recycling, we tested whether ICOS-YF maintained its expression pattern on the cell surface. As shown in Fig. S2A, WT and YF-mutant ICOS

displayed an identical inducible expression pattern. Thus, the tyrosine-to-phenylalanine mutation does not alter the expression pattern of ICOS and all the phenotypic outcomes should be attributable to the altered signaling capacities of the mutant ICOS.

In vitro binding assays using GST fusion proteins have shown that the Tyr 181 residue of ICOS is critical for recruiting PI3K (Nurieva et al., 2007). Consistently, anti-ICOS immunoprecipitates from WT $CD4^+$ T cell blasts contained the regulatory subunit of PI3K, p85α (Fig. S2B, WT). There was a basal level of p85α associated with ICOS which increased upon ligation of TCR or ICOS. The TCR-independent ICOS-mediated p85α recruitment may reflect a potential antigen-independent function of ICOS on cytoskeletal rearrangement of T cells (Franko and Levine, 2009). However, the amount of p85α was maximal when the T cells were activated by a combination of anti-CD3 and anti-ICOS mAb. Importantly, the ICOS-p85α interaction was completely abrogated when the Tyr 181 was mutated to phenylalanine (Fig. S2B, YF).

It has been shown that ligation of ICOS strongly enhances TCR-mediated activation of AKT and, to some extent, MAPKs (ERK, JNK, and p38) (Arimura et al., 2002; Parry et al., 2007). We examined these signal transduction events in primary T cell blasts derived from WT or ICOS-YF mice. In keeping with the PI3K activation, ICOS engagement dramatically augmented TCR-mediated AKT activation as judged by the increase phosphorylation of AKT at Ser473 in WT T cells (Fig. 1, AKT, WT). The ability of ICOS to enhance TCR-mediated AKT activation was completely abrogated in ICOS-YF T cells (Fig. 1, AKT, YF). ERK phosphorylation was moderately enhanced by ICOS ligation in WT but not in ICOS-YF. This is consistent with the observations that PI3K can activate Ras-MAPK pathway (Okkenhaug et al., 2002; Wells et al., 2007). ICOS did not augment phosphorylation of JNK and p38 in primary $CD4^+$ blasts under our experimental settings (Fig. S3). As shown by others (Arimura et al., 2002; Parry et al., 2003), CD28-costimulation strongly enhanced JNK activation with a moderate level of AKT phosphorylation (Fig. S4).

It was shown that ligation of ICOS can facilitate Ca^{2+} mobilization when TCR signal is suboptimal, possibly through PI3K (Parry et al., 2003; Nurieva et al., 2007). As shown

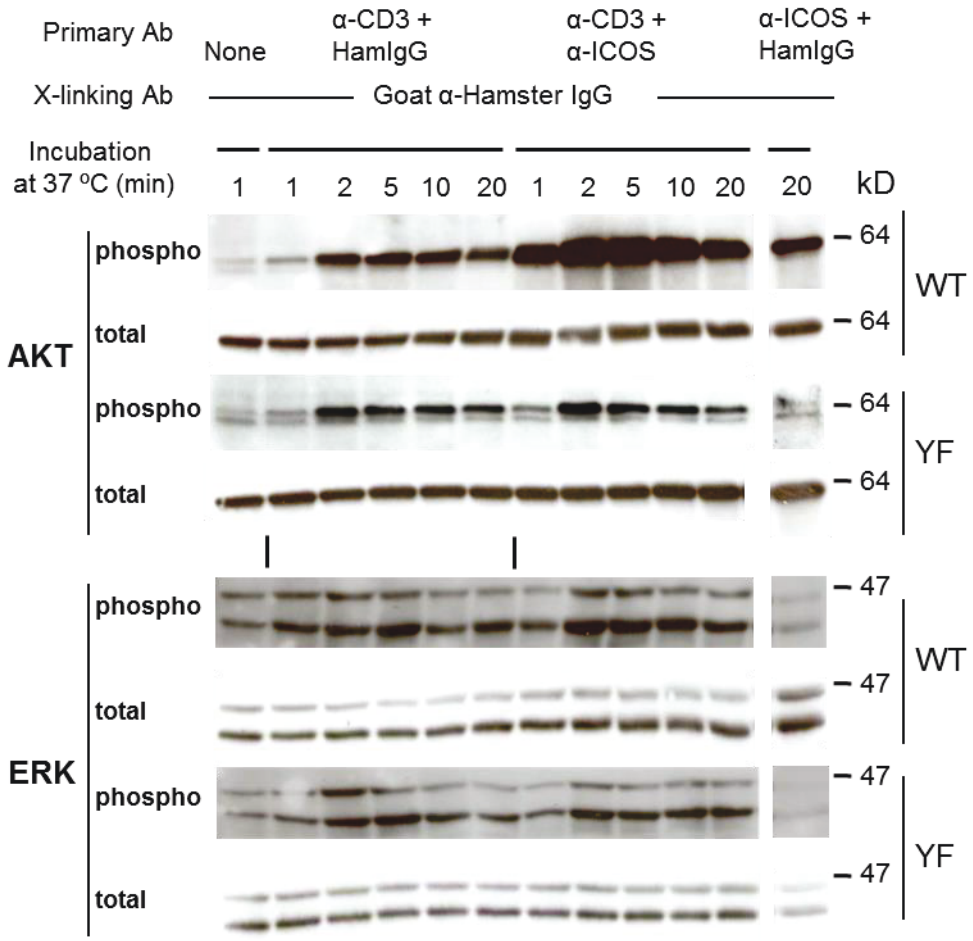

Figure 1. Defective AKT and ERK activation by ICOS-YF. $CD4^+$ T blasts were stimulated with antibodies against CD3 and/or ICOS and the activation of AKT or MAPKs was measured by immunoblotting using phospho-specific antibodies. A representive of three independent experiments is shown.

in Fig. S5, both WT and Y181F mutant ICOS were able to augment TCR mediated Ca^{2+} flux in $CD4^+$ T blasts. Thus, ICOS can augment TCR-mediated Ca^{2+} flux in a PI3K-independent manner.

Collectively, the Y181F mutation selectively disrupts PI3K-dependent signaling pathways, AKT and ERK, without affecting Ca^{2+} signaling.

3.2 Reduced basal serum immunoglobulin levels in ICOS-YF mice

One of the hallmarks of ICOS-deficient mice or humans is a reduction of class-switched immunoglobulins in serum, a reflection of defective GC reaction (Dong et al., 2001; McAdam et al., 2001; Tafuri et al., 2001; Grimbacher et al., 2003). Thus, we quantified the basal serum Ig levels by ELISA from 2-month-old mice of WT, YF, and KO mice (Fig. 2). As previously documented, ICOS-KO mice displayed up to 10-fold reduction in serum concentrations of IgG1, IgG2b, and IgG2c without any difference in IgM compared with WT control. Remarkably, ICOS-YF mice had an identical serum IgG1 level as that of ICOS-KO. Serum IgG2b and IgG2c concentrations in ICOS-YF mice were also reduced to levels close to those of ICOS-KO mice. These results suggest that the ICOS function in supporting Ig class switch critically relies on signaling mechanisms dependent on the Tyr 181.

3.3 Defective GC reaction in Peyer's patches of ICOS mutants

Peyer's patches are part of gut-associated immune tissue in which ongoing humoral immune responses against the intestinal microflora is taking place. It has been shown that, in ICOS-KO mice, the number of PPs is normal but the size and cellularity of PPs are dramatically reduced and the active GCs are not detected (Iiyama et al., 2003). However, the basis of these defects has been unknown. We chose to analyze the PPs of ICOS mutant mice to gain insights into the cellular basis of GC defects. ICOS-YF mice had a normal number of PPs, as do ICOS-KO mice (Fig. S6A). However, the total cellularity of PP was substantially reduced in ICOS-YF mice to a level similar to that of ICOS-KO mice (Fig. S6B). Accordingly, the GC area was greatly reduced in both ICOS-YF and KO mice (Fig. 6C). Flow cytometric analysis revealed that there is a drastic reduction in the percentage of GC B cells over the total B cells (Fig. 3, top two panels). Importantly, the percentages

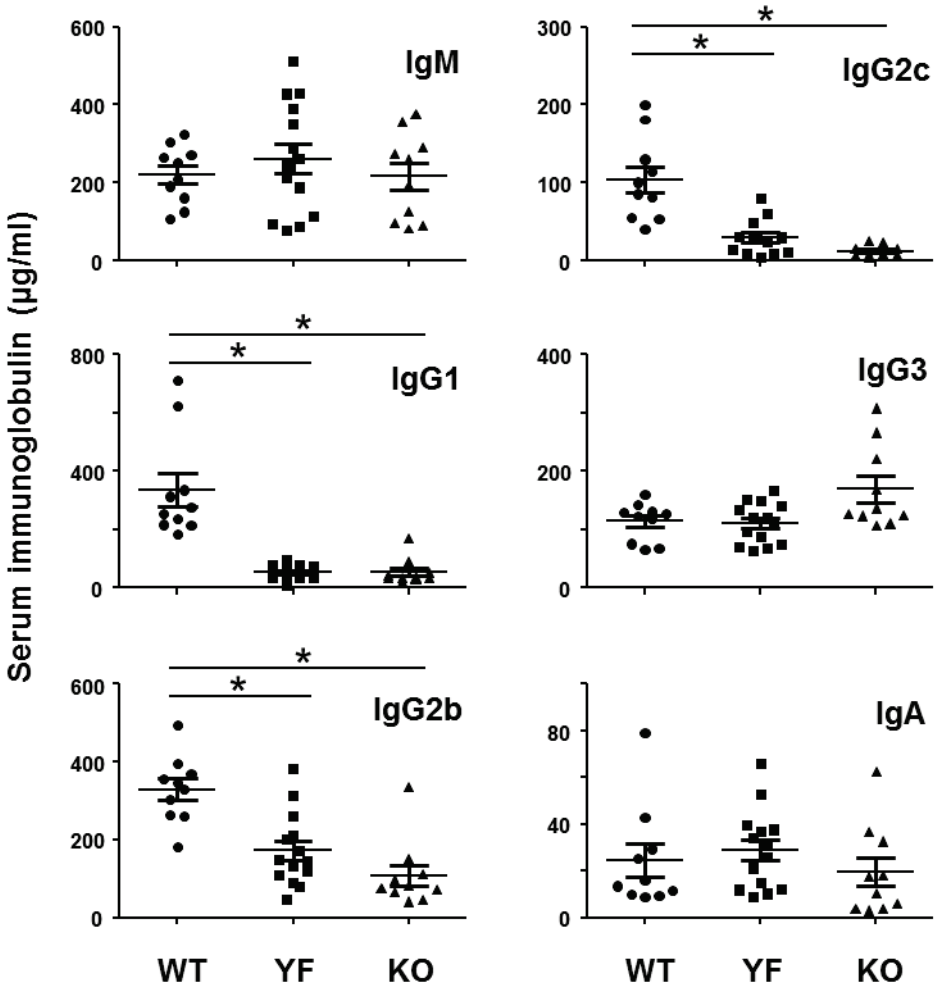

Figure 2. ICOS-YF and KO mice have reduced levels of class-switched immunoglobulins in serum. Sera were obtained from non-immunized mice of ICOS WT, YF, and KO mice at 8-weeks of age. Concentrations of IgM, G1, G2a, G2c, G3, and A were determined by isotype-specific ELISA. Each data point represents a serum Ig level of an individual mouse (n = 10 WT, 14 YF, and 10 KO). * $P < 0.01$

of T_{FH} cells over the total $CD4^+$ T cells in the PPs of ICOS-YF and KO were reduced by 5- to 9-fold compared with that of WT mice (Fig. 3, bottom two panels). Consistent with the GC defects the content of secreted IgA in the feces was dramatically reduced in YF and KO mice (Fig. S6D). This is in contrast to the normal serum IgA levels in ICOS mutant mice (Fig. 2) suggesting that the serum IgA level is mainly controlled by T-independent mechanisms (Cerutti, 2008). Thus, the Tyr 181 motif of ICOS plays a critical role in T_{FH} differentiation and GC reaction in the PP.

3.4 Defective humoral immunity in ICOS mutants

It has been shown that ICOS-KO mice have impaired GC reaction, Ab class switch and affinity maturation upon immunization (Dong et al., 2001; McAdam et al., 2001; Tafuri et al., 2001). We immunized mice with alum-precipitated NP-CGG to examine the role of ICOS-PI3K pathway in humoral immune responses. Both ICOS-YF and KO mice had severely impaired GC reaction along with reduced T_{FH} cells in the spleen (Fig. 4A and 4B). Anti-NP IgG1 antibody titers in serum were substantially reduced in both ICOS-YF and KO mice (Fig. 4C). The difference in anti-NP IgG1 titer was more pronounced in high-affinity Ab (Fig. 4C, NP3) as opposed to the total Ab (Fig. 4C, NP33). It was also clear that the difference in high-affinity Ab titers became bigger upon secondary immunization (Fig. 4C, NP3 1º vs 2º). We assessed affinity maturation process more precisely by measuring anti-NP antibodies after a differential washing step using NaSCN solutions during ELISA assay (SI Method). As depicted in Fig. 4D, a shift from lower to higher affinity anti-NP IgG1 was readily seen in WT mice upon secondary immunization but this was not observed in ICOS-YF and KO mice. Collectively, these data demonstrate that ICOS-YF mice have severely impaired GC reaction, T_{FH} generation, Ab class switch and affinity maturation.

3.5 ICOS promotes expression of IL-21 and IL-4 in a PI3K-dependent manner

The lack of $CXCR5^+CD4^+$ T_{FH} cells during humoral immune reaction in ICOS mutant mice prompted us to examine if ICOS is directly involved in upregulation of CXCR5. When T cells were activated by soluble anti-CD3 Ab in the presence of APCs, there was no difference in the expression levels of OX40 and CXCR5 on $CD4^+$ T cells (Fig. S7A).

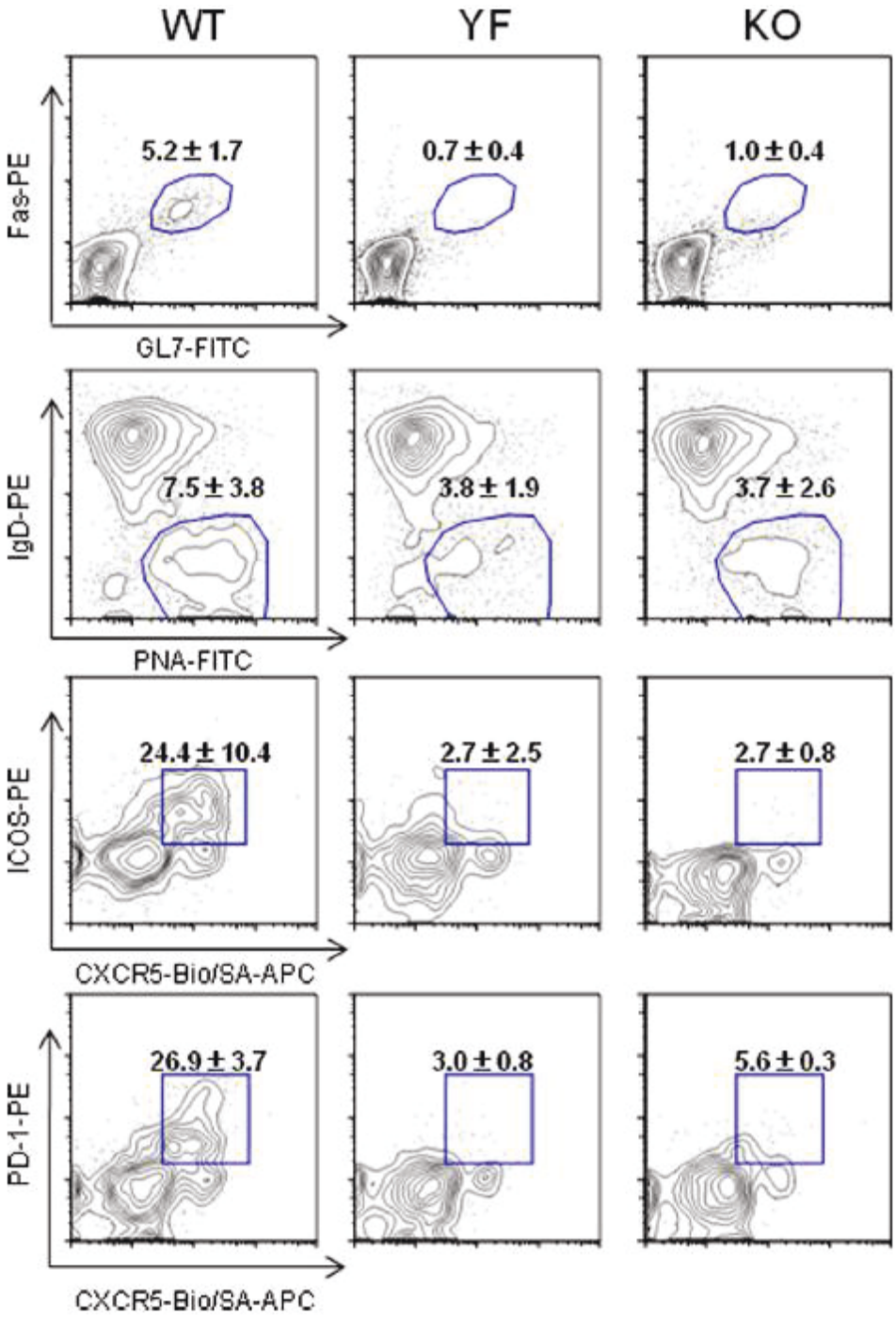

Figure 3. ICOS-YF as well as KO mice have severely impaired humoral immune responses in the PP. GC B cells and T_{FH} cells in the PP were analyzed by flow cytometry. The top two panels were gated on $B220^+$ B cells and the bottom two on $CD4^+$ T cells. Numbers represent mean ± S.D. of data pooled from four mice per genotype.

This result is consistent with the notion that CXCR5 is mainly induced by OX40 whose expression is enhanced by CD28 costimulation (Walker et al., 1999). Thus, ICOS is not required for the induction of CXCR5 and the lack of $CXCR5^+CD4^+$ cells in ICOS mutants probably reflects a failed T_{FH} differentiation program.

Next, we sought to examine the impact of ICOS costimulation on cytokine gene expression in the $CD4^+$ T cells. We activated highly purified $CD4^+$ T cells for two days *in vitro* using antibodies against CD3 and CD28, rested them one day in the absence of stimuli, and then restimulated the cells with a combination of TCR and costimulatory signals. This regimen allowed us to utilize $CD4^+$ T cells with maximal surface ICOS expression within a time frame when primed $CD4^+$ T cells migrate to B cell follicles in secondary lymphoid organs (day 3 post-immunization) (Garside et al., 1998). Under these conditions, ICOS played a dominant role over CD28 in augmentation of IL-21 and IL-4 expression (Fig. 5A). Further, pharmacological inhibition of PI3K activity during the restimulation period negated all the costimulatory impacts of ICOS on IL-21 and IL-4 expression. In contrast, IL-10 expression was marginally increased by ICOS and CD28 but largely unaffected by PI3K inhibition. Consistent with results from PI3K inhibition experiments, ICOS-mediated upregulation of IL-21 and IL-4 was abrogated in ICOS-YF T cells to levels close to those of ICOS-KO T cells (Fig. 5B). In parallel, the differences in cytokine induction capacity of ICOS vs CD28 in the primed $CD4^+$ T cells well correlated with their abilities to activate PI3K: a potent AKT phosphorylation by ICOS but not by CD28 (Fig. S7B). Despite the weak costimulatory activity in cytokine expression, CD28 had a major impact on the secondary expansion of primed $CD4^+$ T cells whereas ICOS played minor contribution (Fig. S7C). Interestingly, the ICOS-mediated proliferation is disrupted in ICOS-YF T cells, suggesting that the ICOS-PI3K pathway may have an additional role in the secondary expansion of primed $CD4^+$ T cells. Taken together, these data show that ICOS-PI3K signaling plays a dominant role in augmenting expression of IL-21 and IL-4 during secondary activation of $CD4^+$ T cells and that CD28 is not able to substitute ICOS.

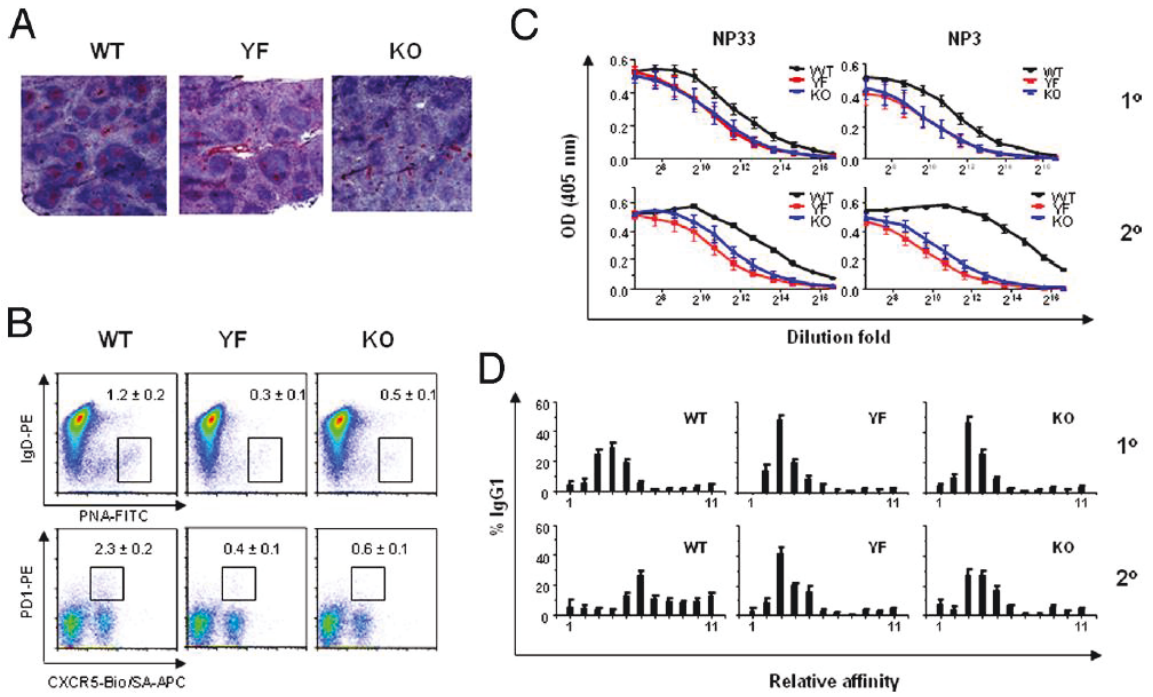

Figure 4. Impaired Ab responses in ICOS-YF and ICOS-KO mice upon immunization. (*A*) Defective GC reaction. Cryosections of spleens from mice immunized with NP_{16}-CGG/alum 12 days before were stained with PNA. (*B*) Decreased GC B cells and T_{FH} cells in ICOS-YF and KO mice. Mice were immunized with NP_{16}-CGG/alum and the splenocytes were analyzed 12 days later. Percentages represent mean ± S.D. of data from 3 mice per genotype. A representative of two independent experiments. (*C*) Impaired class switch. Sera were prepared from immunized mice at day 11 (1°) or day 7 (2°) post-injection and antigen-specific IgG1 were measured by ELISA using NP_{33}- vs NP_3-BSA. Numbers represent mean ± S.D. of data from six mice per genotype. A representative of two independent experiments. (*D*) Defective Ab affinity maturation. Mice were immunized at day 0 and boost injected at day 30. Serum samples were prepared at day 11 after primary injection or day 7 after secondary injection and the anti-NP IgG1 was measured after differential washes with increasing concentrations of NaSCN solution. Each histogram represents mean ± S.D. of data from six mice per genotype. A representative of two independent experiments.

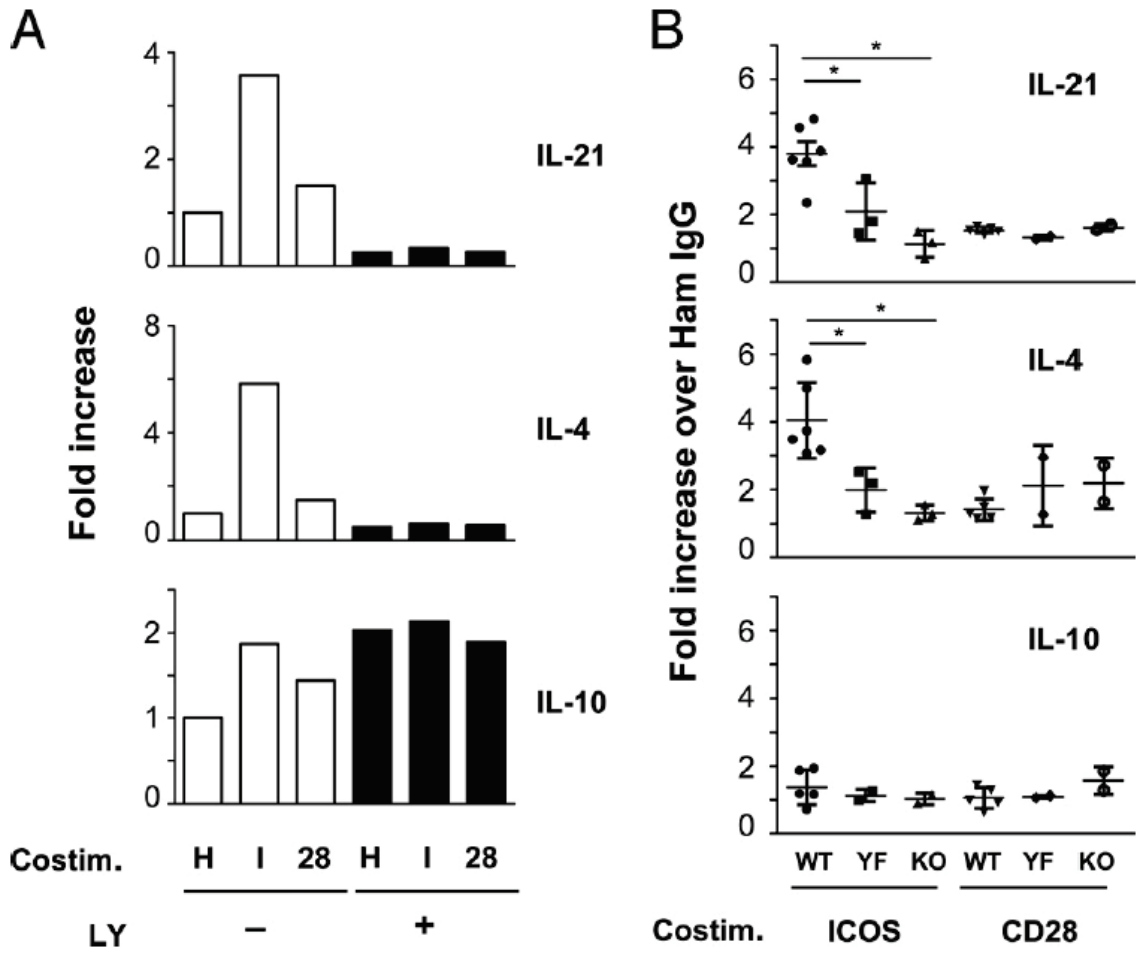

Figure 5. ICOS-YF and ICOS-KO CD4$^+$ T cells have defects in IL-21 and IL-4 expression. (*A*) ICOS induces IL-21 and IL-4 in a PI3K-dependent manner. Pre-activated CD4$^+$ T cells were restimulated with anti-CD3 plus hamster IgG (H), anti-ICOS (I), or anti-CD28 (28) followed by goat anti-hamster IgG for 6 hours without or with LY 294002. The cytokine mRNA levels in the restimulated cells were analyzed by qPCR. A representative of three independent experiments. (*B*) Abrogation of IL-21 and IL-4 induction in ICOS-YF T cells. Cytokine gene expression was analyzed in WT, YF, and KO T cells as described in (*A*). Each data point represents fold increase over hamster IgG control. Data pooled from six independent experiments (n = 5-6 WT, 2-3 YF, and 2-3 KO). * $P < 0.02$.

4. DISCUSSION

In this study we show that ICOS potently activates PI3K in synergy with the TCR. When its ability to activate PI3K is selectively abrogated, ICOS cannot support the generation of T_{FH} cells, GC reaction, Ab class switch and affinity maturation. We found a non-redundant role of ICOS-PI3K pathway in upregulation of IL-21 and IL-4, key cytokines crucial for T_{FH} generation and function.

It has been shown that in murine Th2 clones, ICOS is constitutively bound to PI3K and ICOS ligation further increases PI3K recruitment (Feito et al., 2003). We confirmed these results in activated $CD4^+$ T blasts. The finding that ligation of ICOS without TCR stimulation can activate PI3K signaling cascades explains TCR-independent, yet PI3K-dependent ICOS function in cytoskeletal rearrangement that may have a potential role in T cell adherence and migration (Franko and Levine, 2009). However, it has been shown that interruption of ICOS function does not affect T cell trafficking itself *in vivo* (Smith et al., 2003; Odegard et al., 2009). Regardless, it is clear that co-ligation of the TCR and ICOS gives rise to a maximal PI3K signaling. Biochemical and imaging data have indicated that ICOS is in complex with TCR complexes and it gets recruited into the immunological synapses supporting the view that ICOS probably functions in conjunction with the TCR (Redoglia et al., 1996; Buonfiglio et al., 1999; Fos et al., 2008).

The mechanism by which ICOS enhances TCR-mediated Ca^{2+} mobilization is not clear. It was proposed that the ICOS-mediated PI3K pathway can enhance PLCγ1 function through ITK leading to sustained Ca^{2+} flux (Nurieva et al., 2007). Since ICOS-YF T cells have intact Ca^{2+} flux, we conclude that ICOS can mediate Ca^{2+} flux through a yet unknown mechanism but clearly in a PI3K-independent manner. Although the overall defects in humoral immunity in ICOS-YF mice are very close to those of ICOS-KO mice, we observed marginally higher levels of serum IgG2b and IgG2c in ICOS-YF mice compared to ICOS-KO mice (Fig. 2). Also, pre-activated ICOS-YF $CD4^+$ T cells produced slightly higher levels of IL-21 and IL-4 compared to ICOS-KO counterparts (Fig. 5B). The intact capacity of ICOS-YF to augment TCR-mediated Ca^{2+} flux may explain these residual T cell functions.

Our finding that PI3K plays a key role in ICOS-mediated T_{FH} cells is very relevant to the results that inactivation of the p110δ isoform of PI3K leads to impaired humoral immunity and reduced size of PPs in the gut (Okkenhaug et al., 2002). It will be interesting to see whether lack of T_{FH} underlies these phenotypes and whether the p110δ isoform is the PI3K under the control of ICOS.

Similar to ICOS, CD28 can activate PI3K through its Tyr-based motif (YMNM) in the cytoplasmic tail (Parry et al., 2007). Why is CD28 unable to compensate the lack of ICOS-PI3K signaling pathway? Our data show that it is due to an intrinsic weak capacity of CD28 to activate PI3K compared with that of ICOS. It has been shown that the membrane proximal segment containing the YMFM motif of the ICOS tail is much stronger than its CD28 counterpart (containing the YMNM motif) in the ability to activate PI3K when chimeric receptors were compared in transfected human $CD4^+$ T cells (Parry et al., 2003). The same observation was made in murine $CD4^+$ T cells upon ligation of endogenous CD28 and ICOS (Arimura et al., 2002). Particularly, in $CD4^+$ T cells that rested for one day after 2-day stimulation, CD28 did not evoke any PI3K activity above the TCR stimulation whereas it could still strongly enhance secondary expansion of T cells (Fig. S7B, C). Recent results from CD28 knockin mice reinforced the notion that CD28-mediated PI3K does not play any non-redundant function in T cell proliferation, cytokine production, and survival; it is rather signals emanating from the carboxy terminal proline-rich motif that play more important roles (Dodson et al., 2009).

Deficiency of either CD28 or ICOS results in defective T_{FH} and humoral immunity suggesting distinct roles for the two costimulators (Ferguson et al., 1996; Walker et al., 1999; Dong et al., 2001; McAdam et al., 2001; Tafuri et al., 2001; Linterman et al., 2009a). What differential roles do they play during the process of T_{FH} generation? Based on our results and the data in literature, we propose a model in which CD28 and ICOS support T_{FH} cell differentiation in rather specialized manners. During the first 2-3 days of antigenic exposure, $CD4^+$ T cells interact with dendritic cells in the T cell zone of secondary lymphoid organs (Garside et al., 1998). At this stage antigen-specific T cells rapidly proliferate producing IL-2, upregulate CXCR5 through OX40, and induce ICOS. Since these processes are known to be dependent on CD28 costimulation (Walker et al., 1999;

McAdam et al., 2000; Sharpe and Freeman, 2002), CD28-deficiency may heavily compromise T_{FH} differentiation at this stage. The primed $CD4^+$ T cells then migrate towards B cell follicles and interact with cognate B cells. During this T:B interaction, T cells make helper cytokines such as IL-21 and IL-4. IL-21 may play a key role in facilitating full differentiation of T_{FH} cells (Vogelzang et al., 2008; Nurieva et al., 2009) whereas IL-4 and IL-21 induce B cell proliferation and differentiation (Bryant et al., 2007). Our data show that ICOS provides a unique PI3K-mediated signal to enhance IL-21 and IL-4 at this stage and CD28 cannot substitute ICOS. Therefore, ICOS-deficiency is likely to block this later stage of T_{FH} differentiation. This model is consistent with the finding that a transient activation of CD28 at the early phase of immunization is sufficient for GC formation (Walker et al., 2003). On the other hand, T cells primed in B cell-deficient mice showed normal expansion but failed to induce Ab class switch during *in vitro* coculture with B cells suggesting an incomplete helper T differentiation in the absence of B cells (Macaulay et al., 1998).

In sum, we demonstrated here that the function of ICOS in supporting T_{FH} generation and humoral immunity critically relies on the evolutionarily conserved tyrosine-based signaling motif in its cytoplasmic tail. We provided evidence that ICOS can induce key T_{FH} cytokines by activating PI3K through this signaling motif probably when primed T cells contact with B cells to complete the T_{FH} differentiation program.

5. MATERIALS AND METHODS

Animals. ICOS-YF knock-in mice were generated in 129 background and then backcrossed into C57BL/6 background. ICOS-KO mice have been previously described (Tafuri et al., 2001). The animals 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. Details of the reagents are described in Supporting Information.

***In vitro* T cell culture.** All T cells were cultured in RPMI1640 medium (1 X 10^6 cells/ml) supplemented with 10% FBS, glutamine, penicillin, streptomycin, β-mercaptoethanol. Total LN cells were activated by soluble anti-CD3 (1 μg/ml). For preparation of $CD4^+$ T blasts for biochemical analyses and Ca^{2+} flux experiments, $CD4^+$ T cells were positively selected (> 95%) from single cells suspensions of spleen and LN using CD4 selection kit (Stem Cell Technologies) and then activated by culturing with plate-bound anti-CD3 (3 ug/ml) plus soluble anti-CD28 (1 μg/ml) for 2 days and expanded in media containing recombinant IL-2 (100 μg/ml) for 3 days. For experiments described in Fig. 5, S7B, and S7C, splenic $CD4^+$ T cells were negatively selected (> 90%) using the MACS $CD4^+$ T cell isolation kit (Myltenyi). The $CD4^+$ T cells were stimulated for 2 days as described above except that 10 ng/mL of IL-6 was added to enhance IL-21 expression (Dienz et al., 2009). Subsequently, the activated cells were collected, washed once in complete medium and rested for 1 day in complete medium without IL-2 at 1 x 10^6 cells/mL in 6-well plates (2 mL/well) to avoid overcrowding.

Acute T cell activation, lysis, and immunoprecipitaton. The $CD4^+$ T cell blasts were harvested and stimulated by combinations of anti-CD3 (1 μg/ml), anti-ICOS (2 μg/ml), and control hamster IgG. The bound antibodies were cross-linked by goat anti-hamster IgG (20 μg/ml) at 37 ºC. After washing, cells were lysed in lysis buffer (1 % NP40, 20 mM Tris pH 7.4, 137 mM NaCl, 1 mM $Cacl_2$, 1 mM $MgCl_2$, 1 mM PMSF, and 0.1 mM sodium orthovanadate) for 20 min on ice. After clearance of cell debris, lysates were boiled in

Laemmli sample buffer for immunoblot analysis. For immunoprecipitation of ICOS-associated proteins, the lysates were incubated with anti-ICOS antibody (2 μg/ml) for 1 hr at 4 ºC and the immune complexes were recovered by a mixture of protein G-agarose beads (Thermo Scientific) and protein A-agarose beads (Pierce).

T cell restimulation assays. Negatively selected $CD4^+$ T cells were stimulated for two days and rested for one day in media alone. For cytokine qPCR, five million $CD4^+$ T blasts were re-stimulated for 6 hours in 400 μl of media containing Ab cocktails: anti-CD3 (1 μg/mL) plus either hamster IgG, anti-ICOS, or anti-CD28 (2 μg/mL each) followed by goat anti-hamster IgG (20 μg/mL). For PI3K inhibition experiments, cells were pretreated for 1 hour with LY 294002 (50 μM, Calbiochem) and then stimulated with the Abs in the continued presence of the inhibitor. RNA was isolated using the Trizol reagent (Invitrogen). cDNA was prepared from the extracted RNA using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). HGPRT, IL-21, IL-4, and IL-10 TaqMan primers and probes were from Applied Biosystems. Quantitative real-time PCR was performed by using a TaqMan 7300/7500 system and software (Applied Biosystems). Fold expression was calculated using the $\Delta\Delta^{CT}$ method using HGPRT as a reference gene. For proliferation assays, cells were restimulated in U-bottom 96 wells (1 X 10^5 cells/well) with the Abs for 24 hours. For the last 8 hours of incubation, ^{3}H-thymidine was added at 1 μCi/well.

Ca^{2+} flux, immunization of mice, and ELISA. Details are described in Supproting Information.

Statistical analysis. The significance of the data was tested by Student's *t* test.

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REFERENCES

Akiba, H., Takeda, K., Kojima, Y., Usui, Y., Harada, N., Yamazaki, T., Ma, J., Tezuka, K., Yagita, H., and Okumura, K. (2005). The role of ICOS in the CXCR5+ follicular B helper T cell maintenance in vivo. J Immunol 175, 2340-2348.

Arimura, Y., Kato, H., Dianzani, U., Okamoto, T., Kamekura, S., Buonfiglio, D., Miyoshi-Akiyama, T., Uchiyama, T., and Yagi, J. (2002). A co-stimulatory molecule on activated T cells, H4/ICOS, delivers specific signals in T(h) cells and regulates their responses. International immunology 14, 555-566.

Bossaller, L., Burger, J., Draeger, R., Grimbacher, B., Knoth, R., Plebani, A., Durandy, A., Baumann, U., Schlesier, M., Welcher, A.A.*, et al.* (2006). ICOS deficiency is associated with a severe reduction of CXCR5+CD4 germinal center Th cells. J Immunol 177, 4927-4932.

Bryant, V.L., Ma, C.S., Avery, D.T., Li, Y., Good, K.L., Corcoran, L.M., de Waal Malefyt, R., and Tangye, S.G. (2007). Cytokine-mediated regulation of human B cell differentiation into Ig-secreting cells: predominant role of IL-21 produced by CXCR5+ T follicular helper cells. J Immunol 179, 8180-8190.

Buonfiglio, D., Bragardo, M., Bonissoni, S., Redoglia, V., Cauda, R., Zupo, S., Burgio, V.L., Wolff, H., Franssila, K., Gaidano, G.*, et al.* (1999). Characterization of a novel human surface molecule selectively expressed by mature thymocytes, activated T cells and subsets of T cell lymphomas. European journal of immunology 29, 2863-2874.

Cerutti, A. (2008). The regulation of IgA class switching. Nature reviews 8, 421-434.

Dienz, O., Eaton, S.M., Bond, J.P., Neveu, W., Moquin, D., Noubade, R., Briso, E.M., Charland, C., Leonard, W.J., Ciliberto, G.*, et al.* (2009). The induction of antibody production by IL-6 is indirectly mediated by IL-21 produced by CD4+ T cells. The Journal of experimental medicine 206, 69-78.

Dodson, L.F., Boomer, J.S., Deppong, C.M., Shah, D.D., Sim, J., Bricker, T.L., Russell, J.H., and Green, J.M. (2009). Targeted knock-in mice expressing mutations of CD28 reveal an essential pathway for costimulation. Molecular and cellular biology 29, 3710-3721.

Dong, C., Juedes, A.E., Temann, U.A., Shresta, S., Allison, J.P., Ruddle, N.H., and Flavell, R.A. (2001). ICOS co-stimulatory receptor is essential for T-cell activation and function. Nature 409, 97-101.

Dullaers, M., Li, D., Xue, Y., Ni, L., Gayet, I., Morita, R., Ueno, H., Palucka, K.A., Banchereau, J., and Oh, S. (2009). A T cell-dependent mechanism for the induction of human mucosal homing immunoglobulin A-secreting plasmablasts. Immunity 30, 120-129.

Feito, M.J., Vaschetto, R., Criado, G., Sanchez, A., Chiocchetti, A., Jimenez-Perianez, A., Dianzani, U., Portoles, P., and Rojo, J.M. (2003). Mechanisms of H4/ICOS costimulation: effects on proximal TCR signals and MAP kinase pathways. European journal of immunology 33, 204-214.

Ferguson, S.E., Han, S., Kelsoe, G., and Thompson, C.B. (1996). CD28 is required for germinal center formation. J Immunol 156, 4576-4581.

Fos, C., Salles, A., Lang, V., Carrette, F., Audebert, S., Pastor, S., Ghiotto, M., Olive, D., Bismuth, G., and Nunes, J.A. (2008). ICOS ligation recruits the p50alpha PI3K regulatory subunit to the immunological synapse. J Immunol 181, 1969-1977.

Franko, J.L., and Levine, A.D. (2009). Antigen-independent adhesion and cell spreading by inducible costimulator engagement inhibits T cell migration in a PI-3K-dependent manner. Journal of leukocyte biology 85, 526-538.

Garside, P., Ingulli, E., Merica, R.R., Johnson, J.G., Noelle, R.J., and Jenkins, M.K. (1998). Visualization of specific B and T lymphocyte interactions in the lymph node. Science (New York, N.Y 281, 96-99.

Greenwald, R.J., Freeman, G.J., and Sharpe, A.H. (2005). The B7 family revisited. Annual review of immunology 23, 515-548.

Grimbacher, B., Hutloff, A., Schlesier, M., Glocker, E., Warnatz, K., Drager, R., Eibel, H., Fischer, B., Schaffer, A.A., Mages, H.W.*, et al.* (2003). Homozygous loss of ICOS is associated with adult-onset common variable immunodeficiency. Nature immunology 4, 261-268.

Hu, Y.L., Metz, D.P., Chung, J., Siu, G., and Zhang, M. (2009). B7RP-1 blockade ameliorates autoimmunity through regulation of follicular helper T cells. J Immunol 182, 1421-1428.

Hutloff, A., Dittrich, A.M., Beier, K.C., Eljaschewitsch, B., Kraft, R., Anagnostopoulos, I., and Kroczek, R.A. (1999). ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. Nature 397, 263-266.

Iiyama, R., Kanai, T., Uraushihara, K., Totsuka, T., Nakamura, T., Miyata, T., Yagita, H., Kushi, A., Suzuki, K., Tezuka, K., and Watanabe, M. (2003). The role of inducible co-stimulator (ICOS)/B7-related protein-1 (B7RP-1) interaction in the functional development of Peyer's patches. Immunology letters 88, 63-70.

Johnston, R.J., Poholek, A.C., DiToro, D., Yusuf, I., Eto, D., Barnett, B., Dent, A.L., Craft, J., and Crotty, S. (2009). Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. Science (New York, N.Y 325, 1006-1010.

Linterman, M.A., Rigby, R.J., Wong, R., Silva, D., Withers, D., Anderson, G., Verma, N.K., Brink, R., Hutloff, A., Goodnow, C.C., and Vinuesa, C.G. (2009a). Roquin differentiates the specialized functions of duplicated T cell costimulatory receptor genes CD28 and ICOS. Immunity 30, 228-241.

Linterman, M.A., Rigby, R.J., Wong, R.K., Yu, D., Brink, R., Cannons, J.L., Schwartzberg, P.L., Cook, M.C., Walters, G.D., and Vinuesa, C.G. (2009b). Follicular helper T cells are required for systemic autoimmunity. The Journal of experimental medicine 206, 561-576.

Lohning, M., Hutloff, A., Kallinich, T., Mages, H.W., Bonhagen, K., Radbruch, A., Hamelmann, E., and Kroczek, R.A. (2003). Expression of ICOS in vivo defines CD4+ effector T cells with high inflammatory potential and a strong bias for secretion of interleukin 10. The Journal of experimental medicine 197, 181-193.

Luxton, R.W., and Thompson, E.J. (1990). Affinity distributions of antigen-specific IgG in patients with multiple sclerosis and in patients with viral encephalitis. Journal of immunological methods 131, 277-282.

Macaulay, A.E., DeKruyff, R.H., and Umetsu, D.T. (1998). Antigen-primed T cells from B cell-deficient JHD mice fail to provide B cell help. J Immunol 160, 1694-1700.

McAdam, A.J., Chang, T.T., Lumelsky, A.E., Greenfield, E.A., Boussiotis, V.A., Duke-Cohan, J.S., Chernova, T., Malenkovich, N., Jabs, C., Kuchroo, V.K.*, et al.* (2000). Mouse inducible costimulatory molecule (ICOS) expression is enhanced by CD28 costimulation and regulates differentiation of CD4+ T cells. J Immunol 165, 5035-5040.

McAdam, A.J., Greenwald, R.J., Levin, M.A., Chernova, T., Malenkovich, N., Ling, V., Freeman, G.J., and Sharpe, A.H. (2001). ICOS is critical for CD40-mediated antibody class switching. Nature 409, 102-105.

Nurieva, R.I., Chung, Y., Hwang, D., Yang, X.O., Kang, H.S., Ma, L., Wang, Y.H., Watowich, S.S., Jetten, A.M., Tian, Q., and Dong, C. (2008). Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. Immunity 29, 138-149.

Nurieva, R.I., Chung, Y., Martinez, G.J., Yang, X.O., Tanaka, S., Matskevitch, T.D., Wang, Y.H., and Dong, C. (2009). Bcl6 mediates the development of T follicular helper cells. Science (New York, N.Y 325, 1001-1005.

Nurieva, R.I., Chuvpilo, S., Wieder, E.D., Elkon, K.B., Locksley, R., Serfling, E., and Dong, C. (2007). A costimulation-initiated signaling pathway regulates NFATc1 transcription in T lymphocytes. J Immunol 179, 1096-1103.

Odegard, J.M., DiPlacido, L.D., Greenwald, L., Kashgarian, M., Kono, D.H., Dong, C., Flavell, R.A., and Craft, J. (2009). ICOS controls effector function but not trafficking receptor expression of kidney-infiltrating effector T cells in murine lupus. J Immunol 182, 4076-4084.

Okkenhaug, K., Bilancio, A., Farjot, G., Priddle, H., Sancho, S., Peskett, E., Pearce, W., Meek, S.E., Salpekar, A., Waterfield, M.D.*, et al.* (2002). Impaired B and T cell antigen receptor signaling in p110delta PI 3-kinase mutant mice. Science (New York, N.Y 297, 1031-1034.

Parry, R.V., Riley, J.L., and Ward, S.G. (2007). Signalling to suit function: tailoring phosphoinositide 3-kinase during T-cell activation. Trends in immunology 28, 161-168.

Parry, R.V., Rumbley, C.A., Vandenberghe, L.H., June, C.H., and Riley, J.L. (2003). CD28 and inducible costimulatory protein Src homology 2 binding domains show distinct regulation of phosphatidylinositol 3-kinase, Bcl-xL, and IL-2 expression in primary human CD4 T lymphocytes. J Immunol 171, 166-174.

Redoglia, V., Dianzani, U., Rojo, J.M., Portoles, P., Bragardo, M., Wolff, H., Buonfiglio, D., Bonissoni, S., and Janeway, C.A., Jr. (1996). Characterization of H4: a mouse T lymphocyte activation molecule functionally associated with the CD3/T cell receptor. European journal of immunology 26, 2781-2789.

Reinhardt, R.L., Liang, H.E., and Locksley, R.M. (2009). Cytokine-secreting follicular T cells shape the antibody repertoire. Nature immunology 10, 385-393.

Schwenk, F., Baron, U., and Rajewsky, K. (1995). A cre-transgenic mouse strain for the ubiquitous deletion of loxP-flanked gene segments including deletion in germ cells. Nucleic acids research 23, 5080-5081.

Sharpe, A.H., and Freeman, G.J. (2002). The B7-CD28 superfamily. Nature reviews 2, 116-126.

Smith, K.M., Brewer, J.M., Webb, P., Coyle, A.J., Gutierrez-Ramos, C., and Garside, P. (2003). Inducible costimulatory molecule-B7-related protein 1 interactions are

important for the clonal expansion and B cell helper functions of naive, Th1, and Th2 T cells. J Immunol 170, 2310-2315.

Tafuri, A., Shahinian, A., Bladt, F., Yoshinaga, S.K., Jordana, M., Wakeham, A., Boucher, L.M., Bouchard, D., Chan, V.S., Duncan, G.*, et al.* (2001). ICOS is essential for effective T-helper-cell responses. Nature 409, 105-109.

Vinuesa, C.G., Cook, M.C., Angelucci, C., Athanasopoulos, V., Rui, L., Hill, K.M., Yu, D., Domaschenz, H., Whittle, B., Lambe, T.*, et al.* (2005a). A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity. Nature 435, 452-458.

Vinuesa, C.G., Tangye, S.G., Moser, B., and Mackay, C.R. (2005b). Follicular B helper T cells in antibody responses and autoimmunity. Nature reviews 5, 853-865.

Vogelzang, A., McGuire, H.M., Yu, D., Sprent, J., Mackay, C.R., and King, C. (2008). A fundamental role for interleukin-21 in the generation of T follicular helper cells. Immunity 29, 127-137.

Walker, L.S., Gulbranson-Judge, A., Flynn, S., Brocker, T., Raykundalia, C., Goodall, M., Forster, R., Lipp, M., and Lane, P. (1999). Compromised OX40 function in CD28-deficient mice is linked with failure to develop CXC chemokine receptor 5-positive CD4 cells and germinal centers. The Journal of experimental medicine 190, 1115-1122.

Walker, L.S., Wiggett, H.E., Gaspal, F.M., Raykundalia, C.R., Goodall, M.D., Toellner, K.M., and Lane, P.J. (2003). Established T cell-driven germinal center B cell proliferation is independent of CD28 signaling but is tightly regulated through CTLA-4. J Immunol 170, 91-98.

Wells, V., Downward, J., and Mallucci, L. (2007). Functional inhibition of PI3K by the betaGBP molecule suppresses Ras-MAPK signalling to block cell proliferation. Oncogene 26, 7709-7714.

Yu, D., Rao, S., Tsai, L.M., Lee, S.K., He, Y., Sutcliffe, E.L., Srivastava, M., Linterman, M., Zheng, L., Simpson, N.*, et al.* (2009). The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. Immunity 31, 457-468.

Yu, D., Tan, A.H., Hu, X., Athanasopoulos, V., Simpson, N., Silva, D.G., Hutloff, A., Giles, K.M., Leedman, P.J., Lam, K.P.*, et al.* (2007). Roquin represses autoimmunity by limiting inducible T-cell co-stimulator messenger RNA. Nature 450, 299-303.

SUPPORTING INFORMATION

SI METHODS

Generation of ICOS-YF mice.

We designed a targeting construct such that the exon 3 of the wild type *Icos* gene will be replaced by an engineered exon 3 and a neomycin-resistance (Neo) cassette flanked by two loxP elements (Fig. S1A). The engineered exon 3 contained an adenine-to-thymidine change that results in a tyrosine-to-phenylalanine mutation at amino acid 181 of the cytoplasmic tail of ICOS. Utilizing a novel *EcoRI* site created by the mutation, we identified ES clones (R1, 129 background) that have integrated the mutant allele by Southern blot analysis (Fig. S1B) and subsequently verified the intended mutation by sequencing the PCR products generated from the gene-targeted ES cells (Fig. S1C). We injected the ES cells into the blastocysts (C57BL/6 background) and resulting chimeric mice were backcrossed with C57BL/6 mice. Mice with germline transmitted *Icos (Y181F)-Neo* allele were bred with CMV-Cre transgenic mice (Schwenk et al., 1995)to achieve *in vivo* deletion of Neo cassette (Fig. S1D). We also verified that the mRNA transcribed from $Icos^{y181f}$ locus has the same structure as the WT *Icos* mRNA except for the point mutation by sequencing PCR-amplified cDNA fragments encompassing exon 2 to exon 5 (data not shown).

Antibodies and cytokines.

For flow cytometry or T cell stimulation, the following antibodies were used: Armenian hamster IgG, antibodies against ICOS (mAb C398.4A for stimulation and 5F9 for staining), CD3 (145.2C11), CD4, CD8, CD16/32 (Fc block), Fas, IgD, PD-1 (all purchased from eBioscience) and GL7 (BD). Goat anti-Armenian hamster IgG (Jackson Immunoresearch) or avidin (Calbiochem) were used to crosslink primary antibodies. Biotinylated anti-CXCR5 (BD) was used with streptavidin-APC or -PE (eBioscience). For immunoblots, goat anti-mouse ICOS (Santa Cruz, sc 5748) and rabbit anti-PI3K p85α (Upstate Biotechnology) and antibodies against phospho-specific or total Akt, p44/42 MAPK, JNK, p38 (Cell Signaling Technology) were used with HRP-conjugated anti-goat (Santa Cruz,

sc2020) or anti-rabbit (Biorad) secondary antibodies. Recombinant IL-2 was purchased from R&D Systems and IL-6 from eBioscience.

Ca^{2+} flux.

$CD4^+$ T blasts were loaded with Indo-1 (Invitrogen) at 5 X 10^6 cells/ml in HBSS buffer supplemented with 0.1 % BSA,1 mM $CaCl_2$ and 1 µM $MgCl_2$. Cells were incubated for 1 min at RT with biotinylated antibodies: 0.1 ug/ml of anti-CD3 plus 1 µg/ml anti-ICOS (1 µg/ml) or control hamster IgG. After recording baseline for 30 sec, avidin (14 µg/ml) was added and the mobilization of intracellular Ca^{2+} was monitored by measuring FL4/FL5 on LSR FACS machine (BD). Equal loading of Indo-1 was confirmed by releasing intracellular Ca^{2+} by ionomycin (Sigma-Aldrich, 1 µg/ml).

Immunization of mice.

NP_{16}-CGG (Biosearch Technologies Inc., 1 mg/ml in PBS) was mixed with an equal volume of Imject Alum (Thermo Scientific) for 30 min at room temperature. Mice were injected i.p. with 100 µg (primary) or 50 µg (secondary) of alum-precipitated NP_{16}-CGG.

ELISA.

Various isotypes of mouse Ig were detected using mouse Ig isotyping kit (SouthernBiotech). Fecal extracts were prepared from fresh fecal pellets by vortexing in PBS containing 0.02% NaN_3 and 1mM PMSF (10 µl per mg feces). NP-specific Ab from mice immunized with NP_{16}-CGG was measured using plate-bound NP_{33}- or NP_3-BSA. Affinity profiling ELISA was performed as previously described(Luxton and Thompson, 1990). Anti-NP antibodies in serum samples (1:100 dilution) were allowed to bind to NP_{33}-BSA ELISA plates. After regular washing, bound antibodies were treated for 10 min at RT with NaSCN solutions (Sigma-Aldrich) each column receiving stepwise increments: 0.05, 0.1, 0.25, 0.5, 1, 2, 2.5, 3, 3.5, 4, and 5M. Remaining antibodies were detected by AP-conjugated secondary antibodies.

SI FIGURES

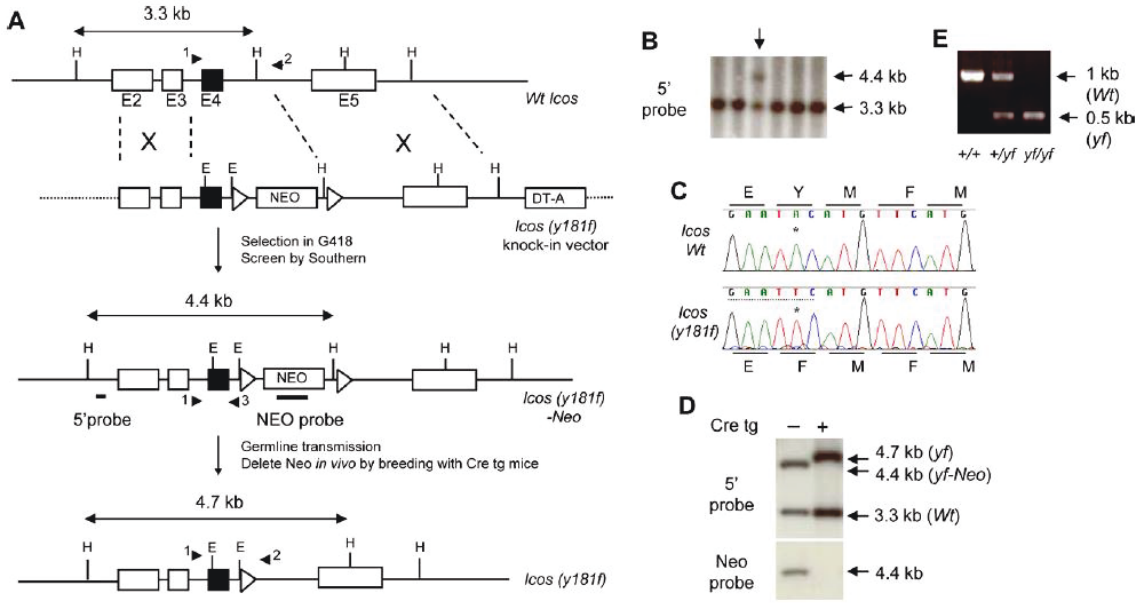

Figure S1. Generation of ICOS-YF mice. (*A*) The targeting vector was constructed with Neo cassette flanked by two loxP elements for positive selection and DT-A for negative selection. The exon 4 containing Tyr181 residue is represented with a filled box, the Southern blot probes are denoted with thick under lines, and PCR primers are shown as arrow heads. (*B*) Southern blot screening of ES clones using *HindIII*-digested ES genomic DNA. (*C*) Genomic DNA from ES cells positive for *Icos (y181f)-Neo* locus was PCR-amplified using primers 1 and 3 as depicted in (*A*) and the nucleotide sequences of the PCR products were determined. Asterisks indicate the A-to-T mutation that results in Tyr-to-Phe mutation with a novel *EcoRI* site. (*D*) Genomic DNAs from mice possessing one copy of *Icos (y181f)-Neo* locus without or with Cre transgene were analyzed by Southern blot after *HindIII* digestion. (*E*) The genotype of F2 progeny of heterozygous interbreeding was determined by PCR using primers 1 and 2 as shown in (*A*).

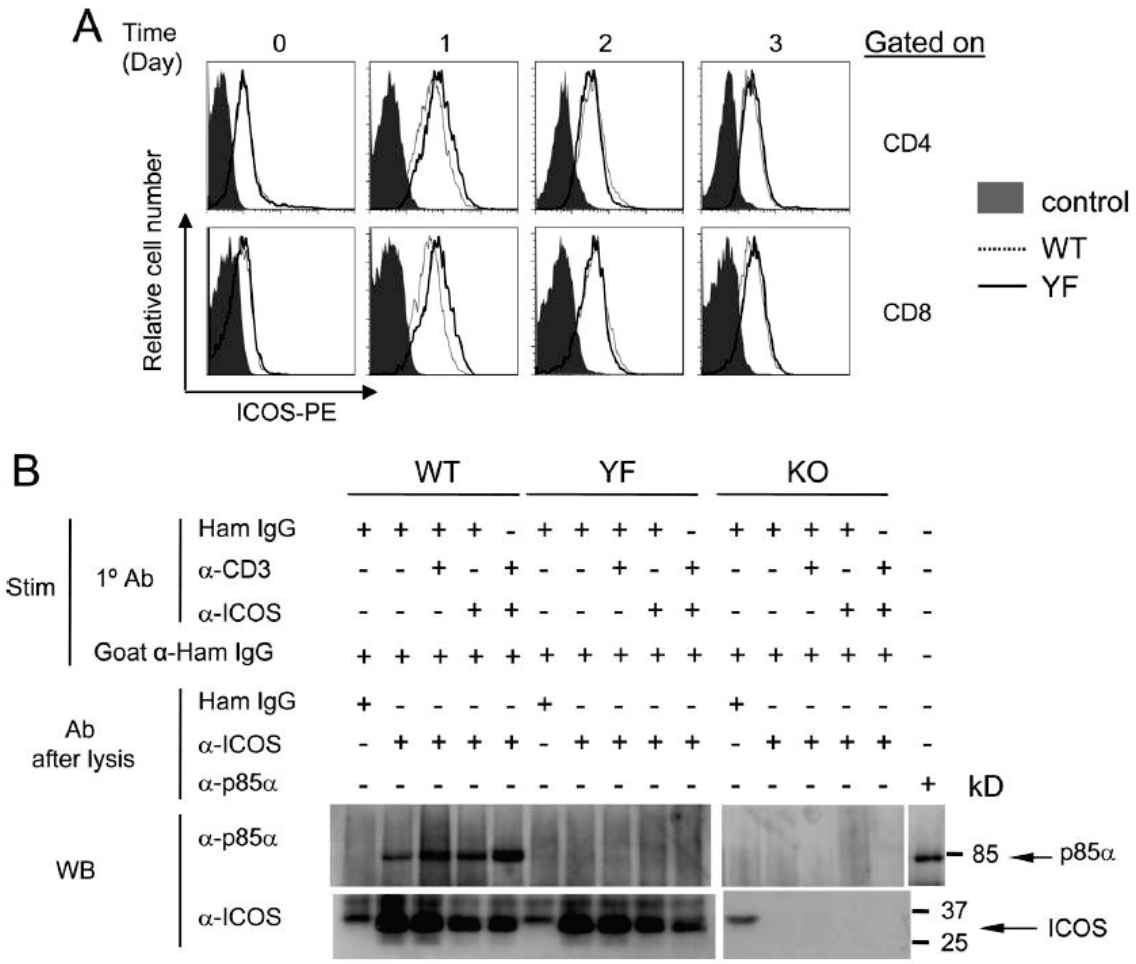

Figure S2. (*A*) Normal expression patterns of ICOS-YF. Total LN cells isolated from WT or ICOS-YF mice were stimulated with soluble anti-CD3 antibody for the indicated periods of time. ICOS expression on CD4+ or CD8+ T cells was assessed by flow cytometry. (*B*) Impaired PI3K recruitment by ICOS-YF. CD4+ T cell blasts were stimulated with antibodies against CD3 and/or ICOS and the immune complexes were recovered by immunoprecipitation. The amounts of p85a subunit of PI3K and the total ICOS protein were assessed by immunoblotting. Data shown in (*A*) and (*B*) are representative of three independent experiments.

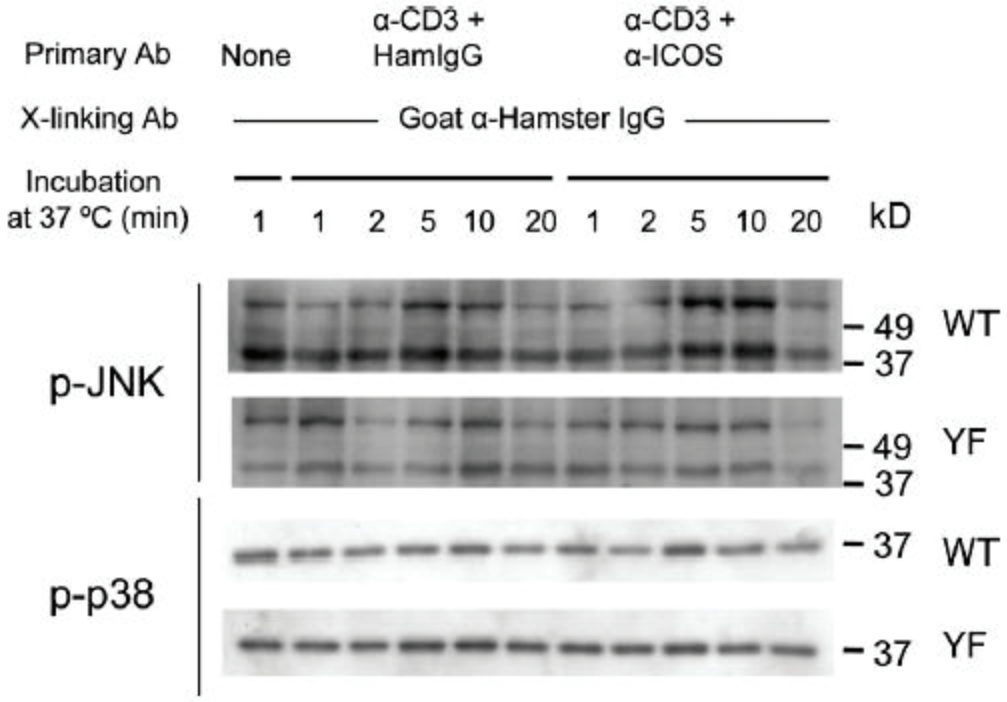

Figure S3. ICOS does not activate JNK and p38. CD4+ T blasts were stimulated with indicated antibodies up to 20 min and the amounts of phospho-JNK and phospho-p38 were assessed by immunoblotting.

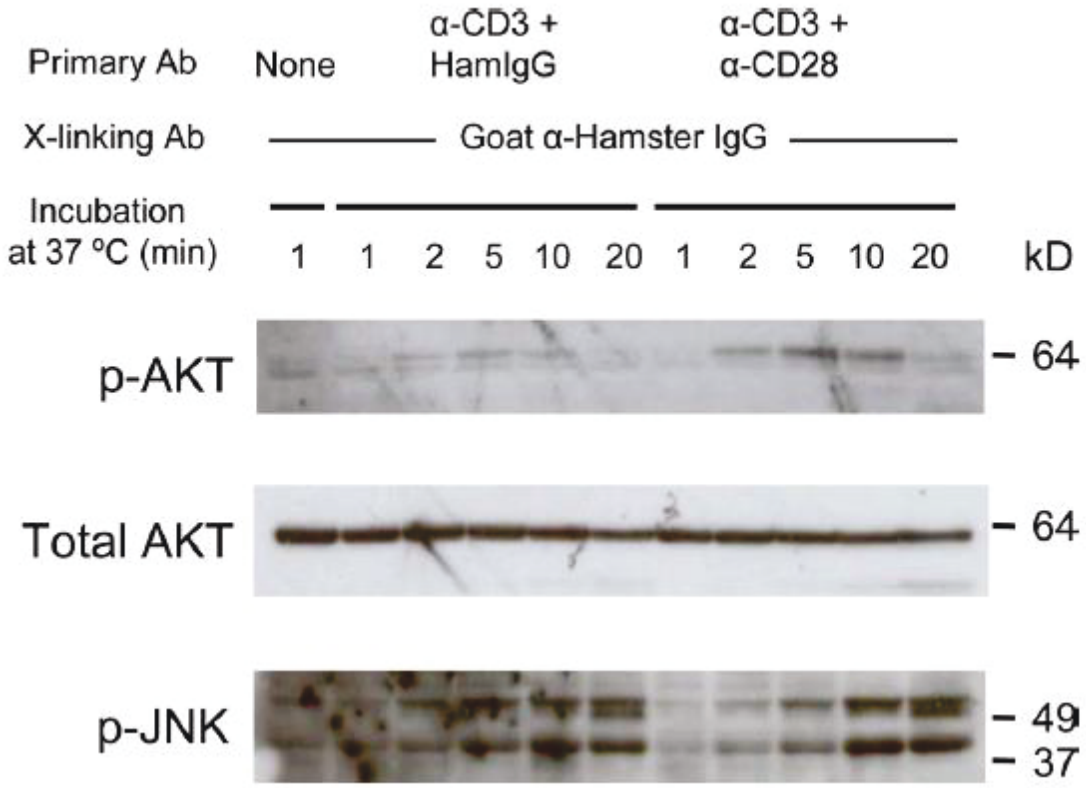

Figure S4. Moderate activation of AKT by CD28. $CD4^+$ T blasts were stimulated with indicated antibodies up to 20 min and the amounts of phospho-AKT, total AKT, and phospho-JNK were assessed by immunoblotting.

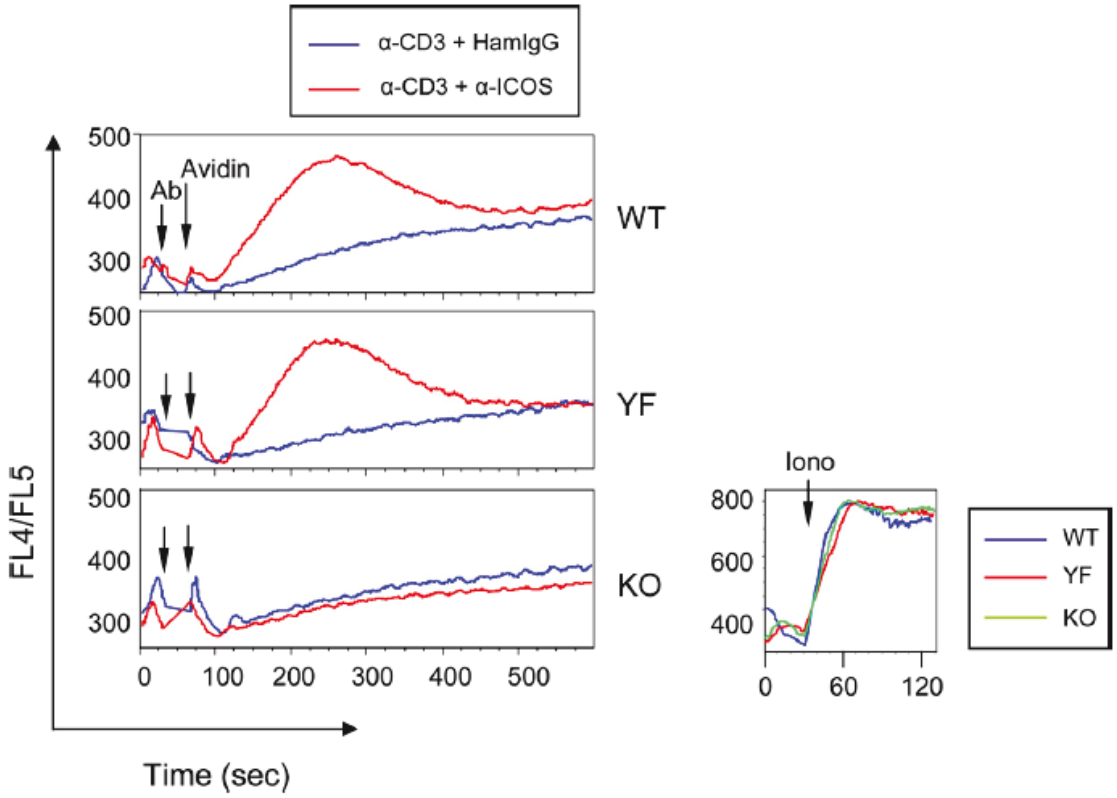

Figure S5. Intact calcium mobilization by ICOS-YF. Indo-1 loaded CD4+ T blasts were stimulated with suboptimal anti-CD3 without or with anti-ICOS and the intracellular calcium flux was monitored by flow cytometry. A representative of four independent experiments is shown.

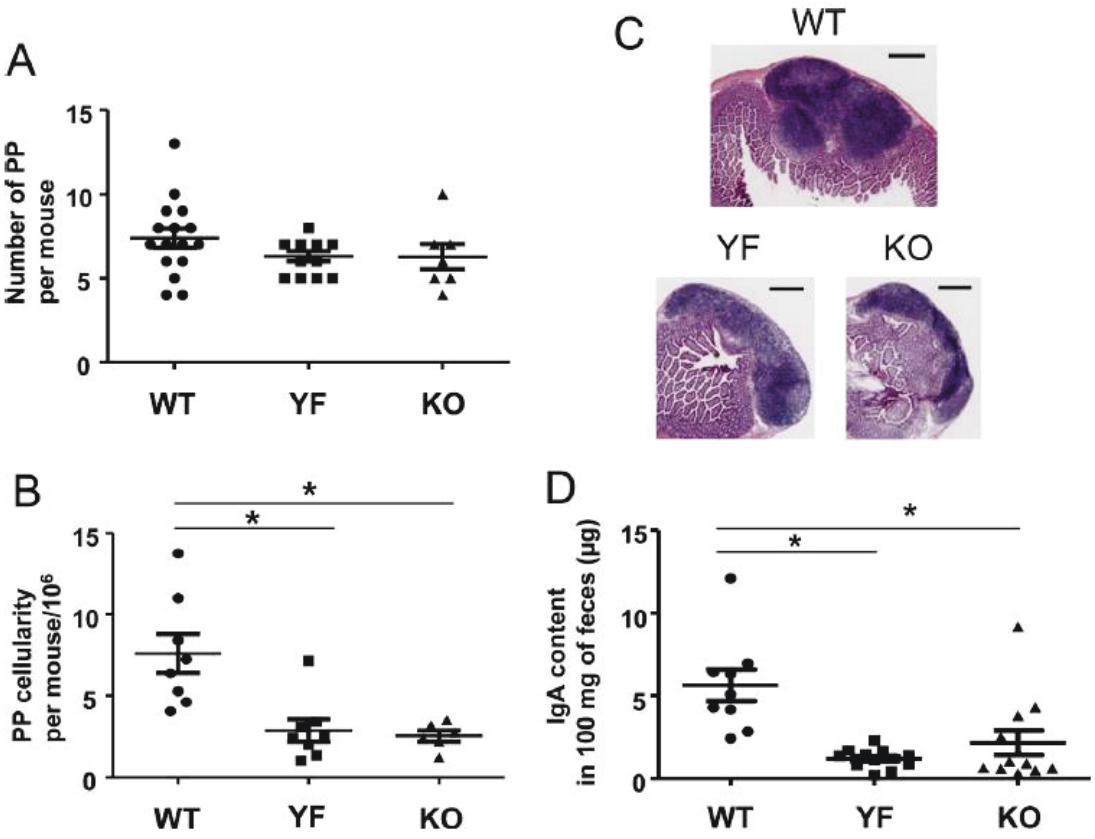

Figure S6. Normal number of PPs with decreased GC reaction in ICOS-YF and KO mice. Each data point represents the number of PPs per mouse (n = 10 WT, 10 YF, and 6 KO). (B) Each data point represents the total number of PP cells collected from a single mouse (n = 8 WT, 8 YF, and 6 KO). * $P < 0.01$. (C) Representative sections of PPs stained with H&E are shown. The scale bars represent 250 µm. (D) Decreased mucosal IgA secretion in the gut. IgA contents in fresh fecal pellets were measured by ELISA. Each data point represents fecal IgA content of an individual mouse (n = 9 WT, 12 YF, and 11 KO). * $P < 0.01$.

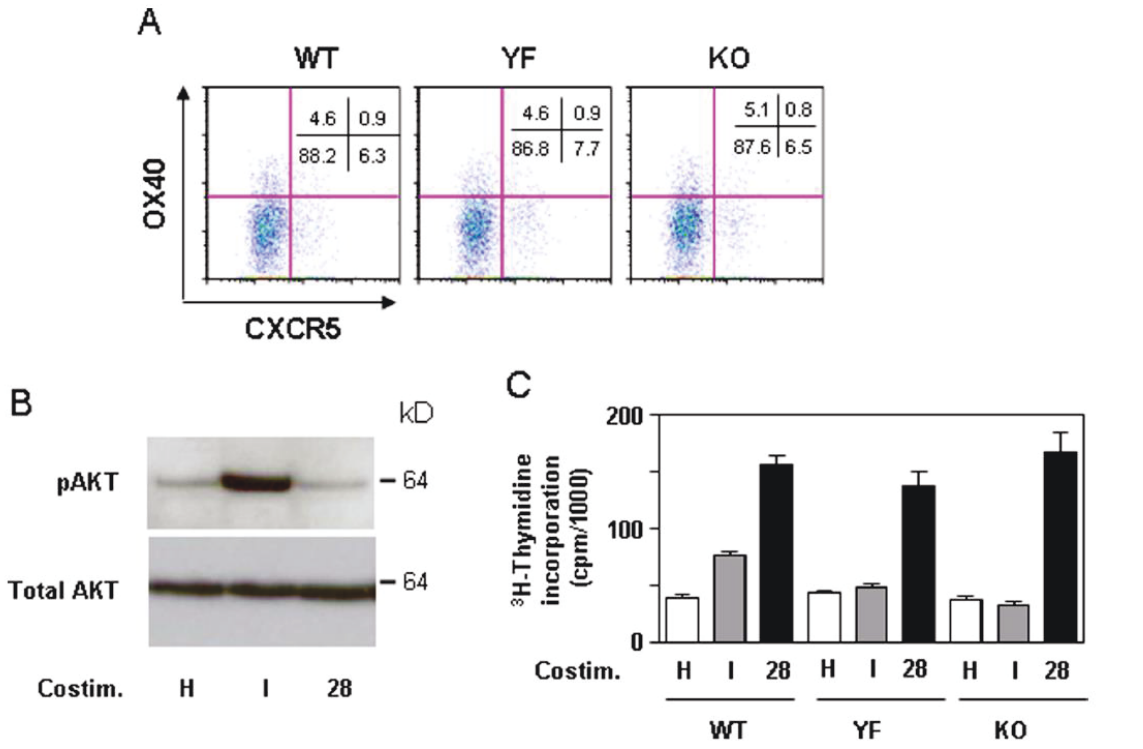

Figure S7. (*A*) Unaltered induction of OX40 and CXCR5 in ICOS-YF and ICOS-KO CD4$^+$ T cells. Total lymph node cells were stimulated with soluble anti-CD3 (1 μg/ml) for 2 days and the levels of OX40 and CXCR5 were analyzed by FACS. A representative of two independent experiments. (*B*) Differential activation of PI3K pathway by ICOS and CD28. Cell lysates were prepared from CD4$^+$ T cells restimulated with anti-CD3 plus hamster IgG (H), anti-ICOS (I), or anti-CD28 (28) followed by goat anti-hamster IgG for 5 min and analyzed by immunoblotting. A representative of two independent experiments. (*C*) CD28 plays a major role in secondary expansion of CD4+ T cells. Cells were restimulated as in (*B*) in 96-well plates for 24 hours. ^{3}H-thymidine was pulsed for the last 8 hours of incubation. A representative of three independent experiments.

Chapter III

Inducible Costimulator (ICOS) facilitates T-dependent B cell differentiation by augmenting IL-4 translation

ICOS plays a crucial role in the development of Tfh cells. However, whether and how ICOS regulates the function of Tfh cells is unknown. In this manuscript, we demonstrate that ICOS-PI3K-mTOR signaling pathways augment formation of IL-4 mRNA polysomes in activated CD4 T cells, and provide evidence that ICOS-mediated IL-4 upregulation plays a crucial role in T cell-dependent B cell differentiation.

Chapter III presents original work to be submitted for publication during the summer of 2013 and shortly after the submission of this thesis.

Inducible Costimulator (ICOS) facilitates T-dependent B cell differentiation by augmenting IL-4 translation

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1. ABSTRACT

The Inducible Costimulator (ICOS) is highly expressed in follicular helper T (Tfh) cells, a subset of CD4 T cells that migrate into the B cell zone and facilitate the germinal center reaction. Although ICOS is known to play a critical role in forming the Tfh cell population during immune responses, its contribution to the effector functions of Tfh cells remains unclear. Here, we demonstrate in activated splenic CD4 T cells that ICOS assists TCR-mediated signal transduction by potentiating the PI3K-AKT-mTOR signaling cascade that leads to hyper-phosphorylation of the key translational regulators, p70S6K and 4E-BP1. Consequently, ICOS costimulation promotes the formation of polysomes on IL-4 mRNA in a PI3K-dependent manner. Consistent with these observations, we provide evidence that IL-4 production is diminished during T cell-B cell co-culture when the ICOS-PI3K signaling axis is disrupted in T cells. We also show data suggesting that IL-4 mRNA translation is repressed through its 5' untranslated region (UTR). Thus, ICOS-PI3K signaling facilitates translation of IL-4 mRNA, at least in part through mTOR signaling and 5' UTR of IL-4 mRNA. We propose that this may ensure targeted delivery of IL-4 to cognate B cells during T cell-B cell interactions in the germinal center.

2. INTRODUCTION

ICOS is a member of the CD28 family of T cell costimulatory receptors (Hutloff et al., 1999; Greenwald et al., 2005). Unlike CD28, however, ICOS is induced in CD4 and CD8 T cells after T cell activation, and is highly enriched in effector/memory T cells. ICOS binds to ICOS ligand (ICOSL), which is expressed in APCs, including B cells, as well as in nonlymphoid cells (Swallow et al., 1999). *In vitro* experiments have shown that in general, ICOS plays similar costimulatory functions to CD28 - the enhancement of T cell proliferation, cytokine production, and survival of activated T cells (Hutloff et al., 1999; Bonhagen et al., 2003; Watanabe et al., 2005). Nonetheless, ICOS has a crucial, non-redundant role *in vivo*, as one of its most important contributions is the ability to support the generation of follicular helper T (Tfh) cells, a subset of CD4 cells that migrate to the B cell zone and facilitate the germinal center reaction in peripheral lymphoid organs (Vinuesa and Cyster, 2011). Thus, in both humans and mice, ICOS-deficiency causes defects in Tfh cell generation, germinal center (GC) reactions, and antibody production (Dong et al., 2001; McAdam et al., 2001; Tafuri et al., 2001; Warnatz et al., 2006).

Although ICOS can potentiate T cell receptor (TCR)-mediated phosphoinositide 3-kinase (PI3K) activation and Ca^{2+} mobilization, the molecular mechanisms and the *in vivo* roles of each signaling component are not fully understood. To address these issues, we recently generated a knock-in strain of mice that possesses a Tyr-to-Phe mutation at Tyr181 in the context of the YMFM PI3K signaling motif in the cytoplasmic tail of ICOS, termed ICOS-YF mice (Gigoux et al., 2009). This mutation abrogates ICOS's ability to bind to PI3K, without affecting cell surface expression patterns and its ability to induce Ca^{2+} flux. Importantly, mice expressing ICOS-YF had defects in Tfh cell generation, GC reactions, antibody class switching, and antibody affinity maturation, similar to mice that are deficient in ICOS (ICOS-KO). Congruent to our results, T cell-specific deletion of the PI3K catalytic subunit p110δ also abrogated Tfh cell generation (Rolf et al., 2010). Thus, ICOS-mediated PI3K signaling plays a critical role in facilitating Tfh cell generation. However, detailed molecular mechanisms are yet to be established as to how ICOS-PI3K signaling regulates Tfh cell differentiation.

Recent evidence indicates that the transcription and translation of the IL-4 gene is segregated by time and by location (King and Mohrs, 2009; Reinhardt et al., 2009; Zaretsky et al., 2009). CD4 T cells possessing IL-4 mRNA, without IL-4 protein secretion, appear early after T

cell priming in the T cell zone. However, CD4 T cells that gain the capacity to secrete IL-4 proteins appear only at the later phase of an immune response in the B cell zone, particularly in germinal centers. These IL-4 secreting cells have been shown to be Tfh cells, as they demonstrate all the criteria of Tfh cells, and are often found in conjugates with cognate B cells (Reinhardt et al., 2009). Presumably, this tight regulation of IL-4 secretion might allow targeted delivery of IL-4 from Tfh cells to cognate B cells during T cell-B cell interactions. These data suggest that there might be a T cell-B cell communication mechanism that acutely elevates IL-4 protein synthesis in Tfh cells. Although restimulation of primed CD4 T cells with TCR ligation alone can augment IL-4 protein synthesis *in vitro* (Scheu et al., 2006), whether or not there is an additional requirement for costimulatory signals *in vivo* is unknown.

Translation of eukaryotic mRNA is predominantly regulated at the level of translation initiation (Sonenberg and Hinnebusch, 2009). The mature mammalian mRNA species have post-transcriptional modifications at both ends: the 5'-cap (7-methyl guanylation) and the 3' poly adenylation tail. For the initiation of translation, the mRNA should be activated by eukaryotic initiation factors (eIFs) and then subsequently loaded with the 40S ribosome subunit charged with the methionyl tRNA specialized for initiation (Met-tRNAi). Both the mRNA activation step and the formation of Met-tRNAi pool are highly regulated processes that can be suppressed by nutrient starvation or stress. However, cellular mRNA species possessing internal ribosome entry site (IRES) are able to maintain their translation in the face of a general inhibition of translational (Johannes and Sarnow, 1998). Importantly, in resting T cells that have been previously activated, the translational initiation machineries are predominantly suppressed (Scheu et al., 2006). These suppression mechanisms can be lifted when the primed T cells receive strong TCR signaling forming the molecular basis of segregated cytokine transcription and translation in time and space (King and Mohrs, 2009; Reinhardt et al., 2009; Zaretsky et al., 2009).

The Ser/Thr kinase mTOR plays a central role in the regulation of mRNA translation in mammalian cells (Ma and Blenis, 2009; Livingstone et al., 2010). Normally, mTOR is activated when cells are exposed to sufficient nutrients (especially amino acids), energy (ATP), and growth factors/mitogens. Activated mTOR phosphorylates downstream translational regulators including p70S6K (S6K) and eIF4 binding proteins (4E-BPs). The S6K kinase is activated by phosphorylation and in turn phosphorylates multiple downstream targets involved in translation

initiation such as eIF4B (Ma and Blenis, 2009). On the other hand, 4E-BPs are inactivated by a series of phosphorylation events to allow its target, eIF4E, to recruit eIF4G and other translational initiation factors to the m^7G 5'-cap structure of mRNA (Ma and Blenis, 2009). These biochemical events ultimately lead to augmented translational initiation and elongation increasing polysomes, mRNA species actively translated by ribosomes. Importantly, one of the key upstream activators of mTOR is the PI3K-AKT signaling pathway that is activated by multiple growth factors, and in activated T cells, by ICOS.

We therefore hypothesize that ICOS may promote IL-4 synthesis in Tfh cells during T cell-B cell interactions by activating the PI3K-Akt-mTOR signaling cascade. Here, we provide biochemical evidence that the ICOS-PI3K signaling axis potentiates the TCR-mediated signaling pathway, culminating in the increase of polysome formation on IL-4 mRNA. Consistent with this, IL-4 is a limiting factor in T-dependent B cell responses when ICOS-PI3K signaling is impaired. Finally, we show that the 5' UTR of IL-4 mRNA can repress expression of a reporter protein. These results suggest the importance of mTOR and 5' UTR elements in ICOS-mediated translational control of IL-4 expression in the context of T-B interation during a germinal center reaction.

3. MATERIALS AND METHODS

Animals ICOS-YF knock-in (*Icos*$^{yf/yf}$) or ICOS-KO (*Icos* $^{-/-}$) mouse strains were previously described (Tafuri et al., 2001; Gigoux et al., 2009). C57BL/6 and OT-II transgenic mice were purchased from Jackson Laboratory. The BAC transgenic AID-GFP reporter mice were provided by Dr. R. Casellas (NCI, Bethesda, USA) (Crouch et al., 2007). 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 Functional grade purified anti-CD3(145.2C11), CD28, ICOS(C398.4A) and control Armenian hamster IgG, as well as staining anti-CD4-PECy7 and CD19-APC were purchased from eBioscience. Goat anti-Armenian hamster antibody was from Jackson Immunoresearch. 7-AAD was purchased from BD Pharmingen. Immunoblot antibodies against AKT, pAKT (S473), 4E-

BP1, p4E-BP1(S65) and pS6K(T389) were purchased from Cell Signalling. HRP-conjugated goat anti-rabbit antibody was purchased from Biorad.

***In vitro* CD4 T cells activation and restimulation** CD4 T cells were isolated from splenocytes using CD4 T cell Isolation Kit II (Miltenyi) according to the manufacturer's instructions and cultured in RPMI1640 medium supplemented with 10% FBS, glutamine, penicillin, streptomycin, β-mercaptoethanol and HEPES. Purified CD4 T cells were activated by culturing with plate-bound anti-CD3 (3μg/mL) and soluble anti-CD28 (2μg/mL) for 2 days and were subsequently expanded in media containing 100U/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 (1μg/mL) plus anti-ICOS (2μg/mL) or anti-CD3 plus hamster IgG (2μg/mL) as control. Immediately after addition of anti-hamster IgG (20μg/mL), the cells were transferred to a water bath at 37° and incubated 1 – 20 min depending on experimental settings.

Immunoblot analysis Restimulation was stopped by adding ice-cold PBS with 10% FBS, 1mM Na3VO4, 1mM EDTA pH 8.0. Cells were lysed in TNE lysis buffer (1% NP-40 in 50mM Tris pH 7.5, 2mM EDTA pH 8.0, 5mM Na4P2O7, 100μM Na3VO4, 5mM NaF, 150 mM NaCl, and protease inhibitor cocktail (Sigma)) for 20 min on ice. Cell debris was removed by centrifugation at 16,000 x g for 30 min and the cleared lysates were boiled in SDS-PAGE sample buffer. The samples were run on a 10% or 15% (for 4E-BP1) SDS-PAGE gels and transferred to Amersham Hyperfilm ECL nitrocellulose membrane (GE Healthcare). Blocking, as well as antibody dilution, was done in 5% fat-free skim milk powder in TBST and detection was achieved with Amersham ECL Plus™ Western Blotting Detection Reagents (GE Healthcare).

Polysome fractionation Restimulated T cells ($1x10^6$ cells/condition) were lysed in hypotonic solution (5mM Tris pH 7.5, 2.5mM MgCl2, 1.5mM KCl, 400U/mL RNaseIN (Promega) and protease inhibitor mix) and immediately adjusted to 100 μg/mL cycloheximide, 2mM DTT, 0.5% Triton X-100 and 0.5% sodium deoxycholate. Cell debris was eliminated by centrifugation at 16,000 x g for 30 min. The cleared lysates were layered on the top of a 10-50% sucrose gradient made in 0.1M HEPES, 0.1M KCl, 5mM MgCl2, 1μg/mL cycloheximide and protease inhibitor mix in 13.2 ml polyallomer centrifuge tubes (Beckman Coulter). After ultracentrifugation at

38000 rpm with a SW 41 Ti rotor (BeckmanCoulter) at 4°C for 2 hours, the tubes were pierced at the bottom using a tube piercer system (Brandel). A series of 1 ml fractions were collected from the top with a fraction collector while the sample solution was steadily pushed up by a chase solution (60% sucrose) fed by a syringe pump. Each collected fractions were divided into halves and then immediately frozen on dry ice and kept at -80 until further analysis.

RNA isolation and cDNA synthesis The fractionated lysates were adjusted to 1% SDS, 10mM EDTA and then treated with Proteinase K (100ug/mL, Invitrogen) for 30 minutes at 37°C. Subsequently, the samples were deprived of proteins with phenol extraction and then nucleic acid was precipitated by cold ethanol in the presence of 0.3M ammonium acetate and Glycoblue© (0.3 M, Ambion). RNA was pelleted by centrifuging and washed twice with 70% ethanol in diethylpyrocarbonate (DEPC)-treated water. RNA pellets were air-dried and dissolved in DEPC-treated water. Equal portions of each fraction were reverse-transcribed using first-strand cDNA synthesis kit and oligo (dT) primers (Invitrogen) according to the manufacturer's instructions.

PCR analysis Quantitative real-time PCR was performed using SYBR green mix (Quanta) and the following primer: *IL-4 forward*, GGAGATGGATGTGCCAAACG; *IL-4 reverse*: CGAGCTCACTCTCT GTGGTGTT; *BiP forward*, GCTGGACTGAATGTCATGAGGAT; and *BiP reverse*, CCAGGCCA TATGCAATAGCA. Relative quantities of transcripts in each fraction were analyzed as the percentage of the total across the total fractions.

T-B coculture and flow cytometry OT-II CD4 T cells were purified and activated for 2 days with anti-CD3 plus anti-CD28 as described above. Activated OT-II T cells were expanded in IL-2 for 1 day and co-cultures were set up with freshly isolated B cells from naive AID-GFP reporter mice loaded with 3 μM OVA323-339 peptides at a ratio of 1:5 (CD4 T cells: B cells). At day 2 of coculture, cells were stained with anti-CD4, anti-CD19, and 7-AAD for flow cytometric analysis. Data were collected with CyAn™ ADP Analyzer (Beckman Coulter) and GFP+ cells were quantified on gated live B cells ($7AAD^{-}CD4^{-}CD19^{+}$) using Flowjo software.

Plasmid construction, transfection and luciferase assay. The 5' UTR of mouse IL-4 gene was amplified from C57BL/6 genomic DNA by PCR and cloned into "pGL3-Promoter" vector

(Promega) between SV40 promoter and firefly luciferase (Fluc) open reading frame using *Hind III* and *NcoI* sites. The 5'UTR-IRF7-Fluc was constructed in an analogous manner (Colina et al., 2008) and used in this study as a positive control. To evaluate the impact of pyrimidine-rich sequences of IL-4 5'UTR in the context of endogenous IL-4 promoter, a IL-4-promoter/5'UTR-Fluc was used (Szabo et al., 1993). Mutant IL-4-promoter/5'UTR-Fluc constructs were generated by site-directed mutagenesis using the GeneArt Site-Directed Mutagenesis System (Invitrogen). For SV40 promoter-driven vectors, MEFs were co-transfected with 5' UTR-IL-4-Fluc and Renilla luciferase control vector (Rluc; Promega) with lipofectamine (Invitrogen). For IL-4 endogenous promoter vectors, EL-4 lymphoma cells were electroporated with the IL-4-promoter/5'UTR-Fluc constructs along with NFATc and Renilla luciferase vectors using Gene Pulser Xcell™ Electroporation System (Bio-Rad). Cell extracts were prepared in passive lysis buffer and assayed for Rluc and Fluc activities using a Dual-Luciferase Reporter Assay System (Promega). Fluc activity was normalized against Rluc activity to control transfection efficiency.

Statistical analysis. We used Student's *t* tests to judge statistical significance.

4. RESULTS

4.1 ICOS costimulation hyperphosphorylates translational regulators

Activation of PI3K is known to enhance mTOR kinase activity through a cascade of events and ultimately lead to phosphorylation of the two key translational regulators: S6K and 4E-BPs (Ma and Blenis, 2009). To assess the role of ICOS-PI3K signaling in the regulation of cap-dependent mRNA translation, we tested if ICOS ligation augments the phosphorylation of S6K and 4E-BP1. CD4 T cells were activated by anti-CD3 and anti-CD28 and expanded in IL-2. Under this condition, ICOS was highly expressed on the T cell surface. The preactivated CD4 T cells were then restimulated with anti-CD3 alone or with anti-CD3 plus anti-ICOS. As we and others have previously shown (Arimura et al., 2002; Gigoux et al., 2009), ICOS potentiated the TCR-mediated activation of AKT, as judged by the phosphorylation of Ser 473 (Fig. 1). ICOS costimulation not only increases the extent of AKT phosphorylation at the peak time (2 min) but also sustained the pAKT level up to 20 min. Consistent with the established signaling cascade, ICOS costimulation augmented the phosphorylation of S6K (at Thr 389) and 4E-BP1 (at Ser 65)

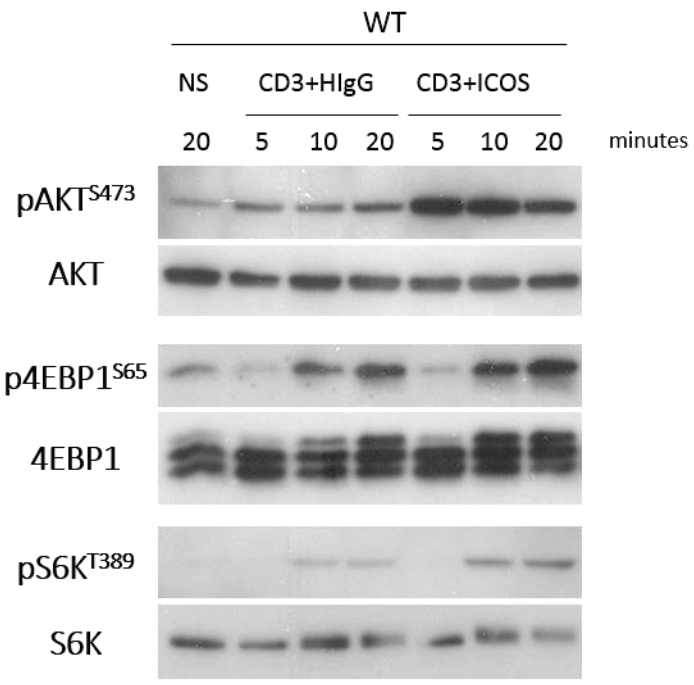

Figure 1. ICOS costimulation potentiates TCR-mediated phosphorylation of S6K and 4E-BP1. Purified WT CD4 T cells were activated *in vitro* with anti-CD3 and -CD28 antibodies, and expanded in IL-2. The cells were then restimulated for indicated periods of time with anti-CD3 or anti-CD3 plus anti-ICOS. Protein extracts were resolved on SDS-PAGE and the indicated proteins were detected by Western blot. A representative of three independent experiments is shown.

in preactivated CD4 T cells (Fig 1). In terms of dynamics of the phosphorylation, the increase of phosphorylated S6K and 4E-BP1 started at 5 min and plateaued at 10-20 min, probably reflecting the time delay between PI3K activation and mTOR activation. In parallel with the phosphorylation of Ser 65 of 4E-BP1, slow migrating hyperphosphorylated 4E-BP1 bands appeared faster when T cells were costimulated by ICOS compared to T cells received TCR stimulus alone. Thus, ICOS-mediated PI3K activation augments TCR-mediated phosphorylation of S6K and hyperphosphorylation of 4E-BP1 during T cell stimulation.

4.2 ICOS costimulation enhances polysome formation on IL-4 mRNA

Given that ICOS costimulation augments TCR-mediated phosphorylation of S6K and 4E-BP1, we hypothesized that ICOS costimulation might enhance the translation of key cytokines. As IL-4 is one of the most important effector cytokines for T-dependent antibody responses, we decided to analyze the formation of polysomes on IL-4 mRNA in response to ICOS costimulation. To this end, we restimulated preactivated CD4 T cells with anti-CD3 with or without anti-ICOS, lysed cells, separated the protein-mRNA complexes on a 10-50 % sucrose gradient, and prepared cDNA from the mRNA isolated from each fraction. The relative amount of IL-4 mRNA in each fraction was subsequently analyzed by qPCR. Consistent with a previous report, IL-4 mRNA was enriched in the non-polysome fraction of control non-restimulated T cells, but shifted towards the polysome fractions upon TCR ligation (Fig. 2A) (Scheu et al., 2006). Importantly, ICOS costimulation led to a much more pronounced shift towards heavier polysome fractions, suggesting that ICOS further enhances the translation of IL-4 transcripts during TCR ligation. However, we observed no increase in IFN-γ mRNA polysome formation after anti-CD3 or anti-CD3 and anti-ICOS costimulation under our experimental settings. Finally, due to the IRES-mediated translation, BiP mRNA has been shown to be insensitive to TCR signaling (Johannes and Sarnow, 1998; Scheu et al., 2006). Consistently, we observed that the changes in the distribution of BiP mRNA were unchanged upon TCR or TCR plus ICOS stimulation compared to IL-4 mRNA. (Fig. 2A). Furthermore, we observed that anti-CD3 and anti-CD3 and anti-ICOS costimulation did not induce dramatic changes in global polysome profiles as indicated by the patterns of ribosomal RNAs in each fraction (Fig. 2B). These results indicate that ICOS costimulation indeed enhances polysome formation on IL-4 mRNA, but not at other transcripts (IFN-γ and BiP) that we analyzed indicating that ICOS can change translation pattern of a subset of genes in activated T cells.

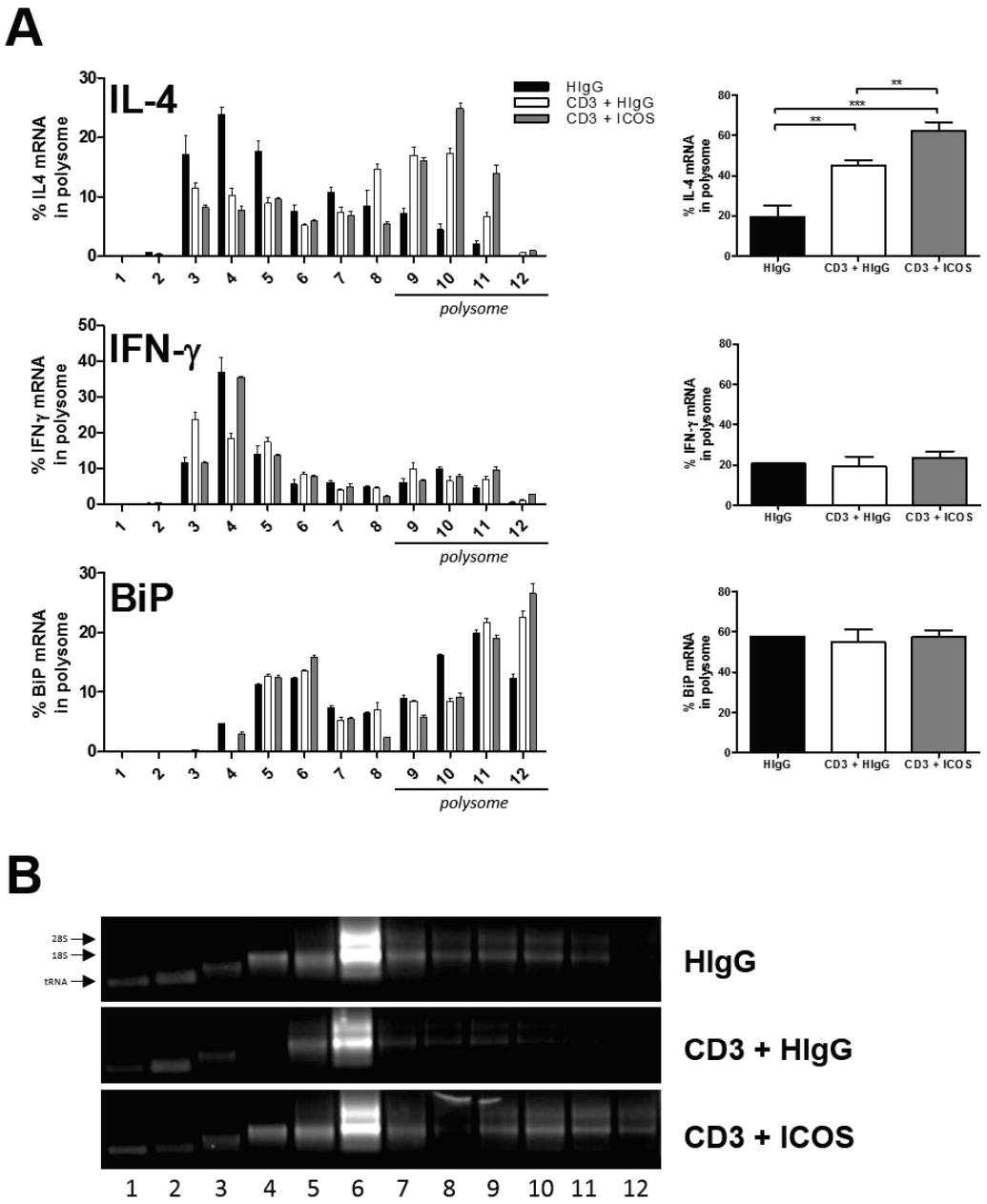

Figure 2. ICOS costimulation enhances polysome formation on IL-4 mRNA, but on IFN-γ or BiP mRNA. Purified WT CD4 T cells were activated *in vitro* with anti-CD3 and anti-CD28 antibodies, and expanded in IL-2. The cells were restimulated for 20 minutes with the indicated antibodies. Lysates were fractionated by sucrose gradient ultracentrifugation and RNA was prepared and analysed as described in Materials and Methods. (A) Relative amounts of IL-4, IFN-γ and BiP mRNA from each fractions are represented by histograms, as measured by quantitative PCR (left panels). The percentage of each mRNA in the polysome fractions (fraction 9-12) over the total (fraction 1-12) is plotted in the right panels (n=4 for IL-4, n=3 for IFN-γ and BiP; ** = $p<0.01$, *** = $p<0.001$). (B) Analysis of RNA species in fractions isolated in (A) by agarose gel electrophoresis. Arrows indicate tRNA and 18S rRNA, and 28S rRNA in each fraction. A representative of four independent experiments is shown.

4.3 ICOS-mediated PI3K signaling is critical for the activation of the translational machinery

We and others previously showed that ICOS recruits the regulatory subunits of PI3K through the SH2 domain binding motif Tyr 181 in the context of Y^{181}MFM located in its cytoplasmic tail (Coyle et al., 2000; Parry et al., 2003; Gigoux et al., 2009). We demonstrated that CD4 T cells with a targeted mutation in Tyr181 (ICOS-YF) selectively lost the ability to activate PI3K, despite the fact that ICOS-YF has normal expression patterns and retains the ability to induce Ca^{2+} flux (Gigoux et al., 2009). We therefore predicted that CD4 T cells from ICOS-YF mice should be defective in enhancing phosphorylation of S6K and 4E-BP1. To test this idea, we prepared CD4 T cell blasts from ICOS-YF mice and assessed the phosphorylation status of S6K and 4E-BP1 by Western blot after restimulation (Fig. 3). We observed that TCR stimulation induced comparable levels of 4E-BP1 and S6K phosphorylation in ICOS-YF CD4 T cell blasts compared to WT counterparts. However, ICOS costimulation did not potentiate TCR-mediated phosphorylation of AKT, S6K, and 4E-BP1. Thus, ICOS-PI3K signaling axis is crucial for regulating S6K and 4E-BP1.

4.4 Ligation of ICOS-YF cannot enhance IL-4 mRNA polysome formation

Given that PI3K activation was necessary for the ICOS-mediated phosphorylation of S6K and 4E-BP1, we predicted that ICOS-YF should not be able to enhance polysome formation of IL-4 mRNA. When ICOS-YF CD4 T cells were stimulated with TCR alone, we observed a shift of IL-4 mRNA towards polysome fractions (Fig. 4). However, when we compared anti-CD3 stimulation alone versus anti-CD3 plus anti-ICOS costimulation, there was no enhancement of polysome formation by ICOS costimulation in ICOS-YF mutants compared to anti-CD3 stimulation alone. Again, there were no observable differences in the polysome profiles of IFN-γ and BiP mRNA (Fig. 4A) as well as global polysomal profiles (Fig. 4B) between anti-CD3 stimulation alone and anti-CD3 plus anti-ICOS costimulation. Therefore, we conclude that the PI3K pathway is a crucial downstream component of ICOS signaling that leads to enhanced polysome formation of IL-4 mRNA.

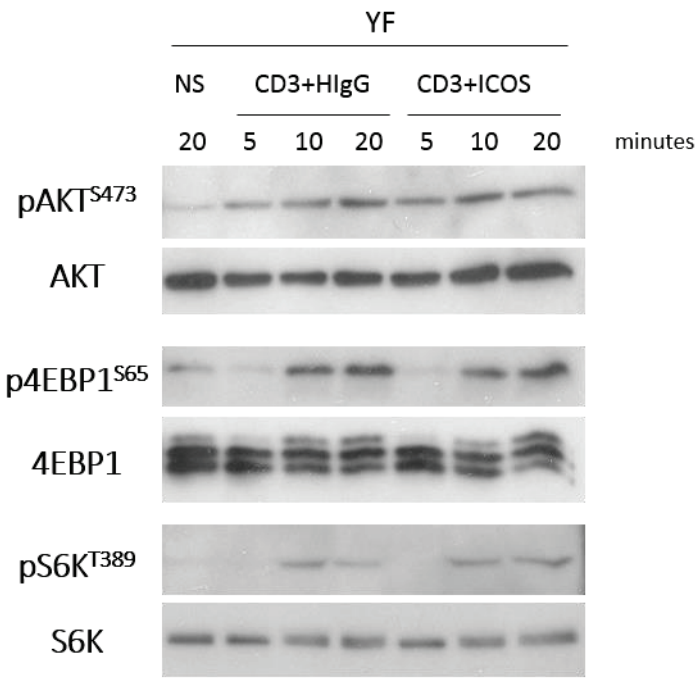

Figure 3. ICOS-YF cannot support hyperphosphorylation of S6K and 4E-BP1. Purified ICOS-YF CD4 T cells were activated *in vitro* with anti-CD3 and anti-CD28 antibodies, and expanded in IL-2. The cells were then restimulated for indicated periods of time with anti-CD3 or anti-CD3 plus anti-ICOS. Protein extracts were resolved on SDS-PAGE and the indicated proteins were detected by Western blot. A representative three independent experiments is shown.

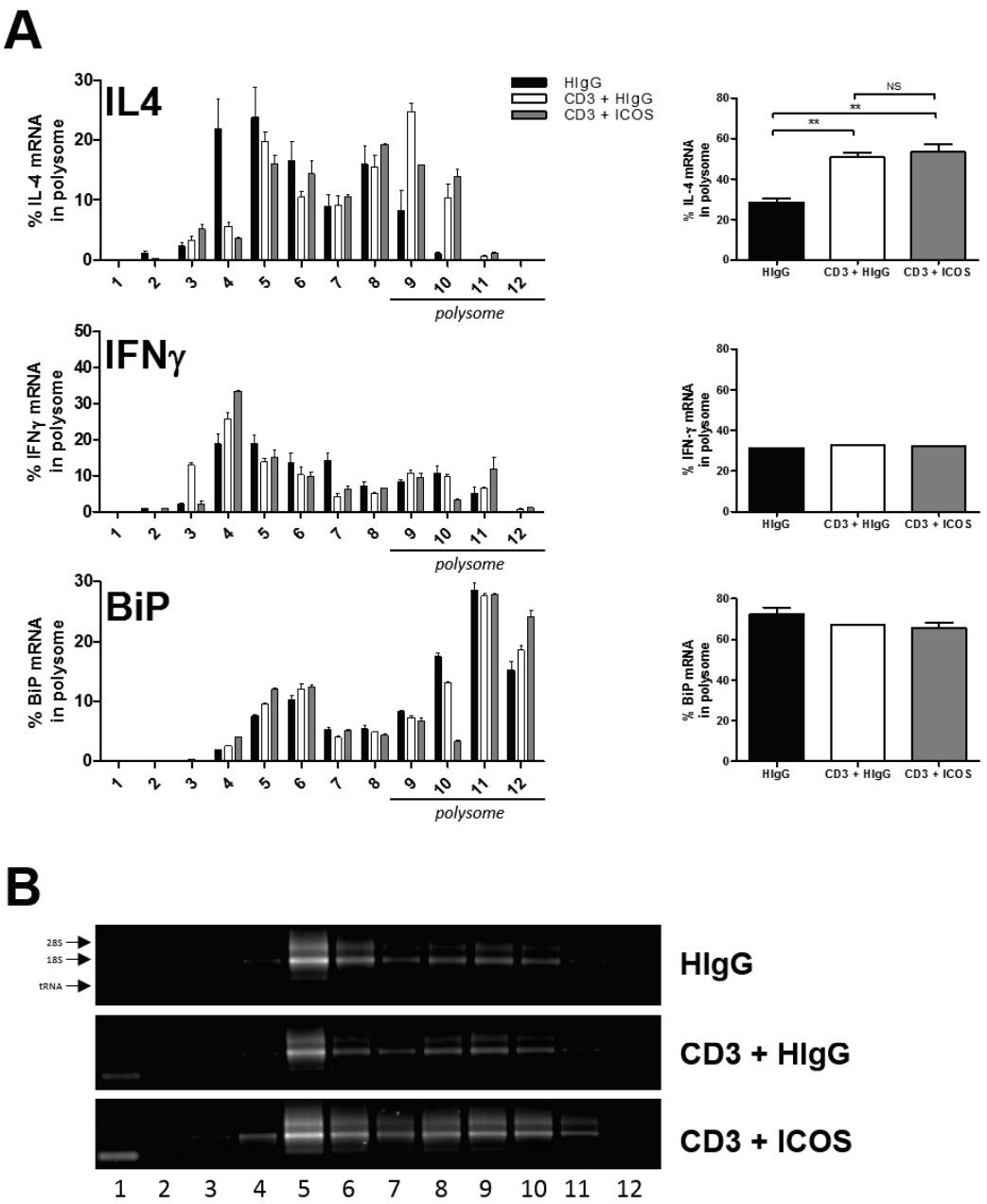

Figure 4. ICOS-YF cannot enhance polysome formation on IL-4 mRNA. Purified ICOS-YF CD4 T cells were activated *in vitro* with anti-CD3 and anti-CD28 antibodies, and expanded in IL-2. The cells were restimulated for 20 minutes with the indicated antibodies. Lysates were fractionated by sucrose gradient ultracentrifugation and RNA was prepared and analysed as described in Materials and Methods. (A) Relative amounts of IL-4, IFN-γ and BiP mRNA from each fractions are represented by histograms, as measured by quantitative PCR (left panels). The percentage of each mRNA in the polysome fractions (fraction 9-12) over the total (fraction 1-12) is plotted in the right panels (n=4 for IL-4, n=3 for IFN-γ and BiP; ** = $p<0.01$). (B) Analysis of RNA species in fractions isolated in (A) by agarose gel electrophoresis. Arrows indicate tRNA and 18S rRNA, and 28S rRNA in each fraction. A representative of four independent experiments is shown.

4.5 Intact ICOS signaling is require for T cell helper function

Next, we sought to determine whether or not the ICOS-mediated augmentation of IL-4 protein synthesis is required for helper T cells to facilitate B cell differentiation during an *in vitro* T cell-B cell co-culture. To this end, we established a T-B co-culture system using *in vitro* activated OT-II transgenic CD4 T cells from ICOS-WT, -YF, or -KO mice combined with naïve B cells loaded with $OVA_{323-339}$ peptides. We used B cells isolated from AID-GFP reporter mice so that we can identify B cells expressing AID-GFP fusion proteins by flow cytometry (Crouch et al., 2007). Since induction of AID is an absolute requirement for antibody class-switch recombination in B cells, the frequency of GFP-positive cells over the total B cells reflects the extent of T-dependent class switching in B cells (Muramatsu et al., 2000; Revy et al., 2000; Crouch et al., 2007). Typically, during a 3 day co-culture with WT CD4 T cells, more than 6% of B cells express AID-GFP (Fig. 5A). As a control, when B cells received the maximum stimulus of LPS plus IL-4 without T cells, approximately 20-25% of B cells became AID-GFP positive. Importantly, when B cells were cultured with ICOS-YF or ICOS-KO T cells, the percentages of AID-GFP^+ cells were reduced by 40-50% from that of the WT CD4 T cell co-culture. These differences were not explained by lower levels of IL-4 mRNA in the mutant T cell blasts, since the amounts of IL-4 mRNA were comparable or marginally higher in ICOS-YF or ICOS-KO T cells in these experiments (data not shown). In addition, AID-GFP expression was dependent on the presence of the OVA peptide (Fig. 5C), confirming that B cell activation was mediated by TCR-dependent T-B interactions, but not through non-specific aggregation.

It is known that T cell "help" to B cells is delivered through cell-cell contact (e.g., CD40L-CD40 interaction), as well as through soluble factors (e.g., IL-4). To determine if alteration of the ICOS signal would completely abrogate T-B interactions or if it would selectively affect the provision of IL-4 to B cells in our experimental system, we supplemented exogenous IL-4 to see if this could rescue the B cell response. Indeed, an optimal level of IL-4 (5 ng/ml) rescued the T-dependent B cell response regardless of the ICOS defects in T cells (Fig. 5B). However, at this concentration of IL-4, CD4 T cells still need to be stimulated with TCR signaling to provide help to B cells since IL-4 in the absence of OVA peptides failed to induce AID-GFP in B cells (Fig. 5C). These data suggest that under our T-B co-culture conditions, intact ICOS-PI3K signaling is required to enhance the supply of IL-4, whereas TCR signaling itself may be sufficient to elicit other signaling events such as CD40L-CD40 interaction and TCR and ICOS signaling work

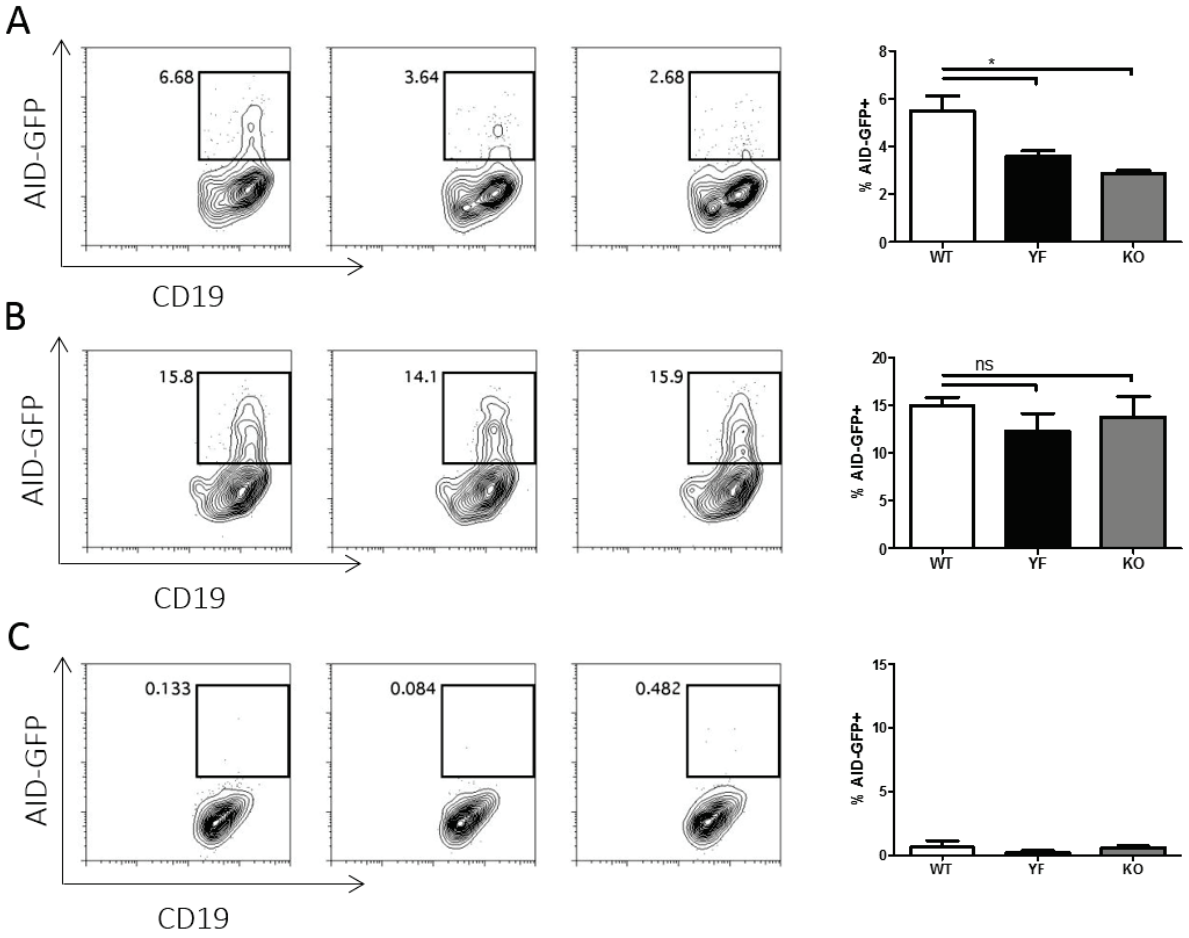

Figure 5. ICOS-PI3K signaling is required for T helper function. OT-II transgenic CD4 T cell blasts with ICOS WT, -YF, or -KO genotypes were prepared and T cell-B cell co-culture was set up as described in Materials and Methods. AID-GFP expression levels in B cells after 3 days of co-culture analyzed by flow cytometry. (A) Co-culture with 3 μM OVA peptide without exogenous IL-4 (n=4, * $p<0.05$). (B) Co-culture with 3 μM OVA peptide and 5 ng/mL of recombinant IL-4. (n=3) (C) Co-culture without OVA peptide and 5 ng/mL of recombinant IL-4. (n=3).

together to induce AID in B cells. We propose that ICOS-PI3K signaling becomes critically important in IL-4 protein synthesis when TCR signaling is limited, as we have shown in these *in vitro* experiments.

4.6 IL-4 mRNA translation is regulated through the 5' UTR independently of the pyrimidine-rich sequences

4E-BPs regulate translation initiation by controlling eIF4E binding to eIF4G (Sonenberg and Hinnebusch, 2009). However, a recent study using the mTOR inhibitor Torin1 and high-resolution transcriptome profiling has shown that approximately 200 mRNA species in mouse embryonic fibroblasts (MEFs) were dependent upon mTOR (Thoreen et al., 2012). The authors found that a disproportionate amount of these mRNAs had pyrimidine-rich 5' terminal oligopyrimidine (TOP) or TOP-like sequences. TOP sequences are pyrimidine-rich sequences at or near the 5' cap structure of mRNAs that inhibit translation through mechanisms which are still unclear (Meyuhas and Dreazen, 2009; Thoreen et al., 2012). However, the translational regulation of TOP mRNAs is fully dependent on PI3K (Tang et al., 2001) and 4E-BP1 (Thoreen et al., 2012). Analysis of murine IL-4 mRNA (NM_021283.2) reveals two pyrimidine-rich sequences in the 5' UTR near the 5' cap (Fig. 4B). We therefore hypothesized that the 5' UTR of IL-4 mRNA regulates the translation of IL-4 mRNA through these pyrimidine-rich sequences. To test this, we first cloned the 5' UTR of IL-4 gene into the SV40 promoter-driven firefly luciferase reporter construct (Fluc). As a positive control, we also used a construct with the 5' UTR of interferon response factor 7 (IRF7) inserted into Fluc, which has previously shown to repress translation (Colina et al., 2008). When transfected in MEFs, the 5' UTR of IL-4 mRNA reduced firefly luciferase activity by about 40% (Fig. 6A). Although significant, the potency of IL-4 5'UTR was much weaker than the IRF7 5' UTR (~85% repression). However, these data suggest that IL-4 translation can be regulated by its 5'UTR.

In order to determine if the pyrimidine-rich sequences of the IL-4 5' UTR are regulating IL-4 mRNA translation, we used a firefly luciferase construct driven by endogenous IL-4 promoter and 5'UTR (IL-4promoter/5'UTR-Fluc) (Szabo et al., 1993). We then used site-directed mutagenesis to mutate the two pyrimidine-rich sequences near the predicted transcription start site to purines (Fig. 6B). We next electroporated the WT or mutant IL-4promoter/5'UTR-Fluc constructs in EL-4 murine lymphoma cells, to best reflect cellular contexts of T cells. In order to

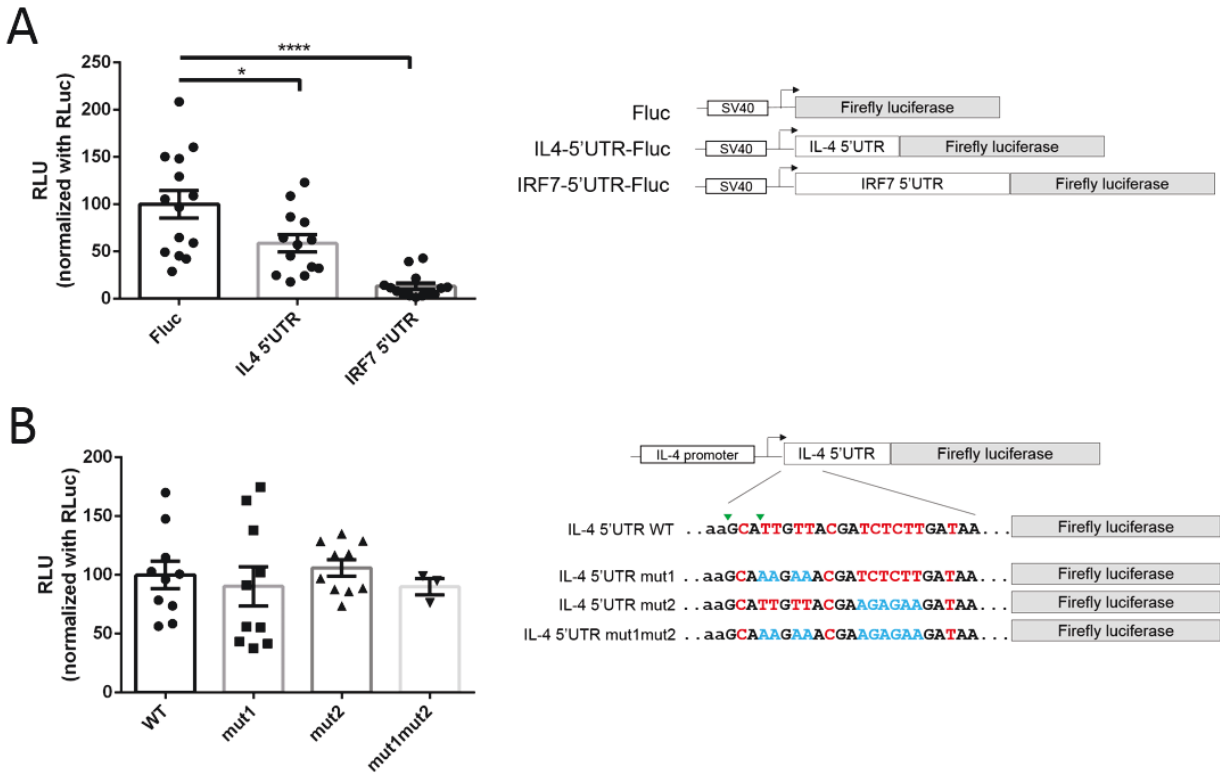

Figure 6. IL-4 5' UTR can repress reporter protein expression in a manner independent of pyrimidine-rich sequences. (A) The 5'UTR of IL-4 (59nt) or IRF7 (443nt) were inserted into a SV40 promoter-driven luciferase reporter construct (right panel). The constructs were transfected into MEFs and luciferase activities were measured as described in Materials and Methods (left panel). Data from four independent experiments. Each data point represents a replicate relative to the median value (set as 100) among the Fluc samples. * = $p<0.05$ (B) Reporter constructs driven by IL-4 promoter (right panel) were transfected into EL-4 cells and luciferase activities were measured as described in Materials and Methods (left panel). Green arrowheads represent predicted transcriptional start sites. Data represent replicates from one (mut1mut2) or three (WT, mut1, mut2) experiments calculated as in (A).

increase the expression of the gene, we co-electroporated a construct encoding for NFATp, which has been shown to bind to the IL-4 promoter and increase transcription (Szabo et al., 1993). However, we observed that mutating the pyrimidine-rich sequences did not increase the firefly luciferase activity, suggesting that the 5' UTR of IL-4 does not regulate IL-4 mRNA translation through these sequences (Fig. 6B). Therefore, we conclude that the 5' UTR of IL-4 regulates the translation of IL-4 mRNA independently of the pyrimidine-rich sequences.

5. DISCUSSION

In this study, we demonstrated that the ICOS-mediated PI3K-AKT signalling pathway leads to the phosphorylation of mTOR targets, S6K and 4E-BP1, in preactivated CD4 T cells. Consequently, the population of IL-4 mRNA that are actively translated in polysomes is increased upon ICOS costimulation. Using a T cell-B cell co-culture system, we provide evidence that IL-4 protein becomes a limiting factor for B cell differentiation when ICOS-PI3K signaling is abrogated in T cells. Thus, we have found a novel costimulatory function of ICOS in the translational upregulation of IL-4 that may facilitate targeted delivery of IL-4 from Tfh cells to cognate B cells during germinal center reactions.

It has been shown that in primed CD4 T cells, translational initiation is globally inhibited, and this inhibition can be lifted when the TCR is ligated by plate-bound peptide-MHC complexes (Scheu et al., 2006). However, the strength of TCR stimulus in this context was probably much greater than physiological levels, and it remains a possibility that costimulation is required for efficient translational initiation in primed T cells exposed to lower, more physiological levels of TCR engagement. Our *in vitro* T-B co-culture system provided an opportunity to optimize the peptide concentration (3 μM) such that the costimulatory requirement was revealed. Of note, during the process of optimization of the T cell- B cell co-culture system, we noticed that excessively high peptide concentrations masks the impact of ICOS mutation on T cells. Therefore, we believe that during an *in vivo* immune reaction in which the TCR signaling strength is weak, it is likely that provision of T cell help to B cells depends on ICOS costimulation.

In addition, it is becoming clear that initial Tfh cell generation is possible in the absence of cognate B cells, but this pool of Tfh cells subsequently collapses shortly after initial expansion (Kerfoot et al., 2011; Xu et al., 2013). Apart from regulating Tfh cell effector function, ICOS-

mediated IL-4 synthesis may also contribute to the maintenance of Tfh cells. Two lines of evidence support this idea: First, in mice lacking ICOSL in a B cell-specific manner, the generation of Tfh cell population was substantially impaired (Nurieva et al., 2008). Second, during a chronic helminthic infection in mice lacking IL-4Rα chain (thus B cells are unresponsive to IL-4), Tfh cells and B cell numbers increase normally during the first week of immune reaction, but rapidly decrease during the second week (King and Mohrs, 2009). It therefore appears that mature Tfh cells provide IL-4 to B cells and receive maintenance signals in return, and the ICOS-ICOSL interaction seems to play an important role in augmenting IL-4 synthesis during this process.

Although we readily detected strong activation of translational regulatory components upon ICOS costimulation, defining the *cis*-acting elements in the IL-4 mRNA turned out to be difficult. We found that the 5'UTR of IL-4 can repress SV40 promoter-mediated luciferase expression in MEFs approximately by two-fold but it was much weaker than that of IRF7 5'UTR. Translational repression through the 5' UTR has been shown to be mediated by a long stretch of sequence possessing secondary structures with high thermostability as exemplified by the 5' UTR of IRF7 (443nt, ΔG= -145.05 kcal/mol) (NM_016850.3; RNAfold) (Colina et al., 2008). However, the 5' UTR of IL-4 mRNA is relatively short (59nt) (NM_021283.2) with predicted secondary structures that would not be considered to be thermally stable (ΔG= -7.70 kcal/mol) (RNAfold). Because of this, we predicted that pyrimidine-rech sequences near the transcription initiation site could serve as translational repression elements as proposed by recent studies (REF). However, mutating these residues did not affect expression of luciferase reporter gene. Therefore, IL-4 mRNA translation is regulated at least in part by its 5' UTR but the precise mechanisms remain unknown.

In summary, we showed here that the evolutionarily conserved PI3K-AKT-mTOR pathway is operating in activated T cells to regulate translational initiation. This pathway links ICOS costimulation to enhanced IL-4 translation, in part through the 5' UTR of IL-4. We propose that this novel ICOS costimulatory function contributes to T cell-dependent B cell responses and may also possibly contribute to the T cell-B cell cross-talk needed to maintain the Tfh cell population.

6. AUTHORSHIP

M. G. performed most experiments and prepared manuscript; A.L. performed polysome experiments; J.L., and J.L. performed qPCR experiments; N.S. provided key reagents and advice; W.-K. S. conceived the project, supervised experiments, interpreted the data, and prepared the manuscript.

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8. REFERENCES

Arimura, Y., Kato, H., Dianzani, U., Okamoto, T., Kamekura, S., Buonfiglio, D., Miyoshi-Akiyama, T., Uchiyama, T., and Yagi, J. (2002). A co-stimulatory molecule on activated T cells, H4/ICOS, delivers specific signals in T(h) cells and regulates their responses. International immunology 14, 555-566.

Bonhagen, K., Liesenfeld, O., Stadecker, M.J., Hutloff, A., Erb, K., Coyle, A.J., Lipp, M., Kroczek, R.A., and Kamradt, T. (2003). ICOS+ Th cells produce distinct cytokines in different mucosal immune responses. European journal of immunology 33, 392-401.

Colina, R., Costa-Mattioli, M., Dowling, R.J., Jaramillo, M., Tai, L.H., Breitbach, C.J., Martineau, Y., Larsson, O., Rong, L., Svitkin, Y.V*., et al.* (2008). Translational control of the innate immune response through IRF-7. Nature 452, 323-328.

Coyle, A.J., Lehar, S., Lloyd, C., Tian, J., Delaney, T., Manning, S., Nguyen, T., Burwell, T., Schneider, H., Gonzalo, J.A*., et al.* (2000). The CD28-related molecule ICOS is required for effective T cell-dependent immune responses. Immunity 13, 95-105.

Crouch, E.E., Li, Z., Takizawa, M., Fichtner-Feigl, S., Gourzi, P., Montano, C., Feigenbaum, L., Wilson, P., Janz, S., Papavasiliou, F.N., and Casellas, R. (2007). Regulation of AID expression in the immune response. The Journal of experimental medicine 204, 1145-1156.

Dong, C., Juedes, A.E., Temann, U.A., Shresta, S., Allison, J.P., Ruddle, N.H., and Flavell, R.A. (2001). ICOS co-stimulatory receptor is essential for T-cell activation and function. Nature 409, 97-101.

Gigoux, M., Shang, J., Pak, Y., Xu, M., Choe, J., Mak, T.W., and Suh, W.K. (2009). Inducible costimulator promotes helper T-cell differentiation through phosphoinositide 3-kinase. Proceedings of the National Academy of Sciences of the United States of America 106, 20371-20376.

Greenwald, R.J., Freeman, G.J., and Sharpe, A.H. (2005). The B7 family revisited. Annual review of immunology 23, 515-548.

Hutloff, A., Dittrich, A.M., Beier, K.C., Eljaschewitsch, B., Kraft, R., Anagnostopoulos, I., and Kroczek, R.A. (1999). ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. Nature 397, 263-266.

Johannes, G., and Sarnow, P. (1998). Cap-independent polysomal association of natural mRNAs encoding c-myc, BiP, and eIF4G conferred by internal ribosome entry sites. Rna 4, 1500-1513.

Kerfoot, S.M., Yaari, G., Patel, J.R., Johnson, K.L., Gonzalez, D.G., Kleinstein, S.H., and Haberman, A.M. (2011). Germinal center B cell and T follicular helper cell development initiates in the interfollicular zone. Immunity 34, 947-960.

King, I.L., and Mohrs, M. (2009). IL-4-producing CD4+ T cells in reactive lymph nodes during helminth infection are T follicular helper cells. The Journal of experimental medicine 206, 1001-1007.

Livingstone, M., Atas, E., Meller, A., and Sonenberg, N. (2010). Mechanisms governing the control of mRNA translation. Physical biology 7, 021001.

Ma, X.M., and Blenis, J. (2009). Molecular mechanisms of mTOR-mediated translational control. Nature reviews. Molecular cell biology 10, 307-318.

McAdam, A.J., Greenwald, R.J., Levin, M.A., Chernova, T., Malenkovich, N., Ling, V., Freeman, G.J., and Sharpe, A.H. (2001). ICOS is critical for CD40-mediated antibody class switching. Nature 409, 102-105.

Meyuhas, O., and Dreazen, A. (2009). Ribosomal protein S6 kinase from TOP mRNAs to cell size. Progress in molecular biology and translational science 90, 109-153.

Muramatsu, M., Kinoshita, K., Fagarasan, S., Yamada, S., Shinkai, Y., and Honjo, T. (2000). Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. Cell 102, 553-563.

Nurieva, R.I., Chung, Y., Hwang, D., Yang, X.O., Kang, H.S., Ma, L., Wang, Y.H., Watowich, S.S., Jetten, A.M., Tian, Q., and Dong, C. (2008). Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. Immunity 29, 138-149.

Parry, R.V., Rumbley, C.A., Vandenberghe, L.H., June, C.H., and Riley, J.L. (2003). CD28 and inducible costimulatory protein Src homology 2 binding domains show distinct regulation of phosphatidylinositol 3-kinase, Bcl-xL, and IL-2 expression in primary human CD4 T lymphocytes. J Immunol 171, 166-174.

Reinhardt, R.L., Liang, H.E., and Locksley, R.M. (2009). Cytokine-secreting follicular T cells shape the antibody repertoire. Nature immunology 10, 385-393.

Revy, P., Muto, T., Levy, Y., Geissmann, F., Plebani, A., Sanal, O., Catalan, N., Forveille, M., Dufourcq-Labelouse, R., Gennery, A.*, et al.* (2000). Activation-induced cytidine deaminase (AID) deficiency causes the autosomal recessive form of the Hyper-IgM syndrome (HIGM2). Cell 102, 565-575.

Rolf, J., Bell, S.E., Kovesdi, D., Janas, M.L., Soond, D.R., Webb, L.M., Santinelli, S., Saunders, T., Hebeis, B., Killeen, N.*, et al.* (2010). Phosphoinositide 3-kinase activity in T cells regulates the magnitude of the germinal center reaction. J Immunol 185, 4042-4052.

Scheu, S., Stetson, D.B., Reinhardt, R.L., Leber, J.H., Mohrs, M., and Locksley, R.M. (2006). Activation of the integrated stress response during T helper cell differentiation. Nature immunology 7, 644-651.

Sonenberg, N., and Hinnebusch, A.G. (2009). Regulation of translation initiation in eukaryotes: mechanisms and biological targets. Cell 136, 731-745.

Swallow, M.M., Wallin, J.J., and Sha, W.C. (1999). B7h, a novel costimulatory homolog of B7.1 and B7.2, is induced by TNFalpha. Immunity 11, 423-432.

Szabo, S.J., Gold, J.S., Murphy, T.L., and Murphy, K.M. (1993). Identification of cis-acting regulatory elements controlling interleukin-4 gene expression in T cells: roles for NF-Y and NF-ATc. Molecular and cellular biology 13, 4793-4805.

Tafuri, A., Shahinian, A., Bladt, F., Yoshinaga, S.K., Jordana, M., Wakeham, A., Boucher, L.M., Bouchard, D., Chan, V.S., Duncan, G.*, et al.* (2001). ICOS is essential for effective T-helper-cell responses. Nature 409, 105-109.

Tang, H., Hornstein, E., Stolovich, M., Levy, G., Livingstone, M., Templeton, D., Avruch, J., and Meyuhas, O. (2001). Amino acid-induced translation of TOP mRNAs is fully dependent on phosphatidylinositol 3-kinase-mediated signaling, is partially inhibited by rapamycin, and is independent of S6K1 and rpS6 phosphorylation. Molecular and cellular biology 21, 8671-8683.

Thoreen, C.C., Chantranupong, L., Keys, H.R., Wang, T., Gray, N.S., and Sabatini, D.M. (2012). A unifying model for mTORC1-mediated regulation of mRNA translation. Nature 485, 109-113.

Vinuesa, C.G., and Cyster, J.G. (2011). How T cells earn the follicular rite of passage. Immunity 35, 671-680.

Warnatz, K., Bossaller, L., Salzer, U., Skrabl-Baumgartner, A., Schwinger, W., van der Burg, M., van Dongen, J.J., Orlowska-Volk, M., Knoth, R., Durandy, A.*, et al.* (2006). Human ICOS deficiency abrogates the germinal center reaction and provides a monogenic model for common variable immunodeficiency. Blood 107, 3045-3052.

Watanabe, M., Hara, Y., Tanabe, K., Toma, H., and Abe, R. (2005). A distinct role for ICOS-mediated co-stimulatory signaling in CD4+ and CD8+ T cell subsets. International immunology 17, 269-278.

Xu, H., Li, X., Liu, D., Li, J., Zhang, X., Chen, X., Hou, S., Peng, L., Xu, C., Liu, W.*, et al.* (2013). Follicular T-helper cell recruitment governed by bystander B cells and ICOS-driven motility. Nature 496, 523-527.

Zaretsky, A.G., Taylor, J.J., King, I.L., Marshall, F.A., Mohrs, M., and Pearce, E.J. (2009). T follicular helper cells differentiate from Th2 cells in response to helminth antigens. The Journal of experimental medicine 206, 991-999.

Chapter IV

General Discussion

1. SUMMARY

ICOS costimulation has been known to play a crucial role in the development of Tfh cells, as well as in the production of key cytokines involved in T cell-mediated B cell help such as IL-4 and IL-21. On the other hand, PI3K has also been shown to be a crucial component of GC reactions, but its role in Tfh cell development remained elusive. As ICOS is a potent inducer of PI3K activity, I hypothesized that ICOS-mediated PI3K activation is crucial for the generation of Tfh cells. To test this, our group generated a knock-in strain of mice with a mutated ICOS PI3K-binding motif (ICOS-YF). Importantly, I show that ICOS-YF mice do not generate any Tfh cells under multiple conditions, demonstrating that the ICOS-PI3K axis is indeed crucial for the differentiation of Tfh cells and the formation of GCs. Additionally, I show that ICOS and CD28 have differential roles, possibly in different stages of Tfh cell development, where ICOS costimulation enhances IL-4 and IL-21 production during T-B interactions, while CD28 costimulation augments IL-2 production and proliferation **(Chapter II)**.

Previous studies have shown that IL-4 translation is heavily regulated and could be induced upon TCR engagement *in vitro*. On the other hand, *in vivo* studies have also shown that IL-4 protein synthesis in LNs is restricted to $ICOS^{hi}$ Tfh cells that localize to the B cell follicles. It is also well established that PI3K activity, through the AKT-mTOR axis, induces cap-dependent translation. I therefore hypothesized that ICOS costimulation, through the PI3K-AKT-mTOR signaling pathway, induces increased IL-4 translation. I demonstrated that ICOS costimulation significantly increases IL-4 translation through a PI3K-dependent mechanism. I also show that ICOS-YF and $ICOS^{-/-}$ CD4 T cells are significantly less efficient at inducing B cell activation, as determined by AID expression, in an *in vitro* T-B co-culture system. Furthermore, the addition of exogenous IL-4 equalized AID expression in B cells, suggesting that it is likely a limited supply of IL-4, as opposed to other components of T cell "help" (e.g., CD40L), that is compromising the efficiency of ICOS mutant T cells to activate B cells in this *in vitro* system **(Chapter III)**.

Thus, I demonstrated that the ICOS-PI3K signaling axis is crucial for both the development and the function of Tfh cells.

2. ICOS SIGNALING: LESSONS LEARNED

My work focused on characterizing the specific effects of PI3K activation in ICOS signaling and its ramifications for Tfh cell differentiation and function. To do so, our group

generated the previously discussed ICOS-YF strain of mice. In the process, I also analyzed certain aspects of ICOS-mediated PI3K activation that had not been previously established and found several counterintuitive observations that require further discussion before moving on to discussing the Tfh cell phenotype.

First, it is generally believed that the TCR complex recruits a Src family kinase to phosphorylate Tyr181 on ICOS (Nurieva et al., 2007) and that this phosphorylation is a necessary step for PI3K recruitment. However, we found that unligated ICOS is abundantly bound to PI3K in activated CD4 T cells, although this recruitment was increased upon TCR and ICOS ligation. PI3K binding to ICOS in activated T cells in the absence of ligation has previously been reported (Coyle et al., 2000; Feito et al., 2003). However, its role in T cell immunity is unclear. It is possible that ICOS-bound PI3K may induce PI(3,4,5)P3 production at steady state, but the rate of conversion is so low that PTEN is able to keep the concentration below a certain threshold. Also, whether or not PI3K which is bound to ICOS in the absence of costimulation is actually functional is also unclear. As we are still lacking a detailed structure/function relationship of the ICOS cytoplasmic tail in non-stimulated CD4 T cells vs. CD4 T cells stimulated with ICOS, it is hard to draw conclusions as to why PI3K is constitutively bound to ICOS, but does not result in strong PI3K activity as determined by levels of AKT phosphorylation.

Second, we found that ICOS stimulation in the absence of TCR engagement induces an increase in the phosphorylation of AKT, but this does not correlate with an increase in the production of IL-4, IL-21 or IL-10 (data not shown) (Beier et al., 2000; Feito et al., 2003) or Ca^{2+} potentiation (data not shown). This is striking as is it is generally believed that ICOS potentiates TCR-mediated signals, but cannot signal in the absence of TCR stimulation. However, our results show that anti-ICOS antibody crosslinking can induce PI3K even in the absence of TCR stimulation. This phenomenon may explain the novel observation that ICOSL expression on non-cognate B cells (ie: without any stimulation to the TCR) is able to induce Tfh cell migration into the B cell follicle in PI3K-dependent but TCR-independent manners (Xu et al., 2013). Furthermore, it had also been previously reported that anti-ICOS-coated culture slides could induce cell polarization with a highly elongated morphology of human activated ($CD45RO^{+}$) CD4 T cells, in a manner dependent upon PI3K/AKT and members of the Rho small GTPase family (Okamoto et al., 2004; Nukada et al., 2006). PI3K is reported to activate the mTORC2 complex

and has important roles for cytoskeletal rearrangement and T cell migration (Gan et al., 2011) and this may be how ICOS induces migration in the absence of TCR signaling. However, the mechanism through which ICOS is able to induce cytoskeletal rearrangement and migration, but not cytokine production and proliferation in the absence of TCR is currently unknown and should be addressed in future studies. This suggests that our understanding of ICOS-mediated PI3K activation is limited and that more structural studies are needed to better understand this relationship.

Third, ICOS is known to be a potent inducer of PI3K activation and assists TCR-mediated Ca^{2+} flux (Nurieva et al., 2007). Since it has been previously suggested that ICOS potentiates TCR-mediated Ca^{2+} signaling by augmenting Itk-PLCγ1 signaling pathways, I predicted that ICOS-mediated Ca^{2+} release would also be abrogated by the ICOS-YF mutation. Surprisingly, I found that ICOS-YF retained its ability to potentiate TCR-mediated Ca^{2+} flux in activated CD4 T cells. Furthermore, we have also recently made the same observations in CD8 T cells (Li et al., 2013). Finally, our group has recently identified a motif that can induce Ca^{2+} signaling potentiation independently of the YMFM motif (Leconte et al; unpublished observations) and we are working to uncover the responsible signaling pathway(s).

In summary, the development of ICOS-YF mice has allowed us to study the specific role of ICOS-mediated PI3K signaling in mice. This has allowed us to demonstrate that ICOS potentiation of TCR-mediated Ca^{2+} signaling is PI3K independent. Therefore, future studies using these mice will help us uncover not only the role of ICOS-mediated PI3K activation, but also the role of ICOS-mediated Ca^{2+} signaling.

3. ICOS PROMOTES THE GENERATION OF Tfh CELLS THROUGH PI3K

Prior to our generation of the ICOS-YF mouse strain, no group had directly looked at the effect of ICOS-mediated PI3K signaling in any model. Previous PI3K studies showed that inhibiting PI3K using pharmacological inhibitors during *in vitro* TCR and ICOS costimulation resulted in the abrogation of IL-4 and IL-10 production, as well as proliferation in human $CD45RO^{+}$ CD4 T cells and mouse Th clones (Feito et al., 2003; Okamoto et al., 2003). However, since the TCR is also able to activate PI3K signaling, completely inhibiting PI3K using pharmacological inhibitors does not accurately reflect the specific effect of ICOS on PI3K

signaling and its downstream effects. Furthermore, administering PI3K inhibitors to live mice is impractical for studying the specific effect of ICOS-mediated PI3K signaling due to the ubiquitous nature of PI3K in various tissues and cell types. For example, recent work by Aaron Marshall and colleagues has shown that p110δ inhibition in immunized mice leads to dramatically higher levels of IgE due to an impact on B cells (Zhang et al., 2012). This is probably due to a dominant effect of the inhibitors on B cells since p110δ inhibition *in vitro* led to increased IgG and IgE production of anti-CD40- and IL-4-treated mouse B cells.

To this point, Vanhaesebroeck's group's development of kinase-dead $p110\delta^{D910A/D910A}$ mice allowed study of this PI3K isoform in the immune system (Okkenhaug et al., 2002). However, due to the importance of PI3K in both T cells and B cells, they were unable to determine whether the resulting defect in GC development was a result of defects in T cells, B cells or both. Recent work by the Martin Turner group has also selectively looked at the role of p110δ in T cells using CD4-cre; $p110\delta^{f/f}$ mice (Rolf et al., 2010) and showed the role of this subunit in Tfh cell differentiation and GC development. However, due to the multiple factors that can activate PI3K in T cells, they were unable to accurately determine the specific function of ICOS-PI3K in Tfh cell development.

3.1 ROLE OF ICOS-PI3K IN Tfh CELL DEVELOPMENT

Multiple studies have shown that ICOS is crucial for the development of Tfh cells in both humans and mice (Akiba et al., 2005; Bossaller et al., 2006; Nurieva et al., 2008), but the underlying molecular mechanisms are not completely understood. In my studies using ICOS-YF mice, I proved that ICOS-mediated PI3K activation is crucial for the generation of Tfh cells *in vivo*. Although not included in Chapter II, I also observed that T cell-specific deletion of PTEN ($PTEN^{f/f}$;Lck-Cre mice) significantly increased the number of Tfh cells and GC B cells in Peyer's patches compared to WT mice (Appendix II). Additionally, crossing ICOS-YF mice with $PTEN^{f/f}$;Lck-Cre mice partially rescued the number of Tfh cells and GC B cells (Appendix II), further demonstrating the importance of PI3K signaling in Tfh cell development. These findings support our study and further validate that the ICOS-PI3K signaling pathways are crucial for the development of Tfh cells.

I next sought to determine how the ICOS-PI3K pathway was responsible for Tfh cell differentiation. When I first confirmed the Tfh cell phenotype in ICOS-YF mice, two studies were

published showing that in the absence of IL-21 or its receptor, development of Tfh cells is diminished and GCs fail to arise after immunization (Nurieva et al., 2008; Vogelzang et al., 2008). Additionally, the authors of the studies observed that ICOS costimulation directly upregulated IL-21 production and that this ICOS-mediated autocrine IL-21 signaling was necessary for Tfh cell generation. I subsequently confirmed that ICOS costimulation did upregulate IL-21 and that this was achieved through a PI3K-dependent mechanism. We therefore proposed that arrested Tfh cell differentiation in ICOS-YF and ICOS$^{-/-}$ mice was due to the inability of their CD4 T cells to induce IL-21 production upon ICOS costimulation, most likely at the stage of T-B interaction. However, shortly after publishing our results in 2009, three studies came out in 2010, which contradicted the initial 2008 IL-21$^{-/-}$ studies. The newer studies showed that lack of IL-21 responsiveness only had a partial effect on Tfh cell differentiation *in vivo* (Avery et al., 2010; Linterman et al., 2010; Zotos et al., 2010). IL-21-deficiency seemed to have a much greater impact on B cells than on Tfh cells, such that B cells failed to differentiate into GC B cells and PCs in the absence of IL-21. A subsequent study demonstrated that IL-6 likely compensates for IL-21-deficiency in Tfh cell differentiation, as IL-21$^{-/-}$ mice had normal Tfh cell differentiation but showed defect only when IL-6 blocking antibodies were injected into immunized mice (Eto et al., 2011). Therefore, it is likely that ICOS-mediated IL-21 production is not the rate-limiting step in ICOS-mediated Tfh cell differentiation, although it may still play a role in this process. However, the production of IL-4 and IL-21 upon ICOS costimulation still likely has important ramifications for T cell-mediated B cell help. Nevertheless, our proposed model from Chapter II should be updated to include current findings.

One candidate mechanism for ICOS-mediated Tfh cell differentiation is through the production of IL-4. Previous studies have suggested that IL-4 acts in an autocrine/paracrine manner to promote Th2 cell differentiation (Kuhn et al., 1991; Kopf et al., 1993). However, a recent study has shown that IL-4-deficient mice still generate near-WT levels of Tfh cells two weeks after helminthic infection (King and Mohrs, 2009). Therefore, ICOS-mediated IL-4 production is likely not the main mechanism of ICOS-induced Tfh cell generation.

While our first manuscript (Gigoux et al., 2009) was in revision, three studies were published confirming that Bcl6 is the master regulator of Tfh cells (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009). Of note, Bcl6 transduction in activated antigen-specific CD4 T cells

further induced Tfh cell differentiation when the cells were transferred back into mice with an ongoing immunological reaction. We therefore speculated that ICOS costimulation could upregulate Bcl6 expression, and that this would in turn lead to Tfh cell differentiation. Although I was unable to include the results in our study before publication, I nevertheless sought to test the effect of ICOS costimulation on the expression of Bcl6 and Tfh cell differentiation *in vitro*. We activated isolated CD4 T cells *in vitro* with anti-CD3 and anti-CD28 in the presence of IL-6, which is thought to reflect what occurs *in vivo* (Eddahri et al., 2009; Eto et al., 2011). We then restimulated the cells with anti-CD3 alone, or anti-CD3 and anti-ICOS together. Interestingly, we found that neither anti-CD3 nor anti-CD3 and anti-ICOS antibody crosslinking of activated CD4 T cells had any significant effect on Bcl6 mRNA expression after 6 hours of stimulation (data not shown). This was not wholly surprising, as a scan of the transcription factor–binding elements in the Bcl6 promoter identified DNA-binding sites for the STAT and Foxo families of transcription factors (Oestreich et al., 2012), none of which are reported to be induced upon ICOS costimulation. In fact, AKT negatively regulates Foxo transcription factors, which might negatively regulate Bcl6 transcription. Likewise, TCR and ICOS costimulation failed to upregulate CXCR5 protein or mRNA upon restimulation of *in vitro* activated CD4 T cells (data not shown). Therefore, *in vitro* ICOS costimulation of activated CD4 T cells did not induce Tfh cell differentiation, as determined by analysis of conventional Tfh cell markers.

A recent study investigated a link between ICOS and Bcl6 by following LCMV-specific TCR transgenic CD4 T cells (SMARTA, SM) that were adoptively transferred into WT recipient mice and infected with LCMV (Choi et al., 2011). They found that, compared to ICOS-WT SM CD4 T cells, the $ICOS^{-/-}$ SM CD4 T cells failed to upregulate Bcl6. However, the authors could not confirm whether this was a direct or indirect result of ICOS-deficiency. This strongly suggests that ICOS does play a role in Bcl6 induction *in vivo* and, in turn, Tfh cell differentiation, but the mechanisms behind this remain to be determined. It is also important to emphasize that *in vitro,* Bcl6 transduction alone is not sufficient to drive Tfh cell differentiation (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009) (and data not shown). This strongly suggests that multiple factors are likely required for full Tfh cell differentiation, and that Bcl6 induction may only be one component of many that are required for the development of Tfh cells. Furthermore, a recent finding has also shown that Bcl6 transduction of $ICOS^{-/-}$ OTII CD4 T cells had no effect on Tfh cell differentiation, GC formation or CD4 T cell migration into B cell follicles when the cells were

adoptively transferred into WT recipient mice and immunized with OVA (Xu et al., 2013). This strongly suggests that the ICOS-PI3K pathway does not induce Tfh cell differentiation solely through Bcl6 upregulation alone. With these findings in mind, I propose three possible mechanisms by which ICOS induces Tfh cell differentiation (Figure 1).

First, ICOS costimulation could increase the expression of an unknown factor(s) (*X)*, where the subsequent activation of *X* would then be required to induce Tfh cell differentiation through Bcl6 upregulation or through the upregulation of an as-of-yet unidentified Tfh cell differentiation factor(s) (Figure 1A). This may explain why ICOS costimulation alone does not lead to an increase in Bcl6 transcription or *in vitro* Tfh cell differentiation. There are a few candidates for the unknown factor(s). The first candidate is c-Maf. Retroviral transduction of c-Maf, which is known to be upregulated by ICOS (Bauquet et al., 2009), in human tonsilar $CD45RO^{-}CXCR5^{-}$ CD4 T cells can lead to a small increase in CXCR5 expression *in vitro* (Kroenke et al., 2012). Although c-Maf transduction did not increase Bcl6 protein levels, it is possible that increased CXCR5 expression allows the pre-Tfh cell to migrate into the B cell follicle where it can receive additional signals, which might induce Bcl6 upregulation. However, since we do not see an increase in CXCR5 expression with ICOS costimulation in our *in vitro* activated mouse CD4 T cells, it is possible that secondary factors are required to synergize with c-Maf to induce Tfh cell differentiation. The second candidate gene is IL-6R, which has recently been shown to induce Bcl6 transcription through STAT1 (Choi et al., 2013). Additionally, Tfh cells have been reported to express higher levels of IL-6R than Th1, Th2 and Th17 cells (Nurieva et al., 2008). Interestingly, it has been shown that the interaction of ICOS and its ligand (ICOSL) on mouse bone marrow-derived dendritic cells induces a p38-MAPK dependent elevation of IL-6 (Tang et al., 2009). Finally, a third candidate could be the microRNA cluster miR-17~92. Two recent studies have identified this cluster to be critical for robust Tfh cell differentiation and function (Baumjohann et al., 2013; Kang et al., 2013). Mechanistically, this microRNA cluster restrained the expression of genes "inappropriate" for the Tfh cell subset, such as *Rora* (Baumjohann et al., 2013). Furthermore, miR-17~92 suppressed the expression of the phosphatase PHLPP2 (PH domain and leucine rich repeat protein phosphatase 2), a major phosphatase for AKT, allowing greater ICOS-PI3K signaling capacity, Tfh cell differentiation and Tfh cell migration into B cell follicles. It is tempting to hypothesize that the ICOS-PI3K axis induces expression of miR-17~92, which would not only direct Tfh cell differentiation, but also further sensitize the Tfh cell to subsequent ICOS-PI3K

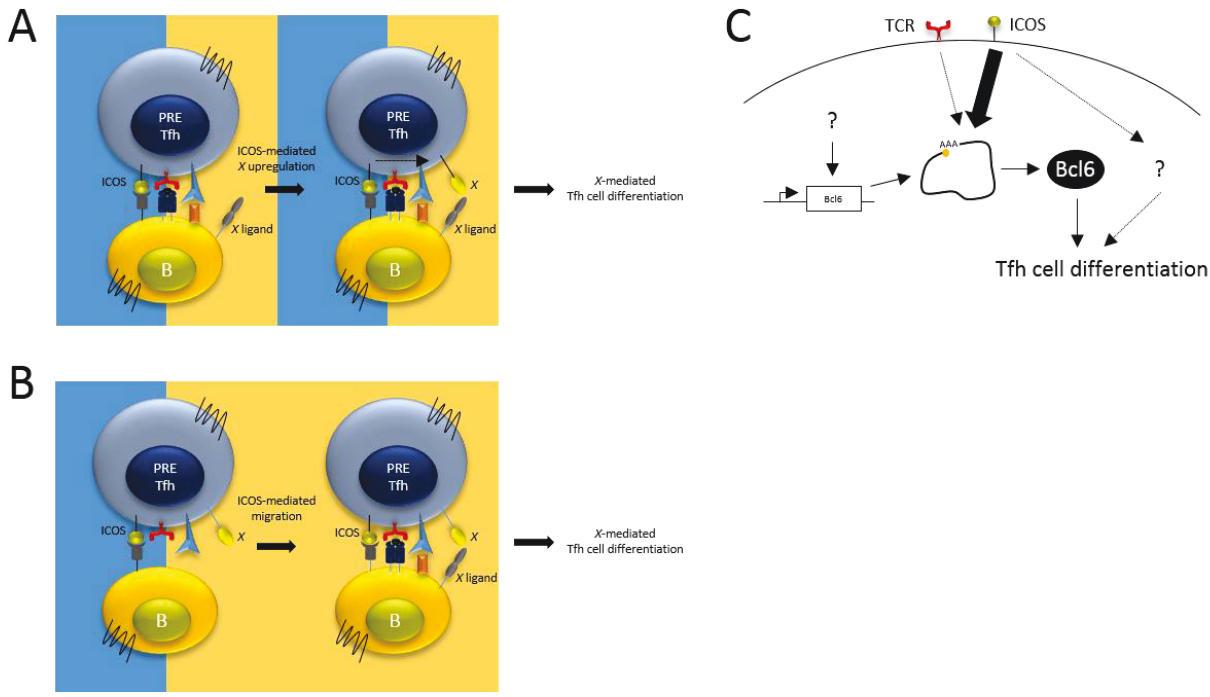

Figure 1. Models for ICOS-mediated Tfh cell differentiation. A) During pre-Tfh cell-cognate B cell interactions at the T-B border (blue = T cell zone, yellow = B cell follicle), ICOS costimulation induces expression of unknown factor *X*. Stimulation of *X* would then lead to Tfh cell differentiation. In this model, *X* is depicted as a transmembrane protein. However, *X* can be a transcription factor or miRNA, which might synergize with an ICOS-independent pathway to induce Tfh cell differentiation. B) ICOSL expression by non-cognate B cell at the T-B border induces the migration of pre-Tfh cells into the B cell follicle. Subsequently, cognate interaction between pre-Tfh cells and B cells in the B cell follicles induces Tfh cell differentiation. C) ICOS-mediated Bcl6 mRNA translation facilitates Tfh cell differentiation. However, Bcl6 protein expression alone is insufficient to induce Tfh cell differentiation in the absence of other unknown ICOS-mediated pathway(s).

signaling, possibly to enhance Tfh cell-mediated B cell help. In summary, c-Maf, IL-6R and miR-17~92 are viable candidates for ICOS-regulated genes that might have the secondary effect of Tfh cell differentiation. Analysis of genes that are regulated by ICOS costimulation at the transcriptional and/or translational level could provide more insight into this issue.

The second possible mechanism through which ICOS might induce Tfh cell differentiation is somewhat related to the first. Assuming that another unknown factor *X* is required to induce Tfh cell differentiation, possibly through an increase in Bcl6 expression, it is possible that ICOS expression allows for the migration of the CD4 T cell to the location for the ligand for *X*, possibly inside the B cell follicles (Figure 1B). A recent study by Hai Qi and colleagues has uncovered a novel TCR-independent role for ICOS in promoting T cell migration into the B cell follicle (Xu et al., 2013). Previous models have proposed that CXCR5 expression by Tfh cells would be sufficient for promoting entry into the B cell follicles (McHeyzer-Williams et al., 2009; Crotty, 2011; Linterman et al., 2012). However, Hai Qi's group found that ectopic transduction of CXCR5 into mouse activated ICOS$^{-/-}$ CD4 T cells was not sufficient to induce CD4 T cell migration into the B cell follicles. Whereas CXCR5 transduction into WT CD4 T cells increased the frequency of the cells inside the B cell follicle, CXCR5-transduced ICOS$^{-/-}$ CD4 T cells collected at the T-B border and were largely absent from the B cell follicle. Furthermore, this retention of cells at the T-B border was not due to CCR7 expression or sensitivity, which might otherwise have explained the retention. Finally, further investigation revealed that the critical source of ICOSL, which promoted T cell migration into the B cell follicle, was provided by non-cognate B cells. Importantly, this ICOS-mediated migration was shown to be dependent upon PI3K. Therefore, it is possible that ICOS-mediated migration allows the Tfh cell to migrate into the B cell follicle and receive the appropriate stimuli from B cells for full Tfh cell differentiation. However, this model does not preclude the importance of ICOS stimulation during cognate B cell interaction in the development of Tfh cells.

The third possible mechanism through which ICOS costimulation might induce Tfh cell differentiation is through an increase in Bcl6 translation (Figure1C). It has been shown that Bcl6 transcription levels do not correlate with Bcl6 protein levels. For example, naïve B cells express Bcl6 mRNA but have undetectable levels of Bcl6 protein. Furthermore, in multiple cases of leukemia, increased Bcl6 expression correlates with a shortening of the 5' UTR of Bcl6 (Deweindt

et al., 1993; Ye et al., 1993; Lo Coco et al., 1994). Since increased length of the 5' UTR can lead to translational repression, this suggests that Bcl6 expression in Tfh cells may be regulated at the translational level, possibly through the ICOS-PI3K axis. Although we observe that ICOS costimulation does not induce the expression of Bcl6 mRNA, preliminary results from our group suggest that ICOS-PI3K partially increases polysome loading on Bcl6 mRNA (data not shown). However, more work is required to determine if this is the case. It is important to note, however, that ectopic expression of Bcl6 in $ICOS^{-/-}$ CD4 T cells was insufficient to induce Tfh cell differentiation or CD4 T cell migration into the B cell follicle (Xu et al., 2013). Therefore, it is unlikely that ICOS-mediated Bcl6 translation would in itself induce Tfh cell differentiation. Alternatively, as we have described in Chapter III, ICOS costimulation could possibly lead to selective translation of another component (*X*) that induces Tfh cell differentiation. A full analysis of the ICOS translatome would provide a list of candidate transcripts. Therefore, ICOS-PI3K axis may induce Tfh cell differentiation through multiple mediators and increased Bcl6 translation can be one of the components.

In summary, although we have shown that Tfh cell differentiation is dependent upon ICOS-mediated PI3K activation, the exact mechanisms have yet to be clarified.

3.2 ICOS AND CD28 HAVE DIFFERENTIAL ROLES IN THE MULTISTEP PROCESS OF Tfh CELL DIFFERENTIATION

As I have already discussed, ICOS costimulation leads to potent PI3K activation, and it was originally believed to be the only pathway downstream of ICOS signaling. As other costimulatory receptors can also activate PI3K, notably CD28, this led to an interesting paradox - why is CD28 unable to compensate for defects in ICOS signaling? There are multiple possible explanations. For example, CD28 ligands are highly restricted to APCs, whereas ICOSL is widely expressed in non-lymphoid tissues as well as cells of hematopoietic lineage, especially under inflammatory conditions (Greenwald et al., 2005). This may explain why the impact of ICOS-deficiency is more pronounced during effector/memory phase of T cell responses. However, defects in ICOS still lead to striking phenotypes, even when CD28 ligands are present, such as a near total absence of Tfh cells in $ICOS^{-/-}$ mice, despite the fact that GC B cells can express CD86 (Victora et al., 2010).

Multiple studies point to the differential roles of CD28 and ICOS in promoting PI3K activity. First, CD28-mediated PI3K activation has been repeatedly shown to be dispensable for most T cell activity *in vivo* (Okkenhaug et al., 2001; Parry et al., 2003; Deane et al., 2007; Gogishvili et al., 2008; Dodson et al., 2009; Pagan et al., 2012). Second, while CD28 is able to bind only to the p85α regulatory subunit of PI3K, ICOS is able to bind to both the p85α and the p50α subunits of PI3K (Fos et al., 2008). This has important repercussions, as p50α is reported to induce stronger PI3K activity than p85α (Inukai et al., 1997; Inukai et al., 2001). Furthermore, the CD28 PI3K-binding domain also recruits Grb2, and this may induce competition with PI3K, further reducing the ability of CD28 to strongly potentiate PI3K activity. ICOS, on the other hand, has no such competition for PI3K (Rudd and Schneider, 2003). This may explain why ICOS induces stronger PI(3,4,5)P3 levels (Parry et al., 2003) and AKT phosphorylation (Okamoto et al., 2003; Fos et al., 2008; Gigoux et al., 2009) than CD28 costimulation. This is likely one reason why ICOS costimulation induces overwhelmingly more IL-4 and IL-21 compared to CD28 costimulation, while CD28 costimulation induces higher levels of IL-2 and proliferation induction.

However, the role of CD28 in ongoing GC reactions is less clear. The CD28 ligand B7.2 has been shown to be upregulated in GC B cells (Victora et al., 2010), and B7.2-deficient mice display impaired Tfh cell maintenance and GC reaction (Salek-Ardakani et al., 2011). Interestingly, the CD28 ligand B7.2 is specifically upregulated on B cells during the transition from centroblasts in the DZ to centrocytes in the LZ during GC reactions (Victora et al., 2010), suggesting that CD28 costimulation may play a role during an ongoing GC reaction. Consistent with the idea that CD28-B7 interaction may become important in T-B communication in the later stage of GC reactions, prolonged survival of GC B cells (by conditional deletion of Fas in GC B cells) leads to an expansion of auto-reactive T cells in a CD28-dependent manner (Hao et al., 2008).

In summary, I propose a model based on our findings and the findings of others, where ICOS and CD28 play differential roles in the multistep process of Tfh cell differentiation. I propose that CD28-mediated IL-2 production is necessary for initial CD4 T cell activation and proliferation in the T cell zone, while ICOS is necessary for full Tfh cell differentiation by B cells at the T-B border (Figure 2) through IL-21 production and other unknown mechanisms.

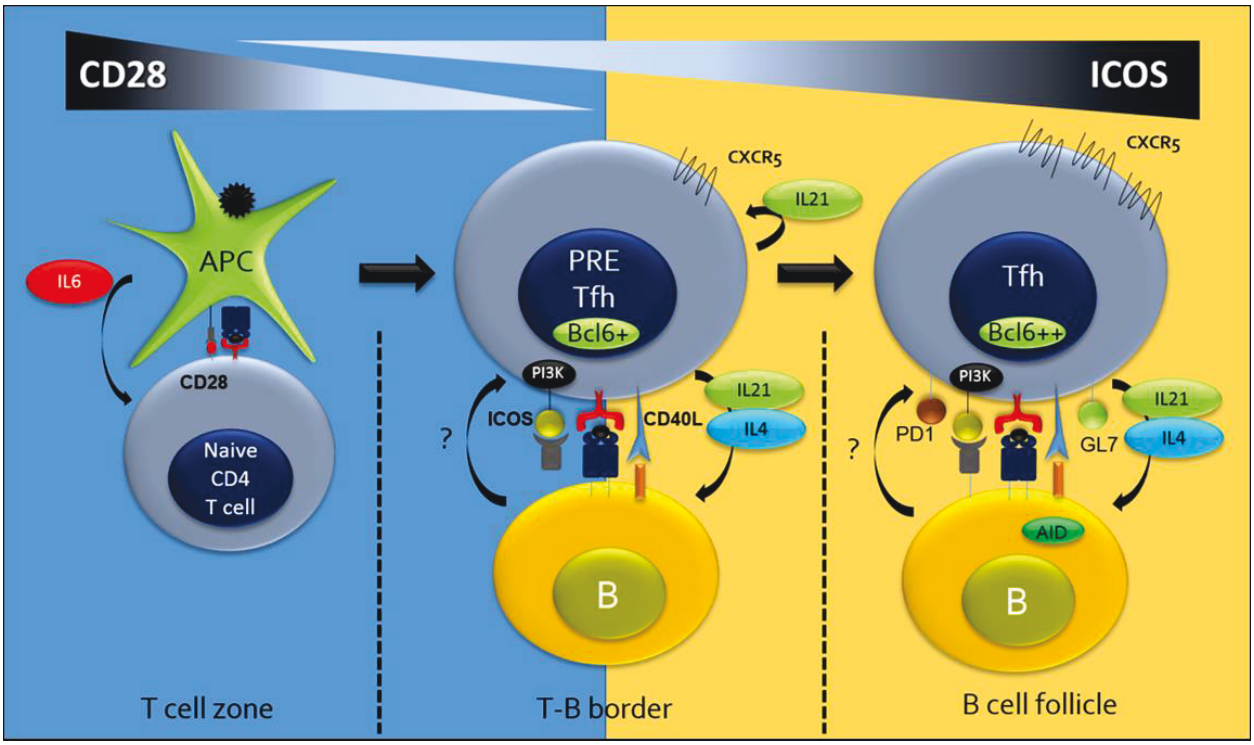

Figure 2. Model of sequential costimulation during Tfh cell differentiation. Naïve CD4 T cells are activated in the T cell zone by APCs through CD28-mediated costimulation. Additional signals through IL-6 (IL-6 in mice, IL-12 in humans) produced by APCs promotes initial pre-Tfh cell program. Upregulation of CXCR5 allows pre-Tfh cell migration to the T-B border where they interact with cognate B cells through a SAP-dependent mechanism (not depicted). Initial activation of B cells by pre-Tfh cells through CD40L, IL-21 and IL-4 expression induces initial GC formation. In this model, ICOS-PI3K signaling provides two functions: ICOS stimulation by non-cognate B cells at the T-B border promotes pre-Tfh cell migration into the B cell follicle; and ICOS costimulation by cognate B cells facilitates full Tfh cell differentiation.

4. ROLE OF ICOS-PI3K IN THE FUNCTION OF Tfh CELLS

Given that ICOS ligation strongly activates the AKT pathway, it is likely that ICOS is necessary to regulate many aspects of the function of Tfh cells. In my work, I focused on one specific aspect of Tfh cell-mediated B cell help; that of IL-4 protein production. A previous study has shown that IL-4 mRNA translation is inhibited in activated polarized Th2 cells, but this inhibition is released upon TCR stimulation (Scheu et al., 2006). We have also shown that activated Th0 unpolarized CD4 T cells also suppress IL-4 translation in the absence of restimulation, and that TCR stimulation induced IL-4 mRNA translation. Importantly, we have shown that the ICOS-PI3K axis, in collaboration with the TCR, can induce the translation of IL-4 mRNA to a much greater extent than TCR stimulation alone. I have also shown that ICOS costimulation induces the phosphorylation of the key translational mediators 4E-BP1 and S6K, which are known targets of mTORC1. This likely has important ramifications for humoral immunity.

It is easy to speculate as to repercussions of increased IL-4 production in response to ICOS costimulation. When Tfh cells come into contact with cognate B cells, their interactions can be very short and have a median contact time of around 20 minutes (Qi et al., 2008; Cannons et al., 2010). In many cases, if the production of IL-4 protein were to depend upon transcription, the conjugated B cells could dislodge before receiving enough IL-4. Therefore, having a pool of IL-4 mRNA already present, but repressing the translation of IL-4 protein until the appropriate time could be an efficient mechanism for quick and targeted IL-4 protein release towards cognate B cells.

What is striking with our findings is that ICOS costimulation does not induce such increased translation in the other analyzed cytokines, specifically IFN-γ. These results partially conflict with another study, which showed that TCR stimulation alone can induce IFN-γ mRNA translation as well as translation of IL-4 mRNA (Scheu et al., 2006). One explanation for this discrepancy is the difference in experimental procedure. While we restimulated Th0 activated CD4 T cells for 20 minutes with soluble antibody crosslinking, Scheu et al. restimulated Th1 polarized CD4 T cells for 3 hours with plate-bound peptide:MHC complexes. It is probable that the length of stimulation has a major impact on translational activity, as a longer stimulation could induce the transcription of factors that may also play a role in translation initiation. I argue that our much shorter restimulation period better represents the immediate effect of ICOS costimulation on

translation in comparison to their much longer restimulation, where transcription of different genes may impact translation.

The mechanisms through which the ICOS-PI3K pathway regulate IL-4 mRNA translation while not affecting IFN-γ translation have yet to be determined. We hypothesized that, as the 5' UTR of IL-4 mRNA is rich in pyrimidines, IL-4 translation would be inhibited by a TOP-like or a PRTE-like motif, and that signaling through the ICOS-PI3K axis would relieve this inhibition. We observed that when the luciferase gene was fused with the IL-4 5' UTR, it inhibited luciferase activity by approximately 40%, suggesting that the 5' UTR partially inhibited IL-4 translation. However, when we mutated the pyrimidine-rich sequences, replacing them with purines, luciferase activity was not affected, suggesting that the inhibition of IL-4 mRNA translation was not through these pyrimidine-rich sequences. This is possibly because the pyrimidine-rich sequences are not bona-fide TOP or PRTE motifs (Biberman and Meyuhas, 1997; Thoreen et al., 2012) and suggests that the pyrimidine-rich sequences have no effect on IL-4 mRNA translation. Another mechanism through which 5' UTRs regulate translation is through a complex and thermally stable secondary structure (Livingstone et al., 2010). However, the predicted secondary structure of the IL-4 mRNA reveals that this is likely not the case (ΔG = -7.70 kcal/mol, RNAfold). Since there are no other known mechanisms through which the 5' UTR regulates translation, it remains unknown how the 5' UTR of IL-4 regulates its translation. Also, since the 5' UTR of IL-4 only weakly inhibited IL-4 mRNA translation, it is also possible that this is not the main mechanism through which IL-4 protein synthesis is regulated.

One possible mechanism through which the ICOS-PI3K pathway could regulate IL-4 mRNA translation is through the dephosphorylation of eIF2α. It has been previously shown that the process of T cell activation induces the phosphorylation of eIF2α while stimulation through the TCR partially dephosphorylates eIF2α (Scheu et al., 2006). eIF2α phosphorylation is associated with global translational repression. Therefore, it is possible that ICOS costimulation further induces eIF2α dephosphorylation, leading to increased IL-4 mRNA translation. During ER stress, eIF2α is dephosphorylated by a complex containing the serine/threonine phosphatase PP1 and its non-enzymatic cofactor GADD34 (Novoa et al., 2001). Alternatively, the GADD34 homologue CReP is constitutively expressed and mediates eIF2α dephosphorylation in unstressed cells in conjunction with PP1 (Jousse et al., 2003). However, there is currently no evidence that

the PI3K pathway induces eIF2α desphorylation through these pathways, although alternate pathways may still exist. Nevertheless, it would be important to determine if ICOS costimulation has an effect on the phosphorylation state of eIF2α.

Another possible mechanism of IL-4 translation regulation is through the 3' UTR of IL-4 mRNA. During transcription, mRNAs are polyadenylated at their 3' end in the nucleus and are then exported to the cytoplasm (Weill et al., 2012). Once mRNAs reach the cytoplasm, the poly-A tail acts synergistically with the 5' cap structure to facilitate translation initiation. This is accomplished by stabilizing the closed-loop formed by the cap structure bound to the translation eIF4F complex. Multiple c*is*-acting regulatory elements in the 3' UTR can assemble mRNA-specific ribonucleoprotein complexes (mRNPs), which can dynamically modulate the length of the poly-A tail in a process termed alternative polyadenylation (APA). This can dramatically affect translation efficiency (Weill et al., 2012). Analysis of the 3' UTR of IL-4 mRNA reveals that it contains two 5′-AUUUA-3′ AU-rich elements (ARE), which can theoretically recruit about 30 ARE-binding proteins (ARE-BP) and induce poly-A tail deadenylation, ultimately inhibiting translation (Halees et al., 2008). However, the 3' UTR of IL-4 also contains two 5′-UUUUAU-3′ or 5′-UUUUAAU-3′ cytoplasmic polyadenylation elements (CPE), which are recognized by CPE-binding proteins (CPEBs) and induce polyadenylation of the poly-A tail as well as increased translation (Pique et al., 2008). Interestingly, analysis of the IFN-γ 3' UTR reveals no such elements. Therefore, it is possible that this is how translation of IL-4, but not that of IFN-γ, is regulated to the extent observed. However, the upstream pathways that regulate APA are not well understood. It remains to be determined if the ICOS-PI3K axis could modulate translation initiation through APA, and this warrants further investigation.

Finally, it is also possible that IL-4 mRNA is sequestered in stress granules (SGs) during initial T cell activation. SGs are RNA granules that form under stress conditions and can be triggered by eIF2α phosphorylation (Anderson and Kedersha, 2008). Specific mRNAs can be actively recruited to these SGs and this either induces their degradation or prevents mRNA translation. TCR stimulation has been shown to induce the disassembly of SGs (Scheu et al., 2006) and it is possible that ICOS costimulation may facilitate this process, ultimately promoting increased translation. It is still unclear which specific mRNA transcripts are included in or are exempt from SG recruitment. However, ARE-BPs are highly concentrated in SGs (Carballo et al.,

1998; Stoecklin et al., 2002), suggesting that ARE-containing mRNAs like IL-4 may be specifically targeted. Alternatively, miRNA-mediated translation suppression of IL-4 through its 3' UTR might also be involved in mediating its IL-4 protein production, but no miRNAs have been identified that could mediate this.

5. OUTSTANDING QUESTIONS IN THE FIELD

In light of the experiments performed in this thesis, as well as the recent work of other groups, there are a number of unanswered questions relating to the role of the ICOS-PI3K axis in the immune response. For example, it will be interesting to see whether the ICOS-PI3K pathway plays a role in the development or the function of Tfr cells. Tfr cells are reported to express higher levels of ICOS than Tfh cells (Linterman et al., 2011). Therefore, given that the ICOS-PI3K pathway is crucial in the homeostasis of Tregs (Kornete et al., 2012) and the development of Tfh cells (Gigoux et al., 2009), it likely also plays a role in controlling Tfr cell numbers. However, this has yet to be tested experimentally.

In addition to Tfr cells, ICOS might also play a role in the persistence of memory Tfh cells. Recent work by the McHeyser-Williams group has demonstrated the presence of a population of antigen-specific CD4 helper T cells in draining lymphoid organs over a prolonged period (up to 200 days post-immunization) (Fazilleau et al., 2007; Fazilleau et al., 2009a; Fazilleau et al., 2009b). This lymphoid reservoir of memory cells comes from cells that displayed all the features of Tfh cells, including a higher level of ICOS compared to activated CD4 T cells in the non-draining LN (Fazilleau et al., 2007). However, the role of ICOS signaling in supporting Tfh cell persistence is unknown. It is also unknown whether ICOS signaling is required for the expansion and/or effector function of memory Tfh cells. Therefore, it is tempting to believe that ICOS is essential to support Tfh cell persistence and/or expansion of memory Tfh cells.

On a more molecular level, we are still lacking a good understanding of the downstream effect of ICOS-PI3K costimulation. Most studies that analyze genes regulated by ICOS have done so on a highly limited basis. For example, a small sample of cytokines (ex: IL-2, IL-4, IL-10, IL-17, IL-21 and IFN-γ) are usually the only genes measured after ICOS costimulation. Furthermore, we are the first to report a role for ICOS in the translation of a specific mRNA (ie: IL-4). However, ICOS likely regulates a much larger pool of genes and mRNAs than the ones reported thus far. Therefore, one of the main outstanding questions is which genes and mRNAs are regulated by

ICOS. In order to address this, gene micro-array and translatome analyses of ICOS-costimulated T cell experiments should be conducted. Furthermore, in the case of the translatome, *in silico* analysis of ICOS-regulated mRNAs could help uncover consensus sequences in target mRNAs and might lead to a greater understanding of selective mRNA translation, which is still poorly understood.

Recently, the field of cell metabolism has undergone a sudden resurgence in importance, especially relating to lymphocyte metabolism (Wang and Green, 2012; Pearce and Pearce, 2013). In T cells, glucose is crucial for the production of ATP (Greiner et al., 1994). In naïve T cells, most of the ATP is produced through oxidative phosphorylation (OXPHOS). However, in activated T cells, the dominant pathway for ATP production is, paradoxically, through aerobic glycolysis, which is marked by the conversion of glucose-derived pyruvate to lactate, even in the presence of oxygen. This phenomenon is called the "Warburg effect" (Vander Heiden et al., 2009). As activated T cells are expected to require more energy than naïve T cells, it is striking that they shift away from OXPHOS and towards aerobic glycolysis. Therefore, it is speculated that activated T cells require aerobic glycolysis substrates, which would otherwise be consumed during OXPHOS, for many of their functions. However, the ramifications of this effect in T cells are poorly understood. A previous study in T cells has found that CD28 costimulation increases glucose uptake, as well as aerobic glycolysis, in a manner dependent upon PI3K/AKT (Frauwirth et al., 2002). Thus, as ICOS is a more potent inducer of PI3K than CD28, it might play a more important role in mediating activated T cell metabolism. As techniques allowing for the study of metabolomics become increasingly well established, we will soon be able to determine if ICOS plays a significant role in mediating the immune system through changes in metabolic pathways.

6. CONCLUDING REMARKS AND SIGNIFICANCE OF WORK PRESENTED: OUR PROPOSED MODEL

A healthy immune response produces high affinity antibodies against dangerous foreign antigens. Tfh cells efficiently control the B cell response so that only B cells that produce the highest affinity antibody survive to differentiate into antibody-producing PCs. Based on the two studies presented in this thesis and work done by others, we propose a model that highlights the unique role of the ICOS-PI3K pathway in the development and function of Tfh cells. Upon antigen exposure, naïve CD4 T cells are activated in the T cell zone of secondary lymphoid organs by

DCs. The DCs provide signals that activate the TCR and CD28 costimulation, which induces T cell activation and high ICOS expression. The exact molecular mechanisms that drive initiation of pre-Tfh cell differentiation, rather than other Th subset differentiation, are still unclear. However, pre-Tfh cell differentiation correlates with high TCR-peptide:MHC-II affinity, as well as STAT3 activation in mice, and STAT4 and STAT3 activation in humans. Subsequent to CXCR5 and Bcl6 upregulation in the CD4 T cells, the newly polarized pre-Tfh cells migrate to the T-B border and search for cognate B cells. This process is facilitated by non-cognate B cells providing TCR-independent ICOS-PI3K signaling to maintain high motility of pre-Tfh cells. Here, cognate B cells induce ICOS-PI3K activation in the pre-Tfh cell, which promotes the production of IL-4 and IL-21. This production of both B cell activation factors induces initial B cell differentiation into centrocytes and the beginning of a GC reaction. Additionally, ICOS-PI3K costimulation induces full Tfh cell differentiation, partially through increased IL-21, but also through an unknown mechanism. Full Tfh cell differentiation is followed by entry of the T cell into the LZ of the GC, where it selects high affinity B cells for survival. Finally, the aforementioned ICOS-PI3K-mediated IL-4 production is mediated by both increased IL-4 mRNA transcription and translation, which further enhances B cell activation. It is likely that ICOS-mediated translation targets other mRNAs, and finding these will further advance our understanding of the molecular mechanisms of T cell-mediated B cell help.

REFERENCES

Akiba, H., Takeda, K., Kojima, Y., Usui, Y., Harada, N., Yamazaki, T., Ma, J., Tezuka, K., Yagita, H., and Okumura, K. (2005). The role of ICOS in the CXCR5+ follicular B helper T cell maintenance in vivo. J Immunol 175, 2340-2348.

Anderson, P., and Kedersha, N. (2008). Stress granules: the Tao of RNA triage. Trends in biochemical sciences 33, 141-150.

Avery, D.T., Deenick, E.K., Ma, C.S., Suryani, S., Simpson, N., Chew, G.Y., Chan, T.D., Palendira, U., Bustamante, J., Boisson-Dupuis, S*., et al.* (2010). B cell-intrinsic signaling through IL-21 receptor and STAT3 is required for establishing long-lived antibody responses in humans. The Journal of experimental medicine 207, 155-171.

Baumjohann, D., Kageyama, R., Clingan, J.M., Morar, M.M., Patel, S., de Kouchkovsky, D., Bannard, O., Bluestone, J.A., Matloubian, M., Ansel, K.M., and Jeker, L.T. (2013). The microRNA cluster miR-17 approximately 92 promotes TFH cell differentiation and represses subset-inappropriate gene expression. Nature immunology 14, 840-848.

Bauquet, A.T., Jin, H., Paterson, A.M., Mitsdoerffer, M., Ho, I.C., Sharpe, A.H., and Kuchroo, V.K. (2009). The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells. Nature immunology 10, 167-175.

Beier, K.C., Hutloff, A., Dittrich, A.M., Heuck, C., Rauch, A., Buchner, K., Ludewig, B., Ochs, H.D., Mages, H.W., and Kroczek, R.A. (2000). Induction, binding specificity and function of human ICOS. European journal of immunology 30, 3707-3717.

Biberman, Y., and Meyuhas, O. (1997). Substitution of just five nucleotides at and around the transcription start site of rat beta-actin promoter is sufficient to render the resulting transcript a subject for translational control. FEBS letters 405, 333-336.

Bossaller, L., Burger, J., Draeger, R., Grimbacher, B., Knoth, R., Plebani, A., Durandy, A., Baumann, U., Schlesier, M., Welcher, A.A*., et al.* (2006). ICOS deficiency is associated with a severe reduction of CXCR5+CD4 germinal center Th cells. J Immunol 177, 4927-4932.

Cannons, J.L., Qi, H., Lu, K.T., Dutta, M., Gomez-Rodriguez, J., Cheng, J., Wakeland, E.K., Germain, R.N., and Schwartzberg, P.L. (2010). Optimal Germinal Center Responses Require A Multistage T Cell:B Cell Adhesion Process Involving Integrins, SLAM-Associated Protein, and CD84. Immunity.

Carballo, E., Lai, W.S., and Blackshear, P.J. (1998). Feedback inhibition of macrophage tumor necrosis factor-alpha production by tristetraprolin. Science (New York, N.Y 281, 1001-1005.

Choi, Y.S., Eto, D., Yang, J.A., Lao, C., and Crotty, S. (2013). Cutting edge: STAT1 is required for IL-6-mediated Bcl6 induction for early follicular helper cell differentiation. J Immunol 190, 3049-3053.

Choi, Y.S., Kageyama, R., Eto, D., Escobar, T.C., Johnston, R.J., Monticelli, L., Lao, C., and Crotty, S. (2011). ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. Immunity 34, 932-946.

Coyle, A.J., Lehar, S., Lloyd, C., Tian, J., Delaney, T., Manning, S., Nguyen, T., Burwell, T., Schneider, H., Gonzalo, J.A*., et al.* (2000). The CD28-related molecule ICOS is required for effective T cell-dependent immune responses. Immunity 13, 95-105.

Crotty, S. (2011). Follicular Helper CD4 T Cells (T(FH)). Annual review of immunology.

Deane, J.A., Kharas, M.G., Oak, J.S., Stiles, L.N., Luo, J., Moore, T.I., Ji, H., Rommel, C., Cantley, L.C., Lane, T.E., and Fruman, D.A. (2007). T-cell function is partially maintained in the absence of class IA phosphoinositide 3-kinase signaling. Blood 109, 2894-2902.

Deweindt, C., Kerckaert, J.P., Tilly, H., Quief, S., Nguyen, V.C., and Bastard, C. (1993). Cloning of a breakpoint cluster region at band 3q27 involved in human non-Hodgkin's lymphoma. Genes, chromosomes & cancer 8, 149-154.

Dodson, L.F., Boomer, J.S., Deppong, C.M., Shah, D.D., Sim, J., Bricker, T.L., Russell, J.H., and Green, J.M. (2009). Targeted knock-in mice expressing mutations of CD28 reveal an essential pathway for costimulation. Molecular and cellular biology 29, 3710-3721.

Eddahri, F., Denanglaire, S., Bureau, F., Spolski, R., Leonard, W.J., Leo, O., and Andris, F. (2009). Interleukin-6/STAT3 signaling regulates the ability of naive T cells to acquire B-cell help capacities. Blood 113, 2426-2433.

Eto, D., Lao, C., Ditoro, D., Barnett, B., Escobar, T.C., Kageyama, R., Yusuf, I., and Crotty, S. (2011). IL-21 and IL-6 Are Critical for Different Aspects of B Cell Immunity and Redundantly Induce Optimal Follicular Helper CD4 T Cell (Tfh) Differentiation. PloS one 6, e17739.

Fazilleau, N., Eisenbraun, M.D., Malherbe, L., Ebright, J.N., Pogue-Caley, R.R., McHeyzer-Williams, L.J., and McHeyzer-Williams, M.G. (2007). Lymphoid reservoirs of antigen-specific memory T helper cells. Nature immunology 8, 753-761.

Fazilleau, N., Mark, L., McHeyzer-Williams, L.J., and McHeyzer-Williams, M.G. (2009a). Follicular helper T cells: lineage and location. Immunity 30, 324-335.

Fazilleau, N., McHeyzer-Williams, L.J., Rosen, H., and McHeyzer-Williams, M.G. (2009b). The function of follicular helper T cells is regulated by the strength of T cell antigen receptor binding. Nature immunology 10, 375-384.

Feito, M.J., Vaschetto, R., Criado, G., Sanchez, A., Chiocchetti, A., Jimenez-Perianez, A., Dianzani, U., Portoles, P., and Rojo, J.M. (2003). Mechanisms of H4/ICOS costimulation: effects on proximal TCR signals and MAP kinase pathways. European journal of immunology 33, 204-214.

Fos, C., Salles, A., Lang, V., Carrette, F., Audebert, S., Pastor, S., Ghiotto, M., Olive, D., Bismuth, G., and Nunes, J.A. (2008). ICOS ligation recruits the p50alpha PI3K regulatory subunit to the immunological synapse. J Immunol 181, 1969-1977.

Frauwirth, K.A., Riley, J.L., Harris, M.H., Parry, R.V., Rathmell, J.C., Plas, D.R., Elstrom, R.L., June, C.H., and Thompson, C.B. (2002). The CD28 signaling pathway regulates glucose metabolism. Immunity 16, 769-777.

Gan, X., Wang, J., Su, B., and Wu, D. (2011). Evidence for direct activation of mTORC2 kinase activity by phosphatidylinositol 3,4,5-trisphosphate. The Journal of biological chemistry.

Gigoux, M., Shang, J., Pak, Y., Xu, M., Choe, J., Mak, T.W., and Suh, W.K. (2009). Inducible costimulator promotes helper T-cell differentiation through phosphoinositide 3-kinase. Proceedings of the National Academy of Sciences of the United States of America 106, 20371-20376.

Gogishvili, T., Elias, F., Emery, J.L., McPherson, K., Okkenhaug, K., Hunig, T., and Dennehy, K.M. (2008). Proliferative signals mediated by CD28 superagonists require the exchange factor Vav1 but not phosphoinositide 3-kinase in primary peripheral T cells. European journal of immunology 38, 2528-2533.

Greenwald, R.J., Freeman, G.J., and Sharpe, A.H. (2005). The B7 family revisited. Annual review of immunology 23, 515-548.

Greiner, E.F., Guppy, M., and Brand, K. (1994). Glucose is essential for proliferation and the glycolytic enzyme induction that provokes a transition to glycolytic energy production. The Journal of biological chemistry 269, 31484-31490.

Halees, A.S., El-Badrawi, R., and Khabar, K.S. (2008). ARED Organism: expansion of ARED reveals AU-rich element cluster variations between human and mouse. Nucleic acids research 36, D137-140.

Hao, Z., Duncan, G.S., Seagal, J., Su, Y.W., Hong, C., Haight, J., Chen, N.J., Elia, A., Wakeham, A., Li, W.Y., *et al.* (2008). Fas receptor expression in germinal-center B cells is essential for T and B lymphocyte homeostasis. Immunity 29, 615-627.

Inukai, K., Funaki, M., Anai, M., Ogihara, T., Katagiri, H., Fukushima, Y., Sakoda, H., Onishi, Y., Ono, H., Fujishiro, M., *et al.* (2001). Five isoforms of the phosphatidylinositol 3-kinase regulatory subunit

exhibit different associations with receptor tyrosine kinases and their tyrosine phosphorylations. FEBS letters 490, 32-38.

Inukai, K., Funaki, M., Ogihara, T., Katagiri, H., Kanda, A., Anai, M., Fukushima, Y., Hosaka, T., Suzuki, M., Shin, B.C*., et al.* (1997). p85alpha gene generates three isoforms of regulatory subunit for phosphatidylinositol 3-kinase (PI 3-Kinase), p50alpha, p55alpha, and p85alpha, with different PI 3-kinase activity elevating responses to insulin. The Journal of biological chemistry 272, 7873-7882.

Johnston, R.J., Poholek, A.C., DiToro, D., Yusuf, I., Eto, D., Barnett, B., Dent, A.L., Craft, J., and Crotty, S. (2009). Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. Science (New York, N.Y 325, 1006-1010.

Jousse, C., Oyadomari, S., Novoa, I., Lu, P., Zhang, Y., Harding, H.P., and Ron, D. (2003). Inhibition of a constitutive translation initiation factor 2alpha phosphatase, CReP, promotes survival of stressed cells. The Journal of cell biology 163, 767-775.

Kang, S.G., Liu, W.H., Lu, P., Jin, H.Y., Lim, H.W., Shepherd, J., Fremgen, D., Verdin, E., Oldstone, M.B., Qi, H*., et al.* (2013). MicroRNAs of the miR-17 approximately 92 family are critical regulators of T differentiation. Nature immunology.

King, I.L., and Mohrs, M. (2009). IL-4-producing CD4+ T cells in reactive lymph nodes during helminth infection are T follicular helper cells. The Journal of experimental medicine 206, 1001-1007.

Kopf, M., Le Gros, G., Bachmann, M., Lamers, M.C., Bluethmann, H., and Kohler, G. (1993). Disruption of the murine IL-4 gene blocks Th2 cytokine responses. Nature 362, 245-248.

Kornete, M., Sgouroudis, E., and Piccirillo, C.A. (2012). ICOS-dependent homeostasis and function of Foxp3+ regulatory T cells in islets of nonobese diabetic mice. J Immunol 188, 1064-1074.

Kroenke, M.A., Eto, D., Locci, M., Cho, M., Davidson, T., Haddad, E.K., and Crotty, S. (2012). Bcl6 and Maf Cooperate To Instruct Human Follicular Helper CD4 T Cell Differentiation. J Immunol.

Kuhn, R., Rajewsky, K., and Muller, W. (1991). Generation and analysis of interleukin-4 deficient mice. Science (New York, N.Y 254, 707-710.

Li, J., Heinrichs, J., Leconte, J., Haarberg, K., Semple, K., Liu, C., Gigoux, M., Kornete, M., Piccirillo, C.A., Suh, W.K., and Yu, X.Z. (2013). Phosphatidylinositol 3-Kinase-Independent Signaling Pathways Contribute to ICOS-Mediated T Cell Costimulation in Acute Graft-Versus-Host Disease in Mice. J Immunol.

Linterman, M.A., Beaton, L., Yu, D., Ramiscal, R.R., Srivastava, M., Hogan, J.J., Verma, N.K., Smyth, M.J., Rigby, R.J., and Vinuesa, C.G. (2010). IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses. The Journal of experimental medicine 207, 353-363.

Linterman, M.A., Liston, A., and Vinuesa, C.G. (2012). T-follicular helper cell differentiation and the co-option of this pathway by non-helper cells. Immunological reviews 247, 143-159.

Linterman, M.A., Pierson, W., Lee, S.K., Kallies, A., Kawamoto, S., Rayner, T.F., Srivastava, M., Divekar, D.P., Beaton, L., Hogan, J.J*., et al.* (2011). Foxp3+ follicular regulatory T cells control the germinal center response. Nature medicine 17, 975-982.

Livingstone, M., Atas, E., Meller, A., and Sonenberg, N. (2010). Mechanisms governing the control of mRNA translation. Physical biology 7, 021001.

Lo Coco, F., Ye, B.H., Lista, F., Corradini, P., Offit, K., Knowles, D.M., Chaganti, R.S., and Dalla-Favera, R. (1994). Rearrangements of the BCL6 gene in diffuse large cell non-Hodgkin's lymphoma. Blood 83, 1757-1759.

McHeyzer-Williams, L.J., Pelletier, N., Mark, L., Fazilleau, N., and McHeyzer-Williams, M.G. (2009). Follicular helper T cells as cognate regulators of B cell immunity. Current opinion in immunology 21, 266-273.

Novoa, I., Zeng, H., Harding, H.P., and Ron, D. (2001). Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2alpha. The Journal of cell biology 153, 1011-1022.

Nukada, Y., Okamoto, N., Konakahara, S., Tezuka, K., Ohashi, K., Mizuno, K., and Tsuji, T. (2006). AILIM/ICOS-mediated elongation of activated T cells is regulated by both the PI3-kinase/Akt and Rho family cascade. International immunology 18, 1815-1824.

Nurieva, R.I., Chung, Y., Hwang, D., Yang, X.O., Kang, H.S., Ma, L., Wang, Y.H., Watowich, S.S., Jetten, A.M., Tian, Q., and Dong, C. (2008). Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. Immunity 29, 138-149.

Nurieva, R.I., Chung, Y., Martinez, G.J., Yang, X.O., Tanaka, S., Matskevitch, T.D., Wang, Y.H., and Dong, C. (2009). Bcl6 mediates the development of T follicular helper cells. Science (New York, N.Y 325, 1001-1005.

Nurieva, R.I., Chuvpilo, S., Wieder, E.D., Elkon, K.B., Locksley, R., Serfling, E., and Dong, C. (2007). A costimulation-initiated signaling pathway regulates NFATc1 transcription in T lymphocytes. J Immunol 179, 1096-1103.

Oestreich, K.J., Mohn, S.E., and Weinmann, A.S. (2012). Molecular mechanisms that control the expression and activity of Bcl-6 in TH1 cells to regulate flexibility with a TFH-like gene profile. Nature immunology 13, 405-411.

Okamoto, N., Nukada, Y., Tezuka, K., Ohashi, K., Mizuno, K., and Tsuji, T. (2004). AILIM/ICOS signaling induces T-cell migration/polarization of memory/effector T-cells. International immunology 16, 1515-1522.

Okamoto, N., Tezuka, K., Kato, M., Abe, R., and Tsuji, T. (2003). PI3-kinase and MAP-kinase signaling cascades in AILIM/ICOS- and CD28-costimulated T-cells have distinct functions between cell proliferation and IL-10 production. Biochemical and biophysical research communications 310, 691-702.

Okkenhaug, K., Bilancio, A., Farjot, G., Priddle, H., Sancho, S., Peskett, E., Pearce, W., Meek, S.E., Salpekar, A., Waterfield, M.D*., et al.* (2002). Impaired B and T cell antigen receptor signaling in p110delta PI 3-kinase mutant mice. Science (New York, N.Y 297, 1031-1034.

Okkenhaug, K., Wu, L., Garza, K.M., La Rose, J., Khoo, W., Odermatt, B., Mak, T.W., Ohashi, P.S., and Rottapel, R. (2001). A point mutation in CD28 distinguishes proliferative signals from survival signals. Nature immunology 2, 325-332.

Pagan, A.J., Pepper, M., Chu, H.H., Green, J.M., and Jenkins, M.K. (2012). CD28 promotes CD4+ T cell clonal expansion during infection independently of its YMNM and PYAP motifs. J Immunol 189, 2909-2917.

Parry, R.V., Rumbley, C.A., Vandenberghe, L.H., June, C.H., and Riley, J.L. (2003). CD28 and inducible costimulatory protein Src homology 2 binding domains show distinct regulation of phosphatidylinositol 3-kinase, Bcl-xL, and IL-2 expression in primary human CD4 T lymphocytes. J Immunol 171, 166-174.

Pearce, E.L., and Pearce, E.J. (2013). Metabolic pathways in immune cell activation and quiescence. Immunity 38, 633-643.

Pique, M., Lopez, J.M., Foissac, S., Guigo, R., and Mendez, R. (2008). A combinatorial code for CPE-mediated translational control. Cell 132, 434-448.

Qi, H., Cannons, J.L., Klauschen, F., Schwartzberg, P.L., and Germain, R.N. (2008). SAP-controlled T-B cell interactions underlie germinal centre formation. Nature 455, 764-769.

Rolf, J., Bell, S.E., Kovesdi, D., Janas, M.L., Soond, D.R., Webb, L.M., Santinelli, S., Saunders, T., Hebeis, B., Killeen, N*., et al.* (2010). Phosphoinositide 3-kinase activity in T cells regulates the magnitude of the germinal center reaction. J Immunol 185, 4042-4052.

Rudd, C.E., and Schneider, H. (2003). Unifying concepts in CD28, ICOS and CTLA4 co-receptor signalling. Nature reviews 3, 544-556.

Salek-Ardakani, S., Choi, Y.S., Rafii-El-Idrissi Benhnia, M., Flynn, R., Arens, R., Shoenberger, S., Crotty, S., and Croft, M. (2011). B cell-specific expression of B7-2 is required for follicular Th cell function in response to vaccinia virus. J Immunol 186, 5294-5303.

Scheu, S., Stetson, D.B., Reinhardt, R.L., Leber, J.H., Mohrs, M., and Locksley, R.M. (2006). Activation of the integrated stress response during T helper cell differentiation. Nature immunology 7, 644-651.

Stoecklin, G., Colombi, M., Raineri, I., Leuenberger, S., Mallaun, M., Schmidlin, M., Gross, B., Lu, M., Kitamura, T., and Moroni, C. (2002). Functional cloning of BRF1, a regulator of ARE-dependent mRNA turnover. The EMBO journal 21, 4709-4718.

Tang, G., Qin, Q., Zhang, P., Wang, G., Liu, M., Ding, Q., Qin, Y., and Shen, Q. (2009). Reverse signaling using an inducible costimulator to enhance immunogenic function of dendritic cells. Cell Mol Life Sci 66, 3067-3080.

Thoreen, C.C., Chantranupong, L., Keys, H.R., Wang, T., Gray, N.S., and Sabatini, D.M. (2012). A unifying model for mTORC1-mediated regulation of mRNA translation. Nature 485, 109-113.

Vander Heiden, M.G., Cantley, L.C., and Thompson, C.B. (2009). Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science (New York, N.Y 324, 1029-1033.

Victora, G.D., Schwickert, T.A., Fooksman, D.R., Kamphorst, A.O., Meyer-Hermann, M., Dustin, M.L., and Nussenzweig, M.C. (2010). Germinal center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter. Cell 143, 592-605.

Vogelzang, A., McGuire, H.M., Yu, D., Sprent, J., Mackay, C.R., and King, C. (2008). A fundamental role for interleukin-21 in the generation of T follicular helper cells. Immunity 29, 127-137.

Wang, R., and Green, D.R. (2012). Metabolic checkpoints in activated T cells. Nature immunology 13, 907-915.

Weill, L., Belloc, E., Bava, F.A., and Mendez, R. (2012). Translational control by changes in poly(A) tail length: recycling mRNAs. Nature structural & molecular biology 19, 577-585.

Xu, H., Li, X., Liu, D., Li, J., Zhang, X., Chen, X., Hou, S., Peng, L., Xu, C., Liu, W*., et al.* (2013). Follicular T-helper cell recruitment governed by bystander B cells and ICOS-driven motility. Nature 496, 523-527.

Ye, B.H., Lista, F., Lo Coco, F., Knowles, D.M., Offit, K., Chaganti, R.S., and Dalla-Favera, R. (1993). Alterations of a zinc finger-encoding gene, BCL-6, in diffuse large-cell lymphoma. Science (New York, N.Y 262, 747-750.

Yu, D., Rao, S., Tsai, L.M., Lee, S.K., He, Y., Sutcliffe, E.L., Srivastava, M., Linterman, M., Zheng, L., Simpson, N*., et al.* (2009). The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. Immunity 31, 457-468.

Zhang, T.T., Makondo, K.J., and Marshall, A.J. (2012). p110delta phosphoinositide 3-kinase represses IgE switch by potentiating BCL6 expression. J Immunol 188, 3700-3708.

Zotos, D., Coquet, J.M., Zhang, Y., Light, A., D'Costa, K., Kallies, A., Corcoran, L.M., Godfrey, D.I., Toellner, K.M., Smyth, M.J*., et al.* (2010). IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism. The Journal of experimental medicine 207, 365-378.

Appendices

Appendix I: Original Article

Gigoux, M., Shang, J., Pak, Y., Xu, M., Choe, J., Mak, T.W., and Suh, W.K. (2009). **Inducible costimulator promotes helper T-cell differentiation through phosphoinositide 3-kinase.** *Proceedings of the National Academy of Sciences of the United States of America* 106, 20371-20376.

Inducible costimulator promotes helper T-cell differentiation through phosphoinositide 3-kinase

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The T-cell costimulatory receptors, CD28 and the inducible costimulator (ICOS), are required for the generation of follicular B helper T cells (T_{FH}) and germinal center (GC) reaction. A common signal transducer used by CD28 and ICOS is the phosphoinositide 3-kinase (PI3K). Although it is known that CD28-mediated PI3K activation is dispensable for GC reaction, the role of ICOS-driven PI3K signaling has not been defined. We show here that knock-in mice that selectively lost the ability to activate PI3K through ICOS had severe defects in T_{FH} generation, GC reaction, antibody class switch, and antibody affinity maturation. In preactivated $CD4^+$ T cells, ICOS delivered a potent PI3K signal that was critical for the induction of the key T_{FH} cytokines, IL-21 and IL-4. Under the same settings, CD28 was unable to activate PI3K but supported a robust secondary expansion of T cells. Thus, our results demonstrate a nonredundant function of ICOS-PI3K pathway in the generation of T_{FH} and suggest that CD28 and ICOS play differential roles during a multistep process of T_{FH} differentiation.

CD28 | follicular B helper T-cell | germinal center | ICOS | PI3K

Follicular B helper T cells (T_{FH}) are a subset of $CD4^+$ T cells that facilitates germinal center (GC) reaction, B cell proliferation, and B cell differentiation (1). T_{FH} cells have an ability to migrate into B cell area using chemokine receptor CXCR5, and they abundantly express costimulatory molecules such as ICOS, PD-1, and CD40L. T_{FH} cells can arise in the absence of factors that mediate Th1, Th2, or Th17 differentiation, but depend on Bcl-6 (2–5). T_{FH} cells express a high level of IL-21, which provides a robust stimulus for proliferation and differentiation of B cells (6, 7). IL-21 also plays an indispensible role in the generation of T_{FH} cells, probably by enhancing Bcl-6 expression (3). Recent studies also revealed an exquisite regulation of IL-4 transcription and translation that allows highly targeted secretion of IL-4 by T_{FH} cells while they form conjugates with cognate B cells (8). Thus, IL-21 and IL-4 appear to be crucial for differentiation and/or function of T_{FH} cells.

ICOS is a CD28 family costimulatory receptor that is expressed in recently activated or antigen-experienced T cells (9, 10). By binding to ICOS ligand (ICOS-L) expressed on antigen presenting cells (APCs), ICOS delivers costimulatory signals that augment T-cell proliferation and expression of an array of cytokines including IL-4, IL-10, and IL-21 (10–12). Both in mice and humans, interruption of ICOS-ICOS-L interaction leads to impaired GC reaction, Ab class switch, and affinity maturation (13–16). Recent findings suggested that these defects in humoral immune responses in ICOS-deficiency are due to the lack of T_{FH} cells (17–19). Conversely, dysregulated overexpression of ICOS in *sanroque* mice causes a lupus-like autoimmune disease that is associated with an increased number of T_{FH} cells, spontaneous GC reaction, and augmented IL-21 production (20–22).

The prototype T-cell costimulator CD28 is also required for GC reaction, humoral immunity, and generation of T_{FH} cells (23, 24). It is puzzling that the generation of T_{FH} requires both CD28 and ICOS, although the two costimulators have a seemingly redundant function in activating PI3K (25, 26). Whether CD28-mediated PI3K pathway plays significant roles in T-cell proliferation, cytokine production, and survival has been a matter of hot debate (27). Recent data from knock-in mice showed that CD28-mediated PI3K pathways do not have any obvious nonredundant role in T-cell functions and humoral immune responses (28).

To address the role of ICOS-mediated PI3K signal transduction pathways in the context of the overall ICOS function, we generated a knock-in mouse strain in which the cytoplasmic tail of ICOS cannot recruit PI3K. Here, we show that the generation of T_{FH} cells critically depends on the PI3K signaling initiated by ICOS. Consequently, GC reaction, Ab class switch, and affinity maturation are drastically diminished in the knock-in mice. We find evidence that in preactivated $CD4^+$ T cells, expression of IL-21 and IL-4 is heavily dependent on PI3K and that the dominant activator of PI3K in this context is ICOS, not CD28.

Results

Normal Inducible Expression Pattern of ICOS-YF with Altered Signaling Capacities. We generated knock-in mice, termed ICOS-YF hereafter, possessing a tyrosine-to-phenylalanine mutation at amino acid residue 181 in the cytoplasmic tail of ICOS, a mutation known to abrogate ICOS-mediated PI3K recruitment (29) (details in *SI Text* and Fig. S1). We compared littermates of ICOS-WT (+/+) and ICOS-YF (*yf/yf*) mice along with nonlittermate ICOS-KO (-/-) mice that have <2 weeks of age difference. All of these mice have been backcrossed five generations into C57BL/6.

Since tyrosine residues in the cytoplasmic tails of membrane proteins are often involved in protein trafficking and recycling, we tested whether ICOS-YF maintained its expression pattern on the cell surface. As shown in Fig. S2*A*, WT and YF-mutant ICOS displayed an identical inducible expression pattern. Thus, the tyrosine-to-phenylalanine mutation does not alter the expression pattern of ICOS, and all of the phenotypic outcomes should be attributable to the altered signaling capacities of the mutant ICOS.

In vitro binding assays using GST fusion proteins have shown that the Tyr 181 residue of ICOS is critical for recruiting PI3K (29). Consistently, anti-ICOS immunoprecipitates from WT $CD4^+$ T-cell blasts contained the regulatory subunit of PI3K,

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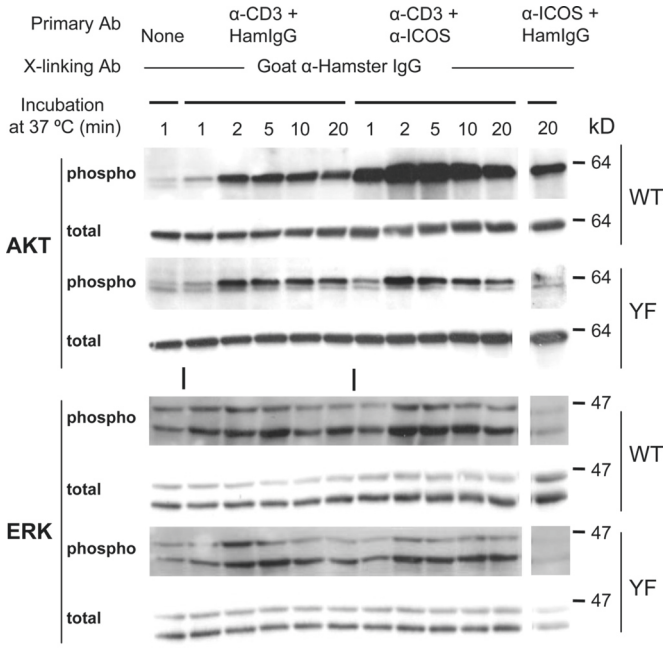

Fig. 1. Defective AKT and ERK activation by ICOS-YF. CD4$^+$ T blasts were stimulated with antibodies against CD3 and/or ICOS, and the activation of AKT or MAPKs was measured by immunoblotting using phospho-specific antibodies. A representative of three independent experiments is shown.

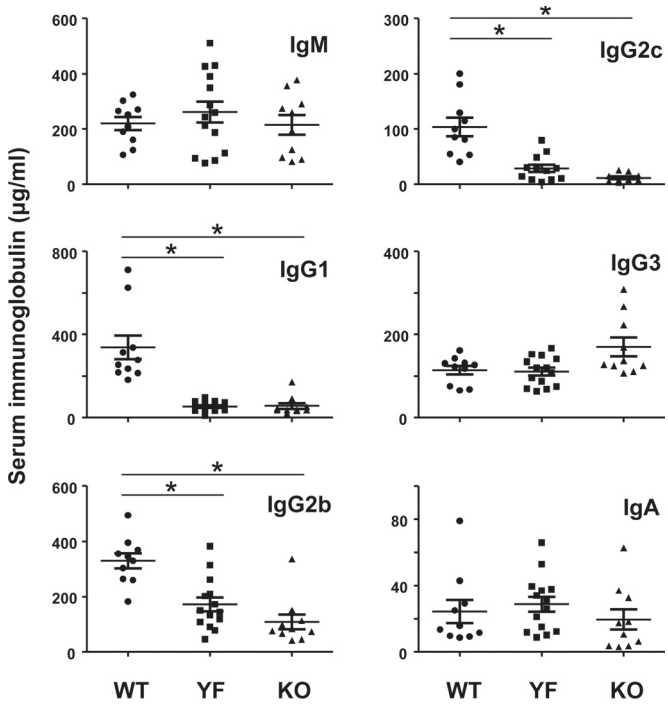

Fig. 2. ICOS-YF and KO mice have reduced levels of class-switched immunoglobulins in serum. Sera were obtained from nonimmunized mice of ICOS WT, YF, and KO mice at 8 weeks of age. Concentrations of IgM, G1, G2a, G2c, G3, and A were determined by isotype specific ELISA. Each data point represents a serum Ig level of an individual mouse (n = 10 WT, 14 YF, and 10 KO). *, $P < 0.01$.

p85α (Fig. S2*B*, WT). There was a basal level of p85α associated with ICOS that increased upon ligation of TCR or ICOS. The TCR-independent ICOS-mediated p85α recruitment may reflect a potential antigen-independent function of ICOS on cytoskeletal rearrangement of T cells (30). However, the amount of p85α was maximal when the T cells were activated by a combination of anti-CD3 and anti-ICOS mAb. Importantly, the ICOS-p85α interaction was abrogated when the Tyr 181 was mutated to phenylalanine (Fig. S2*B*, YF).

It has been shown that ligation of ICOS strongly enhances TCR-mediated activation of AKT and, to some extent, MAPKs (ERK, JNK, and p38) (25, 26). We examined these signal transduction events in primary T-cell blasts derived from WT or ICOS-YF mice. In keeping with the PI3K activation, ICOS engagement dramatically augmented TCR-mediated AKT activation as judged by the increase phosphorylation of AKT at Ser-473 in WT T cells (Fig. 1, AKT, WT). The ability of ICOS to enhance TCR-mediated AKT activation was completely abrogated in ICOS-YF T cells (Fig. 1, AKT, YF). ERK phosphorylation was moderately enhanced by ICOS ligation in WT but not in ICOS-YF. This is consistent with the observations that PI3K can activate Ras-MAPK pathway (31, 32). ICOS did not augment phosphorylation of JNK and p38 in primary CD4$^+$ blasts under our experimental settings (Fig. S3). As shown by others (25, 26), CD28-costimulation strongly enhanced JNK activation with a moderate level of AKT phosphorylation (Fig. S4).

It was shown that ligation of ICOS can facilitate Ca^{2+} mobilization when TCR signal is suboptimal, possibly through PI3K (25, 29). As shown in Fig. S5, both WT and Y181F mutant ICOS were able to augment TCR-mediated Ca^{2+} flux in CD4$^+$ T blasts. Thus, ICOS can augment TCR-mediated Ca^{2+} flux in a PI3K-independent manner.

Collectively, the Y181F mutation selectively disrupts PI3K-dependent signaling pathways, AKT and ERK, without affecting Ca^{2+} signaling.

Reduced Basal Serum Ig Levels in ICOS-YF Mice. One of the hallmarks of ICOS-deficient mice or humans is a reduction of class-switched immunoglobulins in serum, a reflection of defective GC reaction (13–16). Thus, we quantified the basal serum Ig levels by ELISA from 2-month-old mice of WT, YF, and KO mice (Fig. 2). As previously documented, ICOS-KO mice displayed up to 10-fold reduction in serum concentrations of IgG1, IgG2b, and IgG2c without any difference in IgM compared with WT control. Remarkably, ICOS-YF mice had an identical serum IgG1 level as that of ICOS-KO. Serum IgG2b and IgG2c concentrations in ICOS-YF mice were also reduced to levels close to those of ICOS-KO mice. These results suggest that the ICOS function in supporting Ig class switch critically relies on signaling mechanisms dependent on the Tyr 181.

Defective GC Reaction in Peyer's Patches of ICOS Mutants. Peyer's patches (PPs) are part of gut-associated immune tissue in which ongoing humoral immune responses against the intestinal microflora are taking place. It has been shown that, in ICOS-KO mice, the number of PPs is normal, but the size and cellularity of PPs are dramatically reduced, and the active GCs are not detected (33). However, the basis of these defects has been unknown. We chose to analyze the PPs of ICOS mutant mice to gain insights into the cellular basis of GC defects. ICOS-YF mice had a normal number of PPs, as do ICOS-KO mice (Fig. S6*A*). However, the total cellularity of PP was substantially reduced in ICOS-YF mice to a level similar to that of ICOS-KO mice (Fig. S6*B*). Accordingly, the GC area was greatly reduced in both ICOS-YF and KO mice (Fig. S6*C*). Flow cytometric analysis revealed that there is a drastic reduction in the percentage of GC B cells over the total B cells (Fig. 3 *Top*). Importantly, the percentages of T_{FH} cells over the total CD4$^+$ T cells in the PPs of ICOS-YF and KO were reduced by 5- to 9-fold compared with that of WT mice (Fig. 3 *Bottom*). Consistent with the GC defects, the content of secreted IgA in the feces was dramatically reduced

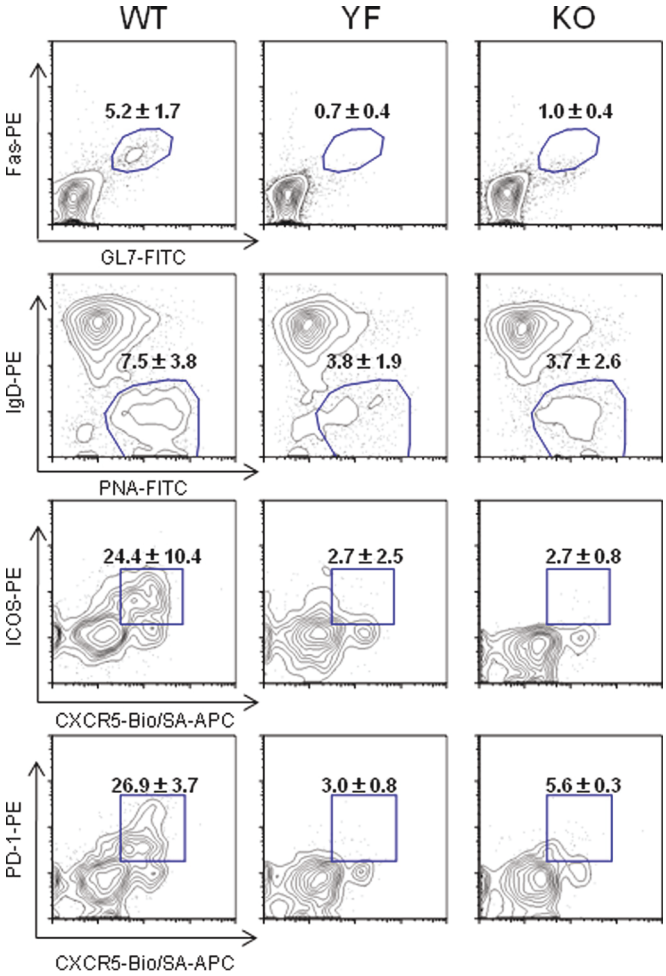

Fig. 3. ICOS-YF as well as KO mice have severely impaired humoral immune responses in the PP. GC B cells and T_{FH} cells in the PP were analyzed by flow cytometry. The (*Top*) two were gated on $B220^+$ B cells and the (*Bottom*) two on $CD4^+$ T cells. Numbers represent mean ± SD of data pooled from four mice per genotype.

in YF and KO mice (Fig. S6*D*). This is in contrast to the normal serum IgA levels in ICOS mutant mice (Fig. 2), suggesting that the serum IgA level is mainly controlled by T-independent mechanisms (34). Thus, the Tyr 181 motif of ICOS plays a critical role in T_{FH} differentiation and GC reaction in the PP.

Defective Humoral Immunity in ICOS Mutants. It has been shown that ICOS-KO mice have impaired GC reaction, Ab class switch, and affinity maturation upon immunization (13–15). We immunized mice with alum-precipitated NP-CGG to examine the role of ICOS-PI3K pathway in humoral immune responses. Both ICOS-YF and KO mice had severely impaired GC reaction along with reduced T_{FH} cells in the spleen (Fig. 4 *A* and *B*). Anti-NP IgG1 antibody titers in serum were substantially reduced in both ICOS-YF and KO mice (Fig. 4*C*). The difference in anti-NP IgG1 titer was more pronounced in high-affinity Ab (Fig. 4*C*, NP3) as opposed to the total Ab (Fig. 4*C*, NP33). It was also clear that the difference in high-affinity Ab titers became bigger upon secondary immunization (Fig. 4*C*, NP3 1 ° vs. 2 °). We assessed affinity maturation process more precisely by measuring anti-NP antibodies after a differential washing step using NaSCN solutions during ELISA (*SI Text*). As depicted in Fig. 4*D*, a shift from lower to higher affinity anti-NP IgG1 was readily seen in WT mice upon secondary immunization, but this was not observed in ICOS-YF and KO mice. Collectively, these data demonstrate that ICOS-YF mice have severely impaired GC reaction, T_{FH} generation, Ab class switch, and affinity maturation.

ICOS Promotes Expression of IL-21 and IL-4 in a PI3K-Dependent Manner. The lack of $CXCR5^+CD4^+$ T_{FH} cells during humoral immune reaction in ICOS mutant mice prompted us to examine if ICOS is directly involved in upregulation of CXCR5. When T cells were activated by soluble anti-CD3 Ab in the presence of APCs, there was no difference in the expression levels of OX40 and CXCR5 on $CD4^+$ T cells (Fig. S7*A*). This result is consistent with the notion that CXCR5 is mainly induced by OX40, whose expression is enhanced by CD28 costimulation (35). Thus, ICOS is not required for the induction of CXCR5, and the lack of $CXCR5^+CD4^+$ cells in ICOS mutants probably reflects a failed T_{FH} differentiation program.

Next, we sought to examine the impact of ICOS costimulation on cytokine gene expression in the $CD4^+$ T cells. We activated highly purified $CD4^+$ T cells for 2 days in vitro using antibodies against CD3 and CD28, rested them 1 day in the absence of stimuli, and then restimulated the cells with a combination of TCR and costimulatory signals. This regimen allowed us to use $CD4^+$ T cells with maximal surface ICOS expression within a time frame when primed $CD4^+$ T cells migrate to B cell follicles in secondary lymphoid organs (day 3 postimmunization) (36). Under these conditions, ICOS played a dominant role over CD28 in augmentation of IL-21 and IL-4 expression (Fig. 5*A*). Further, pharmacological inhibition of PI3K activity during the restimulation period negated all of the costimulatory impacts of ICOS on IL-21 and IL-4 expression. In contrast, IL-10 expression was marginally increased by ICOS and CD28, but largely unaffected by PI3K inhibition. Consistent with results from PI3K inhibition experiments, ICOS-mediated upregulation of IL-21 and IL-4 was abrogated in ICOS-YF T cells to levels close to those of ICOS-KO T cells (Fig. 5*B*). In parallel, the differences in cytokine induction capacity of ICOS vs. CD28 in the primed $CD4^+$ T cells well correlated with their abilities to activate PI3K, a potent AKT phosphorylation by ICOS but not by CD28 (Fig. S7*B*). Despite the weak costimulatory activity in cytokine expression, CD28 had a major impact on the secondary expansion of primed $CD4^+$ T cells, whereas ICOS played minor contribution (Fig. S7*C*). Interestingly, the ICOS-mediated proliferation is disrupted in ICOS-YF T cells, suggesting that the ICOS-PI3K pathway may have an additional role in the secondary expansion of primed $CD4^+$ T cells. Taken together, these data show that ICOS-PI3K signaling plays a dominant role in augmenting expression of IL-21 and IL-4 during secondary activation of $CD4^+$ T cells and that CD28 is not able to substitute ICOS.

Discussion

In this study we show that ICOS potently activates PI3K in synergy with the TCR. When its ability to activate PI3K is selectively abrogated, ICOS cannot support the generation of T_{FH} cells, GC reaction, Ab class switch, and affinity maturation. We found a nonredundant role of ICOS-PI3K pathway in upregulation of IL-21 and IL-4, key cytokines crucial for T_{FH} generation and function.

It has been shown that in murine Th2 clones, ICOS is constitutively bound to PI3K, and ICOS ligation further increases PI3K recruitment (37). We confirmed these results in activated $CD4^+$ T blasts. The finding that ligation of ICOS without TCR stimulation can activate PI3K signaling cascades explains TCR-independent, yet PI3K-dependent ICOS function in cytoskeletal rearrangement that may have a potential role in T-cell adherence and migration (30). However, it has been shown that interruption of ICOS function does not affect T-cell trafficking itself in vivo (38, 39). Regardless, it is clear that coligation of the TCR and ICOS gives rise to a maximal PI3K

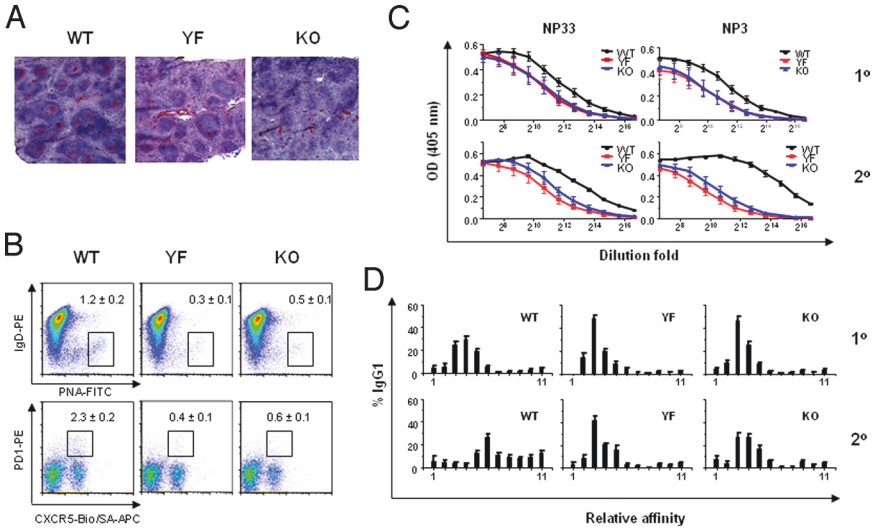

Fig. 4. Impaired Ab responses in ICOS-YF and ICOS-KO mice upon immunization. (*A*) Defective GC reaction. Cryosections of spleens from mice immunized with NP_{16}-CGG/alum 12 days before were stained with PNA. (*B*) Decreased GC B cells and T_{FH} cells in ICOS-YF and KO mice. Mice were immunized with NP_{16}-CGG/alum, and the splenocytes were analyzed 12 days later. Percentages represent mean ± SD of data from three mice per genotype. A representative of two independent experiments. (*C*) Impaired class switch. Sera were prepared from immunized mice at day 11 (1°) or day 7 (2°) postinjection, and antigen-specific IgG1 were measured by ELISA using NP_{33}-BSA vs. NP_3-BSA. Numbers represent mean ± SD of data from six mice per genotype. A representative of two independent experiments. (*D*) Defective Ab affinity maturation. Mice were immunized at day 0 and boost injected at day 30. Serum samples were prepared at day 11 after primary injection or day 7 after secondary injection, and the anti-NP IgG1 was measured after differential washes with increasing concentrations of NaSCN solution. Each histogram represents mean ± SD of data from six mice per genotype. A representative of two independent experiments.

signaling. Biochemical and imaging data have indicated that ICOS is in complex with TCR complexes, and it gets recruited into the immunological synapses, supporting the view that ICOS probably functions in conjunction with the TCR (40–42).

The mechanism by which ICOS enhances TCR-mediated Ca^{2+} mobilization is not clear. It was proposed that the ICOS-mediated PI3K pathway can enhance PLCγ1 function through ITK leading to sustained Ca^{2+} flux (29). Since ICOS-YF T cells have intact Ca^{2+} flux, we conclude that ICOS can mediate Ca^{2+} flux through a yet unknown mechanism, but clearly in a PI3K-independent manner. Although the overall defects in humoral immunity in ICOS-YF mice are very close to those of ICOS-KO mice, we observed marginally higher levels of serum IgG2b and IgG2c in ICOS-YF mice compared to ICOS-KO mice (Fig. 2). Also, preactivated ICOS-YF $CD4^+$ T cells produced slightly higher levels of IL-21 and IL-4 compared to ICOS-KO counterparts (Fig. 5*B*). The intact capacity of ICOS-YF to augment TCR-mediated Ca^{2+} flux may explain these residual T-cell functions.

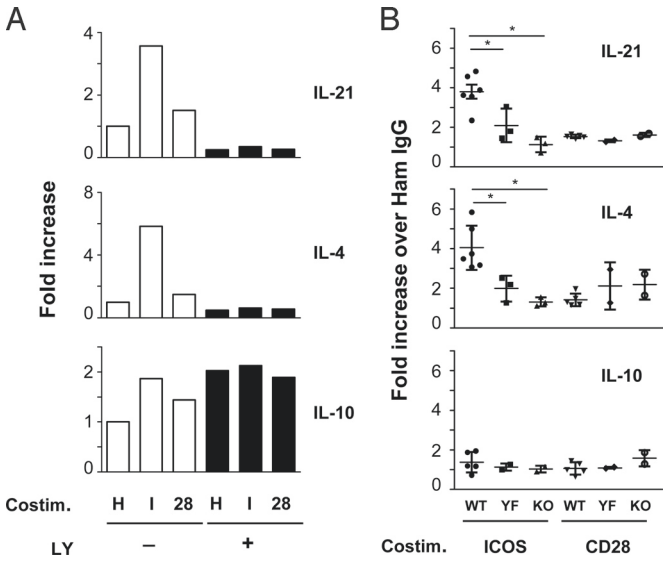

Fig. 5. ICOS-YF and ICOS-KO $CD4^+$ T cells have defects in IL-21 and IL-4 expression. (*A*) ICOS induces IL-21 and IL-4 in a PI3K-dependent manner. Preactivated $CD4^+$ T cells were restimulated with anti-CD3 plus hamster IgG (H), anti-ICOS (I), or anti-CD28 (28) followed by goat anti-hamster IgG for 6 h without or with LY 294002. The cytokine mRNA levels in the restimulated cells were analyzed by qPCR. A representative of three independent experiments. (*B*) Abrogation of IL-21 and IL-4 induction in ICOS-YF T cells. Cytokine gene expression was analyzed in WT, YF, and KO T cells as described in *A*. Each data point represents fold increase over hamster IgG control. Data pooled from six independent experiments (n = 5–6 WT, 2–3 YF, and 2–3 KO). *, $P < 0.02$.

Our finding that PI3K plays a key role in ICOS-mediated T_{FH} cells is very relevant to the results that inactivation of the p110δ isoform of PI3K leads to impaired humoral immunity and reduced size of PPs in the gut (32). It will be interesting to see whether a lack of T_{FH} underlies these phenotypes and whether the p110δ isoform is the PI3K under the control of ICOS.

Similar to ICOS, CD28 can activate PI3K through its Tyr-based motif (YMNM) in the cytoplasmic tail (27). Why is CD28 unable to compensate the lack of ICOS-PI3K signaling pathway? Our data show that it is due to an intrinsic weak capacity of CD28 to activate PI3K compared with that of ICOS. It has been shown that the membrane proximal segment containing the YMFM motif of the ICOS tail is much stronger than its CD28 counterpart (containing the YMNM motif) in the ability to activate PI3K when chimeric receptors were compared in transfected human $CD4^+$ T cells (25). The same observation was made in murine $CD4^+$ T cells upon ligation of endogenous CD28 and ICOS (26). Particularly, in $CD4^+$ T cells that rested for 1 day after a 2-day stimulation, CD28 did not evoke any PI3K activity above the TCR stimulation, whereas it could still strongly enhance secondary expansion of T cells (Fig. S7 *B* and *C*). Recent results from CD28 knock-in mice reinforced the notion that CD28-mediated PI3K does not play any nonredundant function in T-cell proliferation, cytokine production, and survival; it is,

rather, signals emanating from the carboxy terminal proline-rich motif that play more important roles (28).

Deficiency of either CD28 or ICOS results in defective T_{FH} and humoral immunity, suggesting distinct roles for the two costimulators (13–15, 23, 24, 35). What differential roles do they play during the process of T_{FH} generation? Based on our results and the data in literature, we propose a model in which CD28 and ICOS support T_{FH} cell differentiation in rather specialized manners. During the first 2–3 days of antigenic exposure, $CD4^+$ T cells interact with dendritic cells in the T-cell zone of secondary lymphoid organs (36). At this stage, antigen-specific T cells rapidly proliferate producing IL-2, upregulate CXCR5 through OX40, and induce ICOS. Since these processes are known to be dependent on CD28 costimulation (35, 43, 44), CD28-deficiency may heavily compromise T_{FH} differentiation at this stage. The primed $CD4^+$ T cells then migrate toward B cell follicles and interact with cognate B cells. During this T:B interaction, T cells make helper cytokines such as IL-21 and IL-4. IL-21 may play a key role in facilitating full differentiation of T_{FH} cells (3, 12), whereas IL-4 and IL-21 induce B cell proliferation and differentiation (6). Our data show that ICOS provides a unique PI3K-mediated signal to enhance IL-21 and IL-4 at this stage, and CD28 cannot substitute ICOS. Therefore, ICOS-deficiency is likely to block this later stage of T_{FH} differentiation. This model is consistent with the finding that a transient activation of CD28 at the early phase of immunization is sufficient for GC formation (45). On the other hand, T cells primed in B cell-deficient mice showed normal expansion, but failed to induce Ab class switch during in vitro coculture with B cells, suggesting an incomplete helper T differentiation in the absence of B cells (46).

In sum, we demonstrated here that the function of ICOS in supporting T_{FH} generation and humoral immunity critically relies on the evolutionarily conserved tyrosine-based signaling motif in its cytoplasmic tail. We provided evidence that ICOS can induce key T_{FH} cytokines by activating PI3K through this signaling motif, probably when primed T cells contact with B cells to complete the T_{FH} differentiation program.

Materials and Methods

Animals. ICOS-YF knock-in mice were generated in 129 background and then backcrossed into C57BL/6 background. ICOS-KO mice have been described (13). The animals were housed in the Institut de Recherches Cliniques de Montreal (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. Details of the reagents are described in *SI Text*.

In Vitro T-Cell Culture. All T cells were cultured in RPMI1640 medium (1×10^6 cells/mL) supplemented with 10% FBS, glutamine, penicillin, streptomycin, β-mercaptoethanol. Total LN cells were activated by soluble anti-CD3 (1 μg/mL). For preparation of $CD4^+$ T blasts for biochemical analyses and Ca^{2+} flux experiments, $CD4^+$ T cells were positively selected (>95%) from single cells suspensions of spleen and LN using CD4 selection kit (Stem Cell Technologies) and then activated by culturing with plate-bound anti-CD3 (3 μg/mL) plus soluble anti-CD28 (1 μg/mL) for 2 days and expanded in media containing recombinant IL-2 (100 μg/mL) for 3 days. For experiments described in Fig. 5 and Fig. S7 *B* and *C*, splenic $CD4^+$ T cells were negatively selected (>90%) using the MACS $CD4^+$ T-cell isolation kit (Myltenyi). The $CD4^+$ T cells were stimulated for 2 days as described above, except that 10 ng/mL IL-6 were added to enhance IL-21 expression (47). Subsequently, the activated cells were collected, washed once in complete medium, and rested for 1 day in complete medium without IL-2 at 1×10^6 cells/mL in 6-well plates (2 mL/well) to avoid overcrowding.

Acute T-Cell Activation, Lysis, and Immunoprecipitaton. The $CD4^+$ T-cell blasts were harvested and stimulated by combinations of anti-CD3 (1 μg/mL), anti-ICOS (2 μg/mL), and control hamster IgG. The bound antibodies were cross-linked by goat anti-hamster IgG (20 μg/mL) at 37 °C. After washing, cells were lysed in lysis buffer (1% Nonidet P-40, 20 mM Tris pH 7.4, 137 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM PMSF, and 0.1 mM sodium orthovanadate) for 20 min on ice. After clearance of cell debris, lysates were boiled in Laemmli sample buffer for immunoblot analysis. For immunoprecipitation of ICOS-associated proteins, the lysates were incubated with anti-ICOS antibody (2 μg/mL) for 1 h at 4 °C, and the immune complexes were recovered by a mixture of protein G-agarose beads (Thermo Scientific) and protein A-agarose beads (Pierce).

T-Cell Restimulation Assays. Negatively selected $CD4^+$ T cells were stimulated for 2 days and rested for 1 day in media alone. For cytokine qPCR, 5 million $CD4^+$ T blasts were restimulated for 6 h in 400 μL media containing Ab cocktails: Anti-CD3 (1 μg/mL) plus either hamster IgG, anti-ICOS, or anti-CD28 (2 μg/mL each), followed by goat anti-hamster IgG (20 μg/mL). For PI3K inhibition experiments, cells were pretreated for 1 h with LY 294002 (50 μM; Calbiochem) and then stimulated with the Abs in the continued presence of the inhibitor. RNA was isolated using the TRIzol reagent (Invitrogen). cDNA was prepared from the extracted RNA using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). HGPRT, IL-21, IL-4, and IL-10 TaqMan primers and probes were from Applied Biosystems. Quantitative real-time PCR was performed by using a TaqMan 7300/7500 system and software (Applied Biosystems). Fold expression was calculated using the $\Delta\Delta^{CT}$ method using HGPRT as a reference gene. For proliferation assays, cells were restimulated in U-bottom 96 wells (1×10^5 cells/well) with the Abs for 24 h. For the last 8 h of incubation, ^{3}H-thymidine was added at 1 μCi/well.

Ca^{2+} Flux, Immunization of Mice, and ELISA. Details are described in the *SI Text*.

Statistical Analysis. The significance of the data were tested by Student's *t* test.

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1. Vinuesa CG, Tangye SG, Moser B, Mackay CR (2005) Follicular B helper T cells in antibody responses and autoimmunity. *Nat Rev Immunol* 5:853–865.
2. Nurieva RI, et al. (2008) Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity* 29:138–149.
3. Nurieva RI, et al. (2009) Bcl6 mediates the development of T follicular helper cells. *Science* 325:1001–1005.
4. Johnston RJ, et al. (2009) Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science* 325:1006–1010.
5. Yu D, et al. (2009) The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. *Immunity* 31:357–468.
6. Bryant VL, et al. (2007) Cytokine-mediated regulation of human B cell differentiation into Ig-secreting cells: Predominant role of IL-21 produced by CXCR5+ T follicular helper cells. *J Immunol* 179:8180–8190.
7. Dullaers M, et al. (2009) A T cell-dependent mechanism for the induction of human mucosal homing immunoglobulin A-secreting plasmablasts. *Immunity* 30:120–129.
8. Reinhardt RL, Liang HE, Locksley RM (2009) Cytokine-secreting follicular T cells shape the antibody repertoire. *Nat Immunol* 10:385–393.
9. Hutloff A, et al. (1999) ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. *Nature* 397:263–266.
10. Greenwald RJ, Freeman GJ, Sharpe AH (2005) The B7 family revisited. *Annu Rev Immunol* 23:515–548.
11. Lohning M, et al. (2003) Expression of ICOS in vivo defines CD4+ effector T cells with high inflammatory potential and a strong bias for secretion of interleukin 10. *J Exp Med* 197:181–193.
12. Vogelzang A, et al. (2008) A fundamental role for interleukin-21 in the generation of T follicular helper cells. *Immunity* 29:127–137.
13. Tafuri A, et al. (2001) ICOS is essential for effective T-helper-cell responses. *Nature* 409:105–109.
14. McAdam AJ, et al. (2001) ICOS is critical for CD40-mediated antibody class switching. *Nature* 409:102–105.
15. Dong C, et al. (2001) ICOS co-stimulatory receptor is essential for T-cell activation and function. *Nature* 409:97–101.
16. Grimbacher B, et al. (2003) Homozygous loss of ICOS is associated with adult-onset common variable immunodeficiency. *Nat Immunol* 4:261–268.
17. Bossaller L, et al. (2006) ICOS deficiency is associated with a severe reduction of CXCR5+CD4 germinal center Th cells. *J Immunol* 177:4927–4932.
18. Akiba H, et al. (2005) The role of ICOS in the CXCR5+ follicular B helper T cell maintenance in vivo. *J Immunol* 175:2340–2348.

19. Hu YL, et al. (2009) B7RP-1 blockade ameliorates autoimmunity through regulation of follicular helper T cells. *J Immunol* 182:1421–1428.
20. Vinuesa CG, et al. (2005) A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity. *Nature* 435:452–458.
21. Yu D, et al. (2007) Roquin represses autoimmunity by limiting inducible T-cell costimulator messenger RNA. *Nature* 450:299–303.
22. Linterman MA, et al. (2009) Follicular helper T cells are required for systemic autoimmunity. *J Exp Med* 206:561–576.
23. Ferguson SE, Han S, Kelsoe G, Thompson CB (1996) CD28 is required for germinal center formation. *J Immunol* 156:4576–4581.
24. Linterman MA, et al. (2009) Roquin differentiates the specialized functions of duplicated T cell costimulatory receptor genes CD28 and ICOS. *Immunity* 30:228–241.
25. Parry RV, et al. (2003) CD28 and inducible costimulatory protein Src homology 2 binding domains show distinct regulation of phosphatidylinositol 3-kinase, Bcl-x(L), and IL-2 expression in primary human CD4 T lymphocytes. *J Immunol* 171:166–174.
26. Arimura Y, et al. (2002) A co-stimulatory molecule on activated T cells, H4/ICOS, delivers specific signals in T(h) cells and regulates their responses. *Int Immunol* 14:555–566.
27. Parry RV, Riley JL, Ward SG (2007) Signalling to suit function: Tailoring phosphoinositide 3-kinase during T-cell activation. *Trends Immunol* 28:161–168.
28. Dodson LF, et al. (2009) Targeted knockin mice expressing mutations of CD28 reveal an essential pathway for costimulation. *Mol Cell Biol* 29:3710–3721.
29. Nurieva RI, et al. (2007) A costimulation-initiated signaling pathway regulates NFATc1 transcription in T lymphocytes. *J Immunol* 179:1096–1103.
30. Franko JL, Levine AD (2009) Antigen-independent adhesion and cell spreading by inducible costimulator engagement inhibits T cell migration in a PI-3K-dependent manner. *J Leukoc Biol* 85:526–538.
31. Wells V, Downward J, Mallucci L (2007) Functional inhibition of PI3K by the betaGBP molecule suppresses Ras-MAPK signalling to block cell proliferation. *Oncogene* 26:7709–7714.
32. Okkenhaug K, et al. (2002) Impaired B and T cell antigen receptor signaling in p110delta PI 3-kinase mutant mice. *Science* 297:1031–1034.
33. Iiyama R, et al. (2003) The role of inducible co-stimulator (ICOS)/B7-related protein-1 (B7RP-1) interaction in the functional development of Peyer's patches. *Immunol Lett* 88:63–70.
34. Cerutti A (2008) The regulation of IgA class switching. *Nat Rev Immunol* 8:421–434.
35. Walker LS, et al. (1999) Compromised OX40 function in CD28-deficient mice is linked with failure to develop CXC chemokine receptor 5-positive CD4 cells and germinal centers. *J Exp Med* 190:1115–1122.
36. Garside P, et al. (1998) Visualization of specific B and T lymphocyte interactions in the lymph node. *Science* 281:96–99.
37. Feito MJ, et al. (2003) Mechanisms of H4/ICOS costimulation: Effects on proximal TCR signals and MAP kinase pathways. *Eur J Immunol* 33:204–214.
38. Smith KM, et al. (2003) Inducible costimulatory molecule-B7-related protein 1 interactions are important for the clonal expansion and B cell helper functions of naive, Th1, and Th2 T cells. *J Immunol* 170:2310–2315.
39. Odegard JM, et al. (2009) ICOS controls effector function but not trafficking receptor expression of kidney-infiltrating effector T cells in murine lupus. *J Immunol* 182:4076–4084.
40. Redoglia V, et al. (1996) Characterization of H4: A mouse T lymphocyte activation molecule functionally associated with the CD3/T cell receptor. *Eur J Immunol* 26:2781–2789.
41. Buonfiglio D, et al. (1999) Characterization of a novel human surface molecule selectively expressed by mature thymocytes, activated T cells and subsets of T cell lymphomas. *Eur J Immunol* 29:2863–2874.
42. Fos C, et al. (2008) ICOS ligation recruits the p50α PI3K regulatory subunit to the immunological synapse. *J Immunol* 181:1969–1977.
43. Sharpe AH, Freeman GJ (2002) The B7-CD28 superfamily. *Nat Rev Immunol* 2:116–126.
44. McAdam AJ, et al. (2000) Mouse inducible costimulatory molecule (ICOS) expression is enhanced by CD28 costimulation and regulates differentiation of CD4+ T cells. *J Immunol* 165:5035–5040.
45. Walker LS, et al. (2003) Established T cell-driven germinal center B cell proliferation is independent of CD28 signaling but is tightly regulated through CTLA-4. *J Immunol* 170:91–98.
46. Macaulay AE, DeKruyff RH, Umetsu DT (1998) Antigen-primed T cells from B cell-deficient JHD mice fail to provide B cell help. *J Immunol* 160:1694–1700.
47. Dienz O, et al. (2009) The induction of antibody production by IL-6 is indirectly mediated by IL-21 produced by CD4+ T cells. *J Exp Med* 206:69–78.

Supporting Information

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SI Text

Generation of ICOS-YF Mice. We designed a targeting construct such that the exon 3 of the wild-type *Icos* gene will be replaced by an engineered exon 3 and a neomycin-resistance (Neo) cassette flanked by two loxP elements (Fig. S1*A*). The engineered exon 3 contained an adenine-to-thymidine change that results in a tyrosine-to-phenylalanine mutation at amino acid 181 of the cytoplasmic tail of ICOS. Using an *Eco*RI site created by the mutation, we identified ES clones (R1, 129 background) that have integrated the mutant allele by Southern blot analysis (Fig. S1*B*) and subsequently verified the intended mutation by sequencing the PCR products generated from the gene-targeted ES cells (Fig. S1*C*). We injected the ES cells into the blastocysts (C57BL/6 background), and resulting chimeric mice were backcrossed with C57BL/6 mice. Mice with germline transmitted *Icos (Y181F)-Neo* allele were bred with CMV-Cre transgenic mice (1) to achieve in vivo deletion of Neo cassette (Fig. S1*D*). We also verified that the mRNA transcribed from $Icos^{y181f}$ locus has the same structure as the WT *Icos* mRNA, except for the point mutation by sequencing PCR-amplified cDNA fragments encompassing exon 2 to exon 5.

Antibodies and Cytokines. For flow cytometry or T-cell stimulation, the following antibodies were used: Armenian hamster IgG, antibodies against ICOS (mAb C398.4A for stimulation and 5F9 for staining), CD3 (145.2C11), CD4, CD8, CD16/32 (Fc block), Fas, IgD, PD-1 (all purchased from eBioscience), and GL7 (BD). Goat anti-Armenian hamster IgG (Jackson Immunoresearch) or avidin (Calbiochem) were used to cross-link primary antibodies. Biotinylated anti-CXCR5 (BD) was used with streptavidin-APC or -PE (eBioscience). For immunoblots, goat anti-mouse ICOS (sc 5748; Santa Cruz) and rabbit anti-PI3K p85α (Upstate Biotechnology) and antibodies against phospho-specific or total Akt, p44/42 MAPK, JNK, p38 (Cell Signaling Technology) were used with HRP-conjugated anti-goat (sc2020; Santa Cruz) or anti-rabbit (Bio-Rad Laboratories) secondary antibodies. Recombinant IL-2 was purchased from R&D Systems and IL-6 from eBioscience.

Ca^{2+} Flux. $CD4^+$ T blasts were loaded with Indo-1 (Invitrogen) at 5×10^6 cells/mL in HBSS buffer supplemented with 0.1% BSA,1 mM $CaCl_2$, and 1 μM $MgCl_2$. Cells were incubated for 1 min at RT with biotinylated antibodies: 0.1 μg/mL anti-CD3 plus 1 μg/mL anti-ICOS (1 μg/mL) or control hamster IgG. After recording baseline for 30 s, avidin (14 μg/mL) was added, and the mobilization of intracellular Ca^{2+} was monitored by measuring FL4/FL5 on LSR FACS machine (BD). Equal loading of Indo-1 was confirmed by releasing intracellular Ca^{2+} by ionomycin (1 μg/mL; Sigma–Aldrich).

Immunization of Mice. NP_{16}-CGG (1 mg/mL in PBS; Biosearch Technologies) was mixed with an equal volume of Imject Alum (Thermo Scientific) for 30 min at room temperature. Mice were injected i.p. with 100 μg (primary) or 50 μg (secondary) of alum-precipitated NP_{16}-CGG.

ELISA. Various isotypes of mouse Ig were detected using mouse Ig isotyping kit (SouthernBiotech). Fecal extracts were prepared from fresh fecal pellets by vortexing in PBS containing 0.02% NaN_3 and 1 mM PMSF (10 μL/mg feces). NP-specific Ab from mice immunized with NP_{16}-CGG was measured using plate-bound NP_{33}-BSA or NP_3-BSA. Affinity profiling ELISA was performed as described in ref. 2. Anti-NP antibodies in serum samples (1:100 dilution) were allowed to bind to NP_{33}-BSA ELISA plates. After regular washing, bound antibodies were treated for 10 min at RT with NaSCN solutions (Sigma–Aldrich) each column receiving stepwise increments: 0.05, 0.1, 0.25, 0.5, 1, 2, 2.5, 3, 3.5, 4, and 5 M. Remaining antibodies were detected by AP-conjugated secondary antibodies.

1. Schwenk F, Baron U, Rajewsky K (1995) A cre-transgenic mouse strain for the ubiquitous deletion of loxP-flanked gene segments including deletion in germ cells. *Nucleic Acids Res* 23:5080–5081.

2. Luxton RW, Thompson EJ (1990) Affinity distributions of antigen-specific IgG in patients with multiple sclerosis and in patients with viral encephalitis. *J Immunol Methods* 131:277–282.

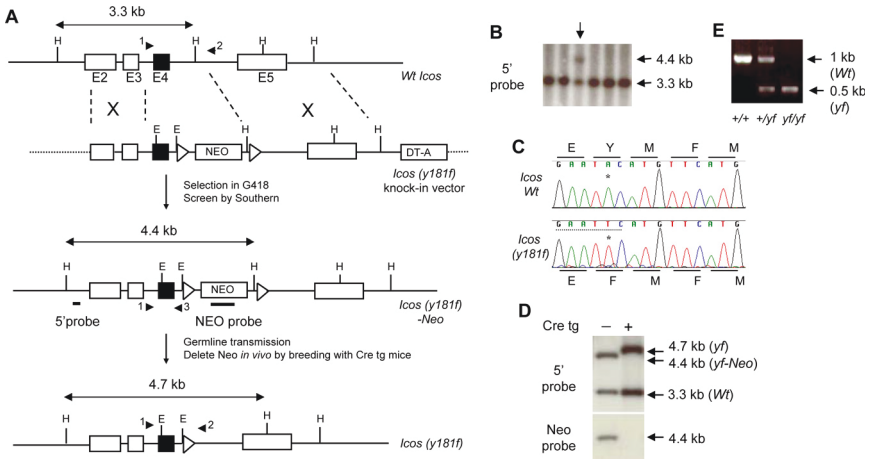

Fig. S1. Generation of ICOS-YF mice. (*A*) The targeting vector was constructed with Neo cassette flanked by two loxP elements for positive selection and DT-A for negative selection. The exon 4 containing Tyr-181 residue is represented with a filled box, the Southern blot probes are denoted with thick underlines, and PCR primers are shown as arrow heads. (*B*) Southern blot screening of ES clones using *Hin*dIII-digested ES genomic DNA. (*C*) Genomic DNA from ES cells positive for *Icos (y181f)-Neo* locus was PCR-amplified using primers 1 and 3 as depicted in *A*, and the nucleotide sequences of the PCR products were determined. Asterisks indicate the A-to-T mutation that results in Tyr-to-Phe mutation with an *Eco*RI site. (*D*) Genomic DNAs from mice possessing one copy of *Icos (y181f)-Neo* locus without or with Cre transgene were analyzed by Southern blot after *Hin*dIII digestion. (*E*) The genotype of F2 progeny of heterozygous interbreeding was determined by PCR using primers 1 and 2 as shown in *A*.

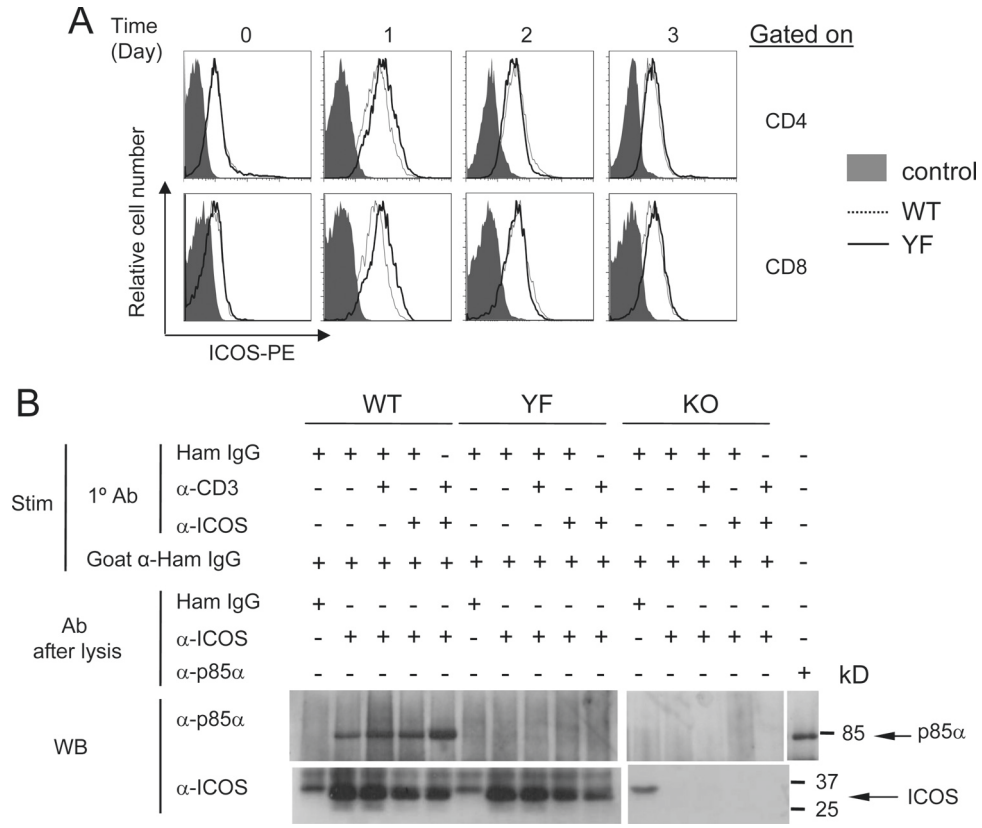

Fig. S2. Normal surface expression but impaired PI3K binding by ICOS-YF. (*A*) Normal expression patterns of ICOS-YF. Total LN cells isolated from WT or ICOS-YF mice were stimulated with soluble anti-CD3 antibody for the indicated periods of time. ICOS expression on $CD4^+$ or CD8+ T cells was assessed by flow cytometry. (*B*) Impaired PI3K recruitment by ICOS-YF. $CD4^+$ T-cell blasts were stimulated with antibodies against CD3 and/or ICOS, and the immune complexes were recovered by immunoprecipitation. The amounts of p85a subunit of PI3K and the total ICOS protein were assessed by immunoblotting. Data shown in *A* and *B* are representative of three independent experiments.

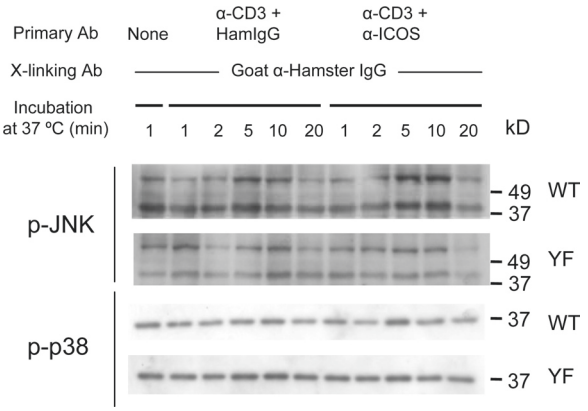

Fig. S3. ICOS does not activate JNK and p38. $CD4^+$ T blasts were stimulated with indicated antibodies up to 20 min, and the amounts of phospho-JNK and phospho-p38 were assessed by immunoblotting.

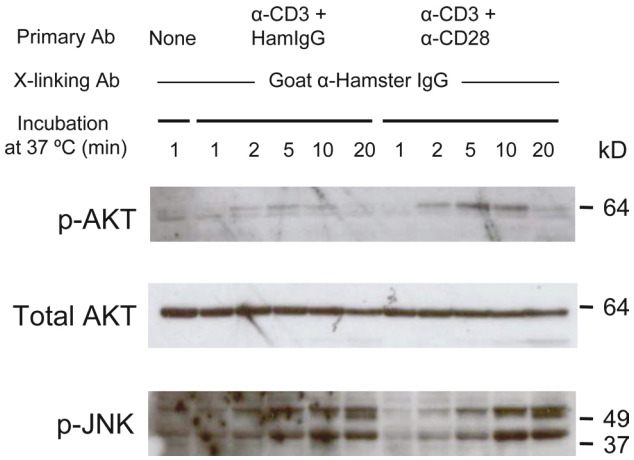

Fig. S4. Moderate activation of AKT by CD28. $CD4^+$ T blasts were stimulated with indicated antibodies up to 20 min, and the amounts of phospho-AKT, total AKT, and phospho-JNK were assessed by immunoblotting.

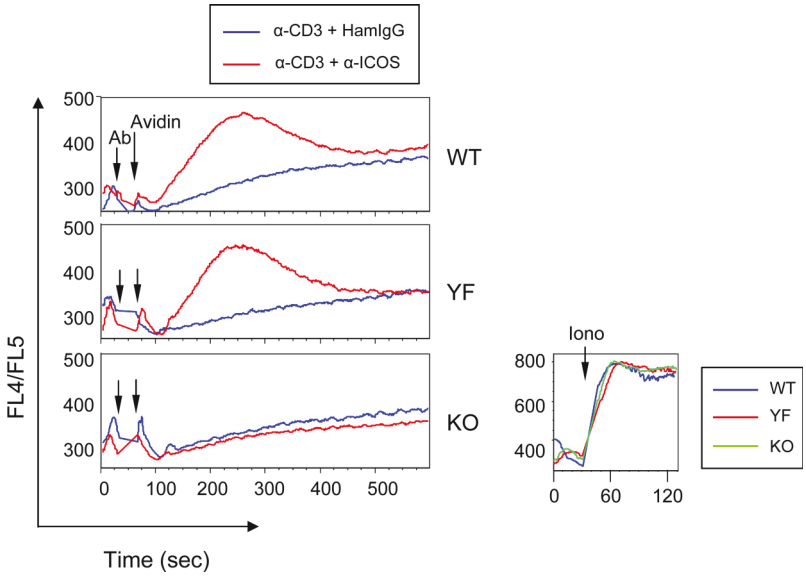

Fig. S5. Intact calcium mobilization by ICOS-YF. Indo-1 loaded $CD4^+$ T blasts were stimulated with suboptimal anti-CD3 without or with anti-ICOS, and the intracellular calcium flux was monitored by flow cytometry. A representative of four independent experiments is shown.

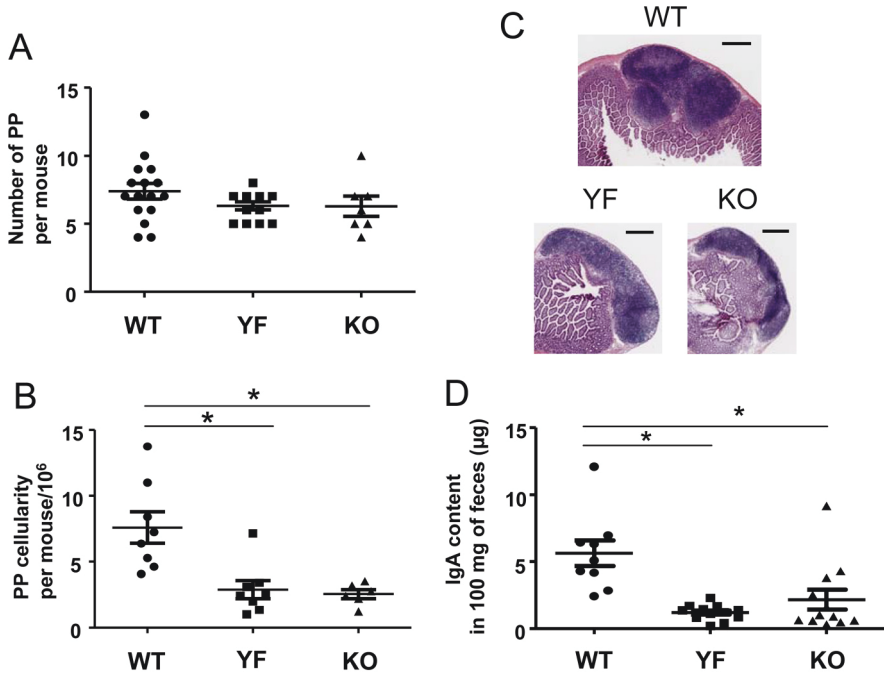

Fig. S6. Normal number of PPs with decreased GC reaction in ICOS-YF and KO mice. (*A*) Each data point represents the number of PPs per mouse ($n = 10$ WT, 10 YF, and 6 KO). (*B*) Each data point represents the total number of PP cells collected from a single mouse ($n = 8$ WT, 8 YF, and 6 KO). *, $P < 0.01$. (*C*) Representative sections of PPs stained with H&E are shown. (Scale bars, 250 μm.) (*D*) Decreased mucosal IgA secretion in the gut. IgA contents in fresh fecal pellets were measured by ELISA. Each data point represents the fecal IgA content of an individual mouse ($n = 9$ WT, 12 YF, and 11 KO). *, $P < 0.01$.

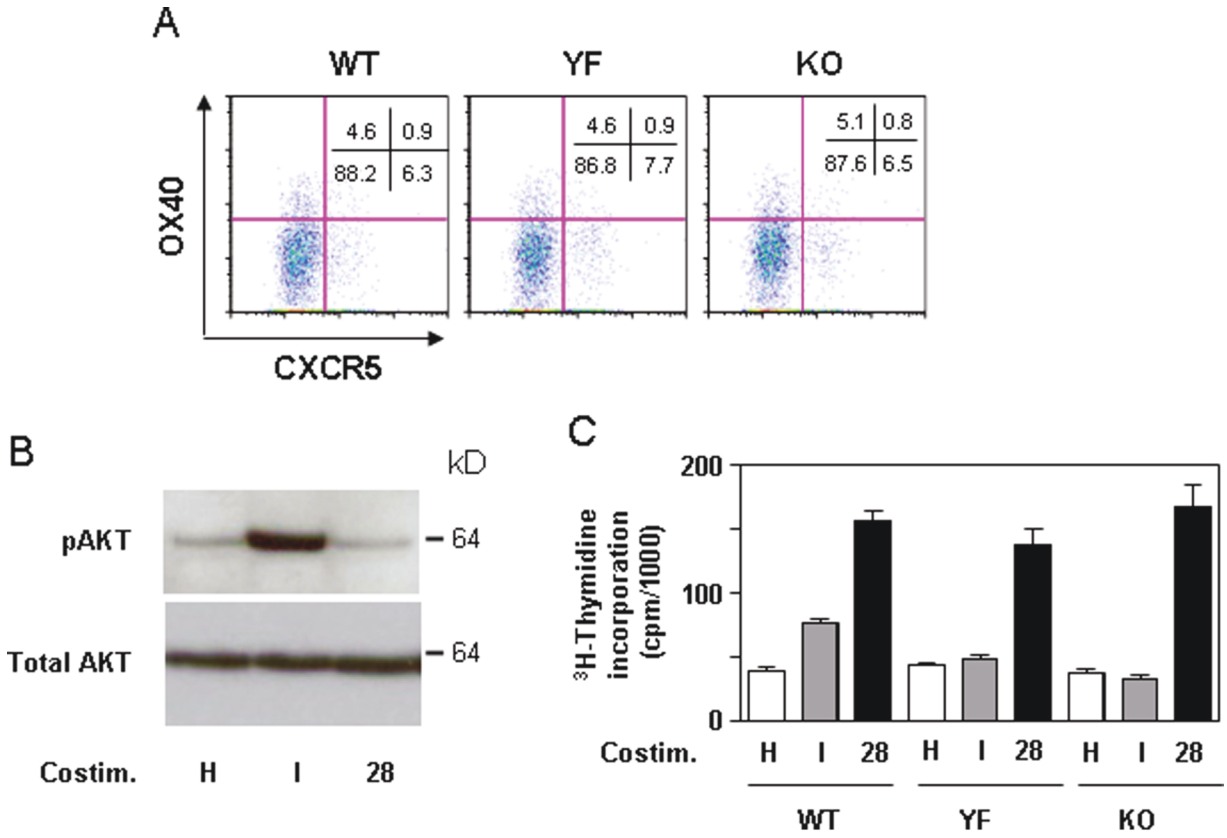

Fig. S7. Differential costimulatory impacts of CD28 and ICOS. (*A*) Unaltered induction of OX40 and CXCR5 in ICOS-YF and ICOS-KO CD4$^+$ T cells. Total lymph node cells were stimulated with soluble anti-CD3 (1 μg/mL) for 2 days, and the levels of OX40 and CXCR5 were analyzed by FACS. A representative of two independent experiments is shown. (*B*) Differential activation of PI3K pathway by ICOS and CD28. Cell lysates were prepared from CD4$^+$ T cells restimulated with anti-CD3 plus hamster IgG (H), anti-ICOS (I), or anti-CD28 (28) followed by goat anti-hamster IgG for 5 min and analyzed by immunoblotting. A representative of two independent experiments is shown. (*C*) CD28 plays a major role in secondary expansion of CD4$^+$ T cells. Cells were restimulated as in *B* in 96-well plates for 24 h. ^{3}H-thymidine was pulsed for the last 8 h of incubation. A representative of three independent experiments is shown.

Appendix II: Increased PI3K signalling results in more Tfh cells

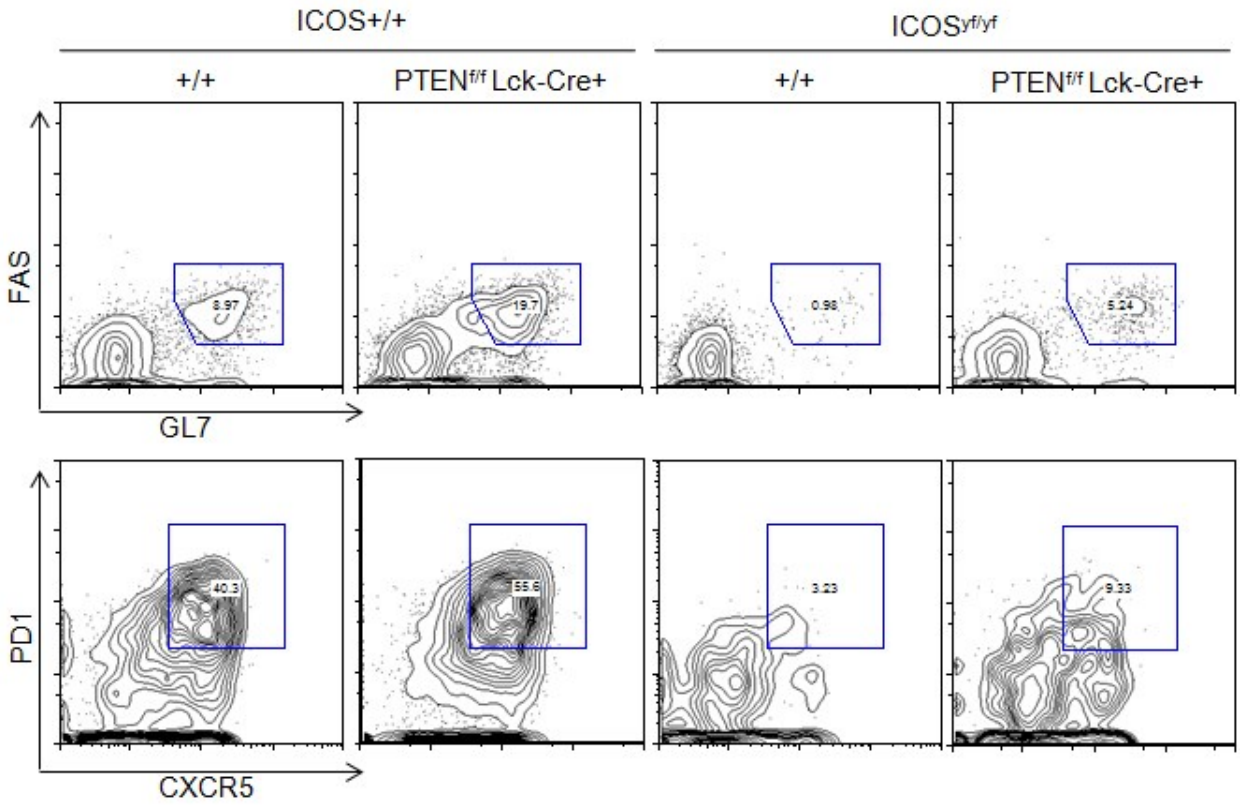

Figure 1. Increased PI3K signalling results in more Tfh cells. Conditionally deleting PTEN in T cells leads to increased GC B cells (top left panels) and Tfh cells (bottom left panels) in the PP of WT, as well as partially rescues the defect in GC formation (top right panels) and Tfh cell differentiations (bottom right panels) in the PP of ICOS-YF mice.