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The role of inducible costimulator in autoimmunity

Par

Vincent Panneton

Département de microbiologie, infectiologie et immunologie

Faculté de médecine

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The role of inducible costimulator in autoimmunity

Présentée par

Vincent Panneton

A été évaluée par un jury composé des personnes suivantes

Dr. Étienne Gagnon
Président-rapporteur

Dr. Woong-Kyung Suh
Directeur de recherche

Dr. Nathalie Arbour
Membre du jury

Dr. Klaus Okkenhaug
Examinateur externe

Abstract

The inducible costimulator (ICOS) is a T cell costimulatory receptor crucial for humoral immunity. In humans, ICOS deficiency is associated with recurrent infections due to defects in T follicular helper (Tfh) differentiation and lack of protective antibodies. Paradoxically, some ICOS-deficient patients were found to exhibit signs of antibody-mediated autoimmunity. These observations are congruent with findings suggesting that ICOS plays a dual role in promoting T effector and T regulatory cell functions. Due to this ambivalence, the role of ICOS in autoimmunity remains unresolved.

Rheumatoid arthritis (RA) is an autoimmune disease characterized by joint inflammation and progressive bone destruction. The number of $ICOS^+$ T cells in the synovial tissues of RA patients was found to be elevated, suggesting a potential involvement of ICOS signaling in the pathogenesis of RA. In the first study presented in Chapter 2, we showed that ICOS is required for the initiation and maintenance of collagen-induced arthritis (CIA), a murine model of RA. We found that key CIA initiation events such as anti-collagen antibody production and inflammatory T cell proliferation were dependent on ICOS-PI3K signaling. Further, we found that ICOS-PI3K signaling promotes maintenance of established CIA. Additionally, we show that glycolysis inhibition ameliorates CIA, thus suggesting potential overlaps between ICOS-PI3K signaling and glucose metabolism in the pathophysiology of autoimmune arthritis.

The initiation of autoimmunity depends on the action of autoimmune effector cells, but also on a failure of regulatory cells. Amongst the latter, T follicular regulatory (Tfr) cells are thought to prevent autoantibody production. In the second study presented in Chapter 3, we used regulatory T cell-specific ICOS knockout (ICOS FC) mice to investigate the impact of ICOS signaling in Tfr cells. We found that ICOS FC mice display a significant reduction in Tfr cell numbers, but no depletion of their T regulatory (Treg) precursors. Further, ICOS FC mice exhibited a rise of extraneous germinal center (GC) B cells numbers and increased production of anti-nuclear antibodies. We also observed disruptions of anti-viral responses and antigen-specific antibody production in ICOS FC

mice, suggesting non-regulatory roles for Tfr cells. We performed single-cell transcriptome analysis of regulatory T cells and observed an accumulation of Tfr precursors in ICOS FC mice suggestive of a Treg-to-Tfr transition defect. Mechanistically, we found that ICOS participates in Tfr differentiation by regulating KLF2 and NFAT2, thereby contributing to the establishment of follicular T cell traits.

In sum, we demonstrate that ICOS promotes the development of autoimmune arthritis by fostering inflammatory T cell responses in a PI3K-dependent manner. In addition, our work shows that ICOS can prevent autoantibody production by supporting Tfr differentiation. Thus, we contribute insights into the dual role of ICOS in the cellular and humoral arms of autoimmunity, providing important parameters to be considered when searching for novel therapeutic targets.

Keywords

ICOS, autoimmunity, rheumatoid arthritis, collagen-induced arthritis, PI3K, T follicular regulatory cell, autoantibody

Résumé

Le costimulateur inductible (ICOS) est un récepteur costimulatoire des cellules T crucial pour l'immunité humorale. Chez l'humain, une déficience de ICOS est associée à des infections récurrentes dû à des défauts de différentiation des lymphocytes T auxiliaires folliculaires (Tfh) et un manque d'anticorps protecteurs. Paradoxalement, certains patients déficients en ICOS développent des signes d'autoimmunité causés par des autoanticorps. Ces observations sont en accord avec des résultats suggérant que ICOS joue un double rôle dans la promotion des fonctions de cellules T effectrices et de cellules T régulatrices.

L'arthrite rhumatoïde (RA) est une maladie autoimmune caractérisée par l'inflammation articulaire et la destruction progressive des os. Dans la première étude présentée au Chapitre 2, nous avons démontré que ICOS est requis pour l'initiation et la maintenance de l'arthrite induite par le collagène (CIA), un modèle murin de la RA. Nous avons démontré que des événements clés de l'initiation de la CIA incluant la production d'anticorps contre le collagène et la prolifération de cellules T inflammatoires sont dépendants de la voie de signalisation ICOS-PI3K. Aussi, nous avons trouvé que la signalisation par ICOS-PI3K promouvoit le maintien de la CIA. De plus, nous avons établi que l'inhibition de la glycolyse réduit la sévérité de la CIA, ce qui suggère un chevauchement entre la signalisation ICOS-PI3K et le métabolisme du glucose dans la pathogenèse de l'arthrite autoimmune.

Dans la deuxième étude présentée au Chapitre 3, nous avons utilisé des souris dont les cellules T régulatrices sont déficientes en ICOS (ICOS FC) pour évaluer l'impact de ICOS dans les cellules Tfr. Nous avons trouvé que les souris ICOS FC possèdent un taux réduit de cellules Tfr, mais aucune diminution des cellules T régulatrices (Treg) précurseurs. De plus, les souris ICOS FC ont un taux élevé de cellules B du centre germinatif (GC) non-spécifiques ainsi qu'une production accrue d'anticorps anti-nucléaires. Nous avons aussi observé une perturbation des réponses anti-virales et de la production d'anticorps spécifiques dans les souris ICOS FC ce qui suggère des rôles non-régulateurs pour les cellules Tfr. Nous avons effectué une analyse de transcriptome de

cellule unique avec des Tregs et nous avons observé une accumulation de précurseurs de Tfr dans les souris ICOS FC, ce qui suggère un défaut de la transition Treg à Tfr. Nos données suggèrent que ICOS participe à la différentiation des cellules Tfr en régulant KLF2 et NFAT2, ce qui contribue à l'établissement de traits folliculaires.

En résumé, nous avons démontré que ICOS promouvoit le développement de l'arthrite autoimmune en soutenant les fonctions des cellules T inflammatoires par la voie de signalisation ICOS-PI3K. De plus, nos résultats prouvent que ICOS peut prévenir la production d'autoanticorps en supportant la différentiation des cellules Tfr. Notre travail contribue à l'avancement des connaissances en ce qui concerne le rôle double de ICOS dans l'immunité cellulaire et humorale et fournit des paramètres importants à considérer lors de la recherche de nouvelles cibles thérapeutiques.

Mots clés

ICOS, autoimmunité, arthrite rhumatoïde, arthrite induite par le collagène, PI3K, cellule T folliculaire régulatrice, autoanticorps

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Chapter 3

List of acronyms and abbreviations

Abbreviation	Full name
3-BrPA	3-bromopyruvate
4E-BP	4E binding protein
ACPA	Anti-citrullinated peptide antibody
ADCC	Antibody-dependent cellular cytotoxicity
AID	Activation-induced cytidine deaminase
AIRE	Autoimmune regulator
ANA	Anti-nuclear antibody
APC	Antigen-presenting cell
APS1	Autoimmune polyendocrine syndrome type I
B7-H2	B7 homolog 2
B7RP-1	B7-related protein 1
BCR	B cell receptor
BLIMP-1	B lymphocyte-induced maturation protein 1
CAIA	Collagen antibody-induced arthritis
CAR	Chimeric antigen receptor
CCR7	C-C motif chemokine receptor 7
CIA	Collagen-induced arthritis
CII	Type II collagen
CNS	Central nervous system
CNS2	Conserved non-coding sequence 2
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte-associated protein 4
CXCR5	C-X-C motif chemokine receptor 5
DAG	Diacylglycerol
DC	Dendritic cell
DMARD	Disease modifying anti-rheumatic drug
DZ	Dark zone

EAE	Experimental autoimmune encephalomyelitis
EBI2	Epstein-Barr virus-induced G-protein coupled receptor 2
EBNA-1	Epstein-Barr virus nuclear antigen 1
EBV	Epstein-Barr virus
eIF4E	Eukaryotic translation initiation factor 4E
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FC	Foxp3-cre
FOXO1	Forkhead box protein O1
FOXP3	Forkhead box P3
G6PI	Glucose-6-phosphate isomerase
GC	Germinal center
GVHD	Graft-versus-host disease
HLA	Human leukocyte antigen
IBD	Inflammatory bowel disease
ICOS	Inducible costimulator
ICOSL	Inducible costimulatory ligand
Ig	Immunoglobulin
IL	Interleukin
IL2Rα	Interleukin-2 receptor alpha chain
ILC	Innate lymphoid cell
Ip	Intraperitoneal
IP3	Inositol trisphosphate
IPEX	Immunodysregulation, polyendocrinopathy, enteropathy X-linked
kDa	Kilodalton
KLF2	Krüppel-like factor 2
LAT	Linker of activated T cells
LN	Lymph node
LZ	Light zone
mAb	Monoclonal antibody

MHC	Major histocompatibility complex
MS	Multiple sclerosis
mTOR	Mammalian target of rapamycin
MTX	Methotrexate
NFAT	Nuclear factor of activated T cells
NIK	NF-κB-inducing kinase
NK	Natural killer (cell)
NOD	Non-obese diabetic
NSAID	Nonsteroidal anti-inflammatory drug
OPN-i	Intracellular osteopontin
PBC	Primary biliary cirrhosis
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5-biphosphate
PLC-γ1	Phospholipase C-gamma 1
PP	Peyer's patch
PTEN	Phosphatase and tensin homolog
RA	Rheumatoid arthritis
RAG	Recombination-activating gene
RF	Rheumatoid factor
RNA	Ribonucleic acid
S.c.	Subcutaneous
S1PR1	Sphingosine-1-phosphate receptor 1
SAP	SLAM-associated protein
SFB	Segmented filamentous bacteria
SLE	Systemic lupus erythematosus
SOSTDC1	Sclerostin domain-containing protein 1
SS	Sjogren's syndrome
STA	Serum transfer arthritis
STIM	Stromal interaction molecule
T1D	Type 1 diabetes

TBK1	TANK-binding kinase 1
TCR	T cell receptor
Tfh	T follicular helper (cell)
Tfr	T follicular regulatory (cell)
TGF-β	Transforming growth factor beta
Th	T helper (cell)
TLR	Toll-like receptor
TM	Transmembrane
TNF-α	Tumor necrosis factor alpha
Treg	T regulatory (cell)
TSDR	Treg-specific demethylated region
UTR	Untranslated region
ZAP70	Zeta-chain-associated protein kinase 70

Author contributions

All chapters of this thesis have been proofread by **Dr. Woong-Kyung Suh** who also gave feedback.

Chapter 1

Figures 1.1, 1.3, and 1.4 have previously been published in *Immunological Reviews*: Panneton V, Chang J, Witalis M, Li J, Suh WK. Inducible T-cell co-stimulator: Signaling mechanisms in T follicular helper cells and beyond. Immunol Rev. 2019 Sep;291(1):91-103. doi: 10.1111/imr.12771. PMID: 31402504.

All figures were prepared by **Vincent Panneton** with input from **Dr. Woong-Kyung Suh**

Chapter 2

A version of Chapter 2 has been published in the *Journal of Immunology*: Panneton V, Bagherzadeh Yazdchi S, Witalis M, Chang J, Suh WK. ICOS Signaling Controls Induction and Maintenance of Collagen-Induced Arthritis. J Immunol. 2018 May 1;200(9):3067-3076. doi: 10.4049/jimmunol.1701305. Epub 2018 Mar 26. PMID: 29581356.

Vincent Panneton performed most of the experiments including subcutaneous immunization, histological analysis, ELISAs, flow cytometry, proliferation assays, and glycolysis inhibition trials. Arthritic mice were monitored by **Vincent Panneton** and animal technicians at the IRCM animal facilities. Oral gavage and intraperitoneal (ip) injections were performed by animal technicians. Histological slides were prepared by technicians at the IRCM histology facility. **Vincent Panneton** and **Dr. Woong-Kyung Suh** conceived the experiments and analyzed the data. All figures were prepared by **Vincent Panneton** with input from **Dr. Woong-Kyung Suh**. The original manuscript was written by **Vincent Panneton** with input from **Dr. Woong-Kyung Suh** and **Mariko Witalis**. **Vincent Panneton**, **Sahar Bagherzadeh Yazdchi**, **Mariko Witalis**, and **Jinsam Chang** performed genotyping. **Sahar Bagherzadeh Yazdchi** assisted with ELISA experiments. **Jinsam Chang** assisted with mouse dissections.

Chapter 3

The results presented in Chapter 3 will be used for a future publication. **Vincent Panneton** performed most of the experiments including flow cytometry, ELISAs, histological analysis, anti-nuclear antibody assays, and Western blots. Intraperitoneal injections were performed by animal technicians at the IRCM animal facilities. Single cell RNA sequencing was performed by **Vincent Panneton**, the IRCM flow cytometry platform, and the IRCM molecular biology platform**. Barbara C. Mindt** performed intranasal influenza infections, mouse dissections, body weight measurements, and anti-viral ELISA assays. **Yasser Bouklouch** analyzed single cell RNA sequencing data with help from **Antoine Bouchard** and input from **Vincent Panneton** and **Dr. Woong-Kyung Suh**. **Jinsam Chang** and **Joanna Li** assisted with mouse dissections. **Jinsam Chang** provided histological samples. **Albert Stancescu** performed ELISAs and assisted with anti-nuclear antibody assays. **Dr. John E. Bradley** and **Dr. Troy D. Randall** provided tetramerized recombinant nucleoproteins conjugated with APC or PE (Flu tetramers**). Dr. Jörg H. Fritz** provided advice and contributed resources. **Vincent Panneton**, **Yasser Bouklouch**, **Jinsam Chang**, **Mariko Witalis**, **Joanna Li**, **Albert Stancescu**, and **Antoine Bouchard** performed genotyping. **Vincent Panneton** and **Dr. Woong-Kyung Suh** conceived and supervised the study. The original manuscript was written by **Vincent Panneton** with input from **Yasser Bouklouch** and **Dr. Woong-Kyung Suh**. All figures were prepared by **Vincent Panneton** with input by **Mariko Witalis**, **Joanna Li**, and **Dr. Woong-Kyung Suh**. The manuscript was proofread by **Vincent Panneton**, **Barbara C. Mindt**, **Mariko Witalis**, **Joanna Li**, **Albert Stancescu**, **Antoine Bouchard**, **Dr. Troy D. Randall**, **Dr. Jörg H. Fritz** and **Dr. Woong-Kyung Suh**.

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Chapter 1: Introduction

1.1 The inducible costimulator

1.1.1 History

The discovery of the inducible costimulator (ICOS) can be traced back to 1996 when Dr. Charles Janeway Jr. and colleagues described a molecule which they named H4 (1). To look for novel T cell costimulatory molecules, they produced monoclonal antibodies (mAbs) by immunizing Armenian hamsters with antigen-specific T cell clones. One of the resulting antibodies (C398.4A) was found to bind a molecule selectively expressed by activated T cells and not by B cells, macrophages, or fibroblasts. Henceforth named H4, this molecule was shown to physically associate with the T cell receptor (TCR) and potentiated TCR-induced T cell proliferation when stimulated by the C398.4A mAb. Thus, H4 was established as a potential T-cell costimulatory molecule.

This study was followed three years later by two reports in *Nature* identifying ICOS as an inducible T cell costimulatory receptor in both mice and humans (2, 3). Using the C398.4A mAb, Buonfiglio *et al*. further showed that H4 and ICOS were indeed the same protein (4). Sequence homology assigned ICOS as a member of the CD28 superfamily, and murine ICOS proteins were found to be ~72% identical to the human ortholog (5). Functionally speaking, ICOS was initially shown to enhance T cell responses to foreign antigens by augmenting proliferation and cytokine production (2). The sole ligand for ICOS, first named B7RP-1, was found to be expressed by B cells and macrophages (3). Subsequent studies referred to this ligand with different nomenclature such as B7h, B7-H2, AILIM-L, GL50 or ICOSL (6-10). The term “ICOSL” will be used in this thesis.

Deletion of the *Icos* gene in mice was initially performed in 2001 by three independent groups (11-13). These reports highlighted profound defects in antibody responses as the main phenotype of $ICOS^{-/-}$ mice. It was later found that ICOS ablation results in differentiation defects of T follicular helper (Tfh) cells, a T cell subset required for germinal center (GC) reactions and for the production of high-affinity class-switched antibodies (14).

1.1.2 Structure and expression patterns

ICOS is a type I transmembrane (TM) glycoprotein comprised of three segments: an extracellular immunoglobulin domain, a TM segment, and a cytoplasmic tail (Figure 1.1). On the cell surface, ICOS exists as a disulfide bond-linked homodimer and has an approximate mass of 55-60 kDa formed of a 27kDa and a 29 kDa chain in humans (2). These two polypeptide chains are encoded as a single 199 amino acid chain which is differentially regulated by post-translational modifications (1, 2). While the impact of such modifications on ICOS remain poorly understood, a study has found that N-linked glycosylation of residue N89 can impact trafficking to the cell surface and may affect ligand binding (15). ICOS-mediated signaling is mostly linked to motifs found in its cytoplasmic tail of ~35 amino acids, although a recent study reported signaling activity mediated by the ICOS TM segment (16, 17).

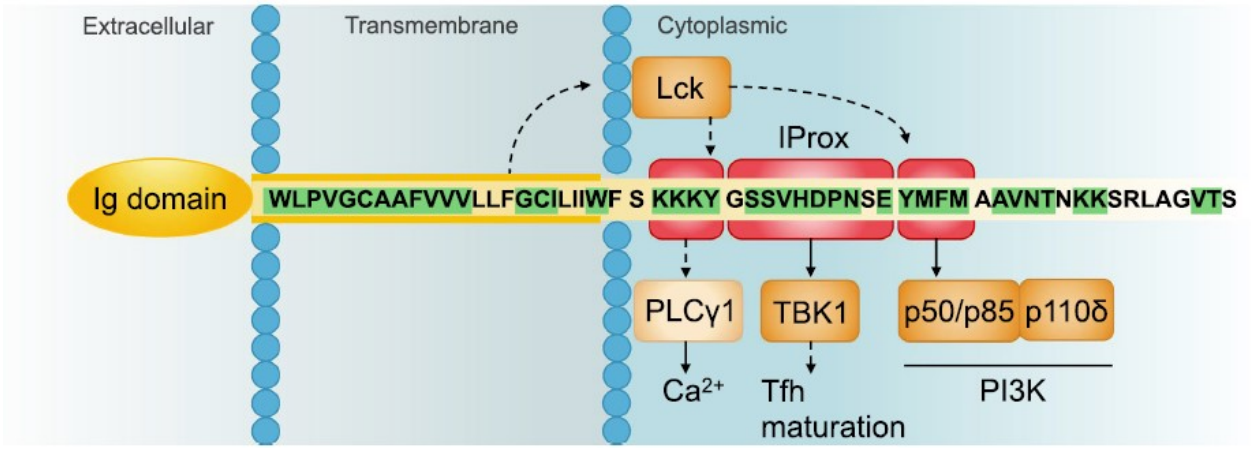

Figure 1.1 Structure of ICOS

Depicted is the polypeptide sequence of the transmembrane segment and cytoplasmic tail of murine ICOS. Evolutionarily conserved residues are highlighted in green. Motifs linked to known signal transducers are in red boxes: KKKY for PLCγ1; IProx for TBK1; YMFM for PI3K. The transmembrane segment of ICOS associates with Lck, facilitating PI3K activation and intracellular calcium release possibly through PLCγ1.

ICOS is predominantly expressed by T cells in anatomical areas linked to humoral immune responses such as the GC light zone, Peyer's patches, and T cell zone of lymph nodes (5, 18). ICOS expression is induced within 24 hours of TCR engagement and can be further enhanced by CD28 costimulation (18, 19). Due to this, ICOS expression is often used as an activation marker in T cells. Mechanisms that govern ICOS expression vary between different T cell subsets. Indeed, initial reports found higher levels of ICOS expression in Th2 cells compared to Th1 cells (19, 20). The Th2 transcription factor GATA-3 was shown to associate with NFATc2 and bind to an *Icos* 3' untranslated region (UTR) (21). In Th1 cells, NFATc2 was instead found to bind with the transcription factor T-bet at the *Icos* promoter region (21). Cytokines such as IL-4, IL-12 and IL-23 were also found to induce ICOS expression, providing an additional layer of complexity (22, 23). Additionally, ICOS was recently shown to be constitutively expressed by innate lymphoid cells (ILCs), challenging the definition of its inducible nature (24).

ICOS expression can be negatively regulated by the RING-type ubiquitin ligase Roquin (25). Specifically, Roquin-dependent mechanisms lead to the degradation of ICOS mRNA. Mice carrying a Roquin-1 point mutation inhibiting its activity (*sanroque*) or mice deficient in Roquin 1 and 2 both show increased ICOS expression along with spontaneous GC reactions and increased Tfh cell numbers (25-27). Negative regulation also occurs at the protein level since ICOS-ICOSL interactions induce the internalization of ICOS (28).

1.1.3 ICOS ligand

ICOSL is comprised of two extracellular immunoglobulin domains, a TM domain, and a cytoplasmic tail (7). It is part of the B7 family of costimulatory ligands which include CD80 and CD86, the CD28 ligands. While ICOSL was found to weakly bind to CD28 and CTLA-4 in humans, ICOS was shown to exclusively bind to ICOSL (3, 29). Consequently, ICOSL mutations cause humoral immune defect phenotypes closely matching those found in ICOS knockout mice (30, 31). Like other B7 family members, ICOSL is expressed on antigen-presenting cells (APCs) such as B cells, macrophages, and dendritic cells

(DCs) (3, 6-10). However, ICOSL was also found to be expressed by non-hematopoietic cells and ILCs, implying non-redundant roles for ICOS and CD28 (5, 6, 32-34).

Unlike CD80 and CD86, ICOSL is constitutively expressed at high levels on B cells (3, 35). This expression is regulated by a non-canonical NF-κB pathway involving the NF-κB-inducing kinase (NIK) and its upstream receptor BAFF (36). Maintenance of ICOSL mRNA levels in B cells requires concomitant B cell receptor (BCR) and CD40 signaling, as BCR stimulation alone results in ICOSL downregulation (37). This suggests that T cell-dependent signaling through CD40 is required for B cells to maintain ICOSL expression. Additionally, ICOS-ICOSL binding triggers the removal of ICOSL from the B cell surface by ADAM family metalloproteinases (28, 38). This shedding can be slowed by Toll-like receptor (TLR) stimulation, specifically by TLR7/8 and TLR9 ligands (39). Thus, it appears that B cell default to ICOSL downregulation during and post-transcription unless they receive stimulation by T cells (CD40) or infection (TLR). This mechanism implies that B cell not receiving sufficient activation signals are prevented from obtaining T cell help through ICOS-ICOSL interactions. This mode of regulation differs from that of CD28 ligands which are normally expressed at low levels during steady state and are not downregulated when presented with experimental settings known to decrease ICOSL levels (35, 39).

Experiments have shown that overexpression of ICOS in T cells paradoxically results in the same phenotype seen in ICOSL-deficient mice (40). Indeed, ICOS overexpression was found to cause ICOSL downregulation at the cell surface of APCs following ICOS-ICOSL interactions. Additionally, mutations in ADAM10 and/or ADAM17 resulted in increased ICOSL expression levels and led to defects in T cell responses (28, 38). In humans, mutations of the genes encoding ICOSL or upstream factors responsible for ICOSL expression such as NIK resulted in hypogammaglobulinemia and recurrent respiratory infections (30, 41). Thus, it seems that ICOS and ICOSL expression levels are tightly regulated and upsetting the balance in either direction can have severe physiological consequences.

1.1.4 ICOS signaling mechanisms

To date, ICOS was found to propagate signals through three different pathways: intracellular calcium flux, phosphoinositide 3-kinase (PI3K), and TANK-binding kinase 1 (TBK1) (Figure 1.1) (20, 42-45). Intracellular calcium flux initiated by TCR engagement can be potentiated by ICOS (44, 46, 47). Initially, TCR signals cause the phosphorylation of ITAMs in CD3 cytoplasmic domains by Src-family kinases which in turn leads to the recruitment of zeta-chain-associated protein kinase 70 (ZAP70) (48). Once activated, ZAP70 can phosphorylate linker for activated T cells (LAT) which then recruits phospholipase C-γ1 (PLC-γ1) to lipid rafts. At the plasma membrane, PLC-γ1 cleaves phosphoinositide-(4,5)-biphosphate (PIP2), causing the release of inositol-(1,4,5)-triphosphate (IP3) and diacyl glycerol (DAG). The increased IP3 concentration is sensed at the endoplasmic reticulum (ER) and causes the release of ER-stored calcium. This triggers a signaling cascade ending in the activation of transcription factors such as nuclear factor of activated T cells (NFAT). Importantly, ICOS can potentiate this process independently from PI3K signaling (46). Mechanistically, we found that ICOS can enhance the activation of PLC-γ1 (47). Moreover, we have shown that a Y170F point mutation in the membrane proximal KKKY motif found in the cytoplasmic tail of ICOS specifically leads to abrogated ICOS-assisted calcium signaling (47).

Like CD28, ICOS possesses a SH2-binding motif (Y^{181}MFM) within its cytoplasmic tail. Phosphorylation of this motif allows for the recruitment of PI3K regulatory subunits p50α and p85α (20, 49). It is likely that a Src family kinase is responsible for the phosphorylation of this tyrosine residue, but it has yet to be formally identified. Introducing a Y181F point mutation within this motif selectively inhibits the ICOS-PI3K pathway and leads to phenotypes reminiscent of ICOS knockout mice such as defects in GC reactions and Tfh cell differentiation (46). This is consistent with a previous study showing that a PI3K catalytic subunit preferentially expressed in leukocytes (p110δ) is required for GC formation (50).

Recently, a third ICOS cytoplasmic tail motif named IProx was described. It resembles TRAF family protein interaction motifs and was shown to mediate the

recruitment of TBK1 upon TCR and ICOS ligation (45). Mutations in the Iprox motif cause Tfh differentiation defects and impaired GC reactions, but the mechanism has not been clarified.

While most research has been focused on the cytoplasmic portion of ICOS, it was shown that the ICOS TM domain possesses signaling capacity (17). Specifically, the ICOS TM segment can recruit Lck which can in turn create a docking site for the p85α subunit of PI3K (44, 46). This implies that Lck could be able to phosphorylate the ICOS Tyr181 residue responsible for PI3K binding. Further, Lck is one of the Src-family kinases responsible for TCR phosphorylation early in the calcium signaling cascade. Replacing the ICOS TM segment with a CD44 TM segment abrogated ICOS-driven impacts on PI3K and calcium signaling (17). Taken together, these data suggest that ICOS possesses distinct signaling capacities requiring the combined action of its TM segment and its cytoplasmic tail.

Interestingly, it was shown that ICOS signaling can induce actin remodelling independently from TCR engagement, resulting in cell elongation and polarization (51, 52). This led to the suggestion that ICOS could act as an adhesion molecule. Further, *in vitro* experiments demonstrated that ICOS signaling can play a role in the transendothelial migration of activated Th1 cells (52). We found that ICOS engagement causes the activation of small GTPases known to regulate actin dynamics (RhoA and Cdc42) independently from TCR signaling (47). While the mechanism remains unclear, we also found that actin polymerization was required for ICOS-driven calcium flux potentiation. Thus, it appears that ICOS can modulate actin remodelling to support some of its other signaling pathways.

1.1.5 Distinctive features of ICOS and CD28 signaling

ICOS is a member of the CD28 family, and as such it presents functional overlaps with CD28 itself. For example, ICOS and CD28 can both enhance T cell proliferation and survival (3, 53-56). Interestingly, ICOS-driven proliferation appears to be IL-2 independent since this cytokine is not expressed in response to ICOS crosslinking (2, 11, 19). This serves as an example of diverging mechanisms between ICOS and CD28. Indeed, several pathways can be differentially regulated by ICOS, providing unique functions during immune responses.

Structurally, ICOS and CD28 both contain a PI3K binding motif in their cytoplasmic tail. However, studies have shown that ICOS can increase PI3K pathway activity to a higher degree than CD28 costimulation (43, 46, 49, 57). It was suggested that differences in the SH2-binding motif (YMFM for ICOS, YMNM for CD28) could explain this discrepancy (58). Further, ICOS preferentially binds to the p50α PI3K regulatory subunit instead of p85α (49). Interestingly, p50α possesses a more potent lipid kinase activity when compared to p85α which could explain how ICOS can mediate stronger PI3K signaling than CD28 (49). Functionally, CD28-PI3K activity was found to be of minimal importance during GC reactions and anti-bacterial responses (57, 59). Mice carrying a point mutation in the PI3K binding motif of CD28 presented largely intact T cell functions *in vivo,* although defects in CD28-induced Bcl-x_L expression and increased propensity to apoptosis were reported (57, 60). In contrast, abrogation of the ICOS-PI3K pathway causes Tfh differentiation defects, reduced autoimmune joint inflammation, acute graft-versus-host disease (GVHD), and impaired anti-tumor as well as anti-chlamydial T cell responses (46, 61-64).

The availability of CD28 and ICOS ligands also dictates separate functions depending on the context of the immune response. Unlike CD80 and CD86 whose expression is limited to APCs, ICOSL was found to be expressed on fibroblasts, endothelial and epithelial cells, muscle fibers and podocytes in certain inflammatory settings (6, 30, 32-34, 52, 65). Therefore, ICOS costimulation can act non-redundantly to CD28 even when utilizing similar signaling pathways. Of note, ILCs express ICOS and

ICOSL but not CD28 or its ligands (24, 66). While the extent of the role of ILCs remains ill defined, they are known to impact other innate and adaptive immune cells. Thus, ICOS can act outside of the realm of T cell costimulation in inflammatory or infectious settings (24, 66-68).

1.2 The germinal center reaction

1.2.1 Overview

Germinal centers are multicellular structures found within the B cell follicles of secondary lymphoid organs such as the spleen and lymph nodes (LNs) (69-71). Typically, GCs will form in response to an immune challenge such as immunization or infection and are normally absent from spleens and LNs under steady state conditions (70). However, some specialized lymphoid organs such as Peyer's patches (PPs) found in the intestinal tract have continuous GC reactions (72). This is presumably due to ongoing responses caused by the gut microbiota. Structurally, GCs develop within B cell follicles and can be subdivided into a dark zone (DZ) and a light zone (LZ) (69, 73). Upon antigenic stimulation, GCs will start to form in about 4-6 days and peak at 10-14 days (69, 71). The main purpose of GCs is to allow the expansion and selection of B cells followed by their differentiation into either antibody-secreting plasma cells or memory B cells. Therefore, successful GC reactions will lead to the production of high-affinity class switched antibodies along with the establishment of long-lasting immunity. In the next sections, the dynamic stages leading to GC reactions and the various cellular components involved will be discussed.

1.2.2 Dynamics

The process underlying GC reactions can be subdivided as follows: 1) Priming, 2) T-B contact, 3) Clonal expansion and somatic hypermutation (SHM), 4) Clonal selection, 5) GC B cell differentiation (Figure 1.2).

The first step leading to GC reactions requires priming of T and B cells. Initially, B cells will be activated by binding either soluble antigens or those presented by APCs such as dendritic cells or macrophages (74). This activation will trigger the upregulation of

homing receptors such as C-C chemokine receptor type 7 (CCR7) and Epstein-Barr virus-induced G-protein coupled receptor 2 (EBI2), allowing their migration towards the T cell zone within secondary lymphoid organs (75, 76). Naïve CD4⁺ T cells also need to be primed by APCs in the T cell zone to eventually become Tfh cells (77). As it is of central importance to this thesis, the differentiation process of Tfh cells will be further discussed in section 1.3.

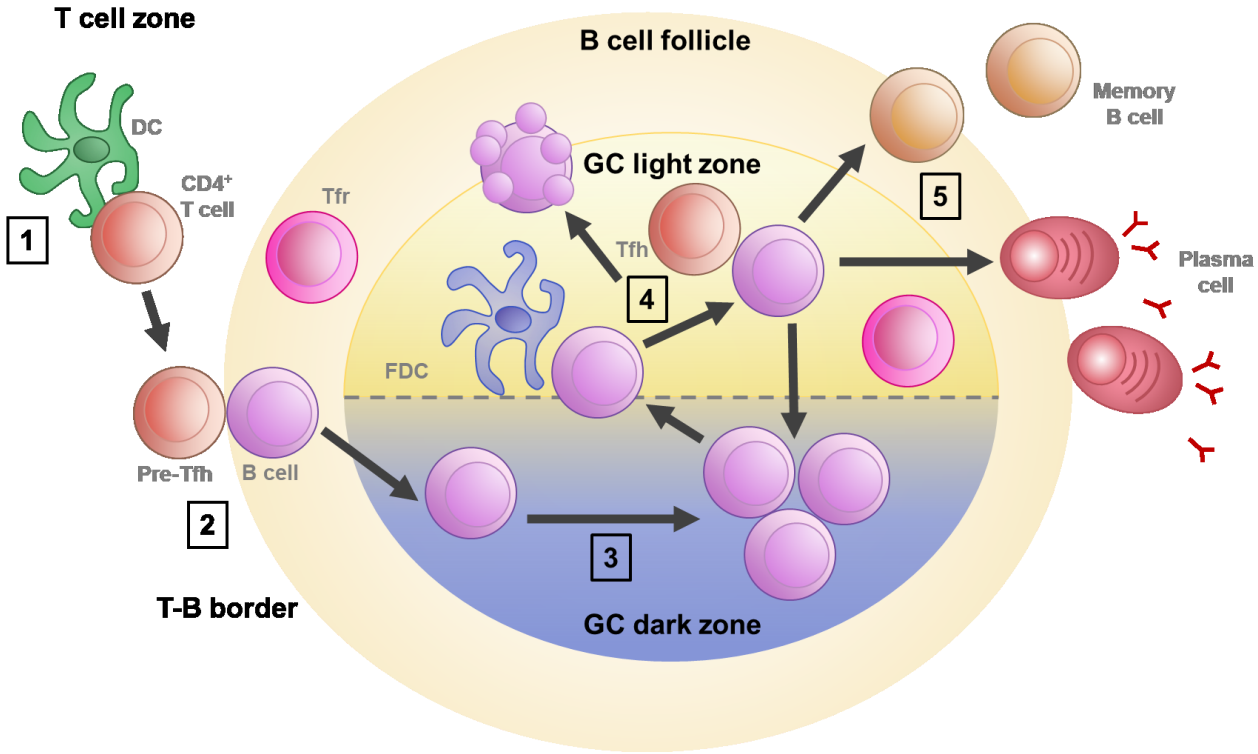

Figure 1.2 The germinal center reaction

1) Priming of naïve CD4+ T cells by DCs in the T cell zone. 2) Migration of pre-Tfh cells to the T-B border and contact with cognate B cells. T follicular regulatory cells can be found at the T-B border and in the GC, providing control over GC reactions. 3) Entry of activated B cells (centroblasts) in the DZ followed by clonal expansion and somatic hypermutation. 4) Migration of GC B cells (centrocytes) into the LZ followed by clonal selection mediated by survival signals from follicular dendritic cells (FDCs) and Tfh cells. Lower affinity GC B cells can migrate back into the DZ to undergo additional rounds of somatic hypermutation. The lowest affinity B cells undergo apoptosis. 5) Differentiation of GC B cells into either memory B cells or antibody-secreting plasma cells

Once activated, $CD4^+$ T cells transiently upregulate the Tfh master transcription factor B cell lymphoma 6 (Bcl6) (78). The C-X-C chemokine receptor type 5 (CXCR5) will also be upregulated, allowing these now pre-Tfh cells to migrate towards the T-B border where cognate interactions with activated B cells can occur (77, 79). At the T-B border, pre-Tfh cells undergo interactions with either bystander B cells or comparatively rare cognate B cells. During this phase, ICOS costimulation plays a critical role in keeping pre-Tfh cells mobile so that they can find the highest affinity B cells (80). Pre-Tfh cells provide signals to their cognate B cells, inducing their return to the B cell follicle to seed a GC (76, 81). Some B cells will instead migrate out of the follicle and differentiate into short-lived plasma cells (82). While they can only produce low-affinity antibodies, these plasma cells bypass the GC and therefore permit an early immune response. B cell engagement causes a second wave of Bcl6 expression in pre-Tfh cells, further reinforcing their differentiation trajectory towards the Tfh lineage (78). Pre-Tfh cells must also downregulate CCR7 at this point to migrate towards the B cell follicle as this receptor would otherwise retain them within the T cell zone (79, 83).

The third stage is where GC reactions start in earnest. B cells begin to proliferate rapidly and form a dark cluster, thus giving its name to the DZ (70). These B cells are referred to as centroblasts and are kept within the DZ by expressing the chemokine receptor CXCR4 (73). At this point, centroblasts upregulate activation induced cytidine deaminase (AID), a key protein in the SHM process (84). By introducing point mutations in the variable and switch regions of the B cell receptor, AID begins the process of antibody optimization and allows class switch recombination (CSR) to occur. The resulting GC B cells, now referred to as centrocytes, migrate towards the LZ to begin the first round of clonal selection (69). In the LZ, centrocytes compete for affinity-dependent survival signals from follicular dendritic cells (FDCs) and Tfh cells. Centrocytes are prone to apoptosis due to low Bcl2 expression, and those of the lowest affinity are therefore removed at this step due to lack of survival signals (85). Depending on their affinity, the remaining centrocytes will either migrate back into the DZ to undergo additional rounds of SHM or differentiate into plasma cells and memory B cells to mediate immune responses (86).

1.3 ICOS signaling in Tfh cells

1.3.1 Induction and maintenance of Tfh signature proteins

The differentiation process of Tfh cells starts with DC-mediated activation in the T cell zone and extends over several days (79). The first characteristic facilitating the transition of naïve $CD4^+$ T cells towards the Tfh lineage is high TCR signaling strength in response to antigens (87). In mice, cytokines including IL-6 and IL-21 must also be provided at this step. Eliminating either of these cytokines individually does not inhibit Tfh differentiation but removing both results in reduced Tfh populations (88). Downstream of IL-6 and IL-21, STAT3 signalling was found to be required for Tfh differentiation (89). Thus, it is likely that these cytokines act in a redundant manner to activate STAT3. In humans, other cytokines including TGF-β, IL-12 and IL-23 appear to be required instead (79). Costimulation through CD28 is also provided to T cells by DCs via CD80/CD86 binding and was found to be critically important for Tfh differentiation (90).

Tfh cells depend on PI3K signaling at multiple stages of their development. Indeed, T cell-specific removal of the PI3K subunit p110δ leads to a lack of Tfh cells (91). Conversely, p110δ activating mutations or enhanced PI3K signaling caused by PTEN deficiency led to elevated Tfh populations (91, 92). As previously discussed, ICOS is one of the most potent costimulatory receptors for the activation of PI3K signaling. Congruently, ICOS-PI3K signaling was shown to be required for Tfh cells since its abrogation by the $ICOS^{Y181F}$ mutation leads to loss of Tfh populations (46). Experiments have shown that ICOS can enhance IL-21 mRNA expression at least in part through the PI3K pathway, suggesting an early ICOS-dependent mechanism in pre-Tfh generation (46, 89, 91). Later during Tfh differentiation, ICOS-PI3K signaling can modulate Bcl6 and Krüppel like factor 2 (KLF2) which are two transcriptional regulators capable of controlling Tfh signature gene expression (Figure 1.3). Downstream of ICOS-PI3K, Akt can phosphorylate Forkhead box protein O1 (FOXO1) thereby preventing its nuclear import (93, 94). This mechanism allows ICOS to promote Tfh identity since nuclear FOXO1 would otherwise decrease Bcl6 mRNA. In addition, ICOS can prevent the ubiquitin-

dependent degradation of Bcl6 (95). Specifically, ICOS promotes the formation of complexes between the PI3K regulatory subunit p85α and intracellular osteopontin (OPN-i). These complexes then migrate to the nucleus and inhibit Bcl6 degradation. Interestingly, OPN-deficient T cell can still produce pre-Tfh cells upon immunization. However, Bcl6 expression was not sustained in these mice and full Tfh differentiation did not occur (95).

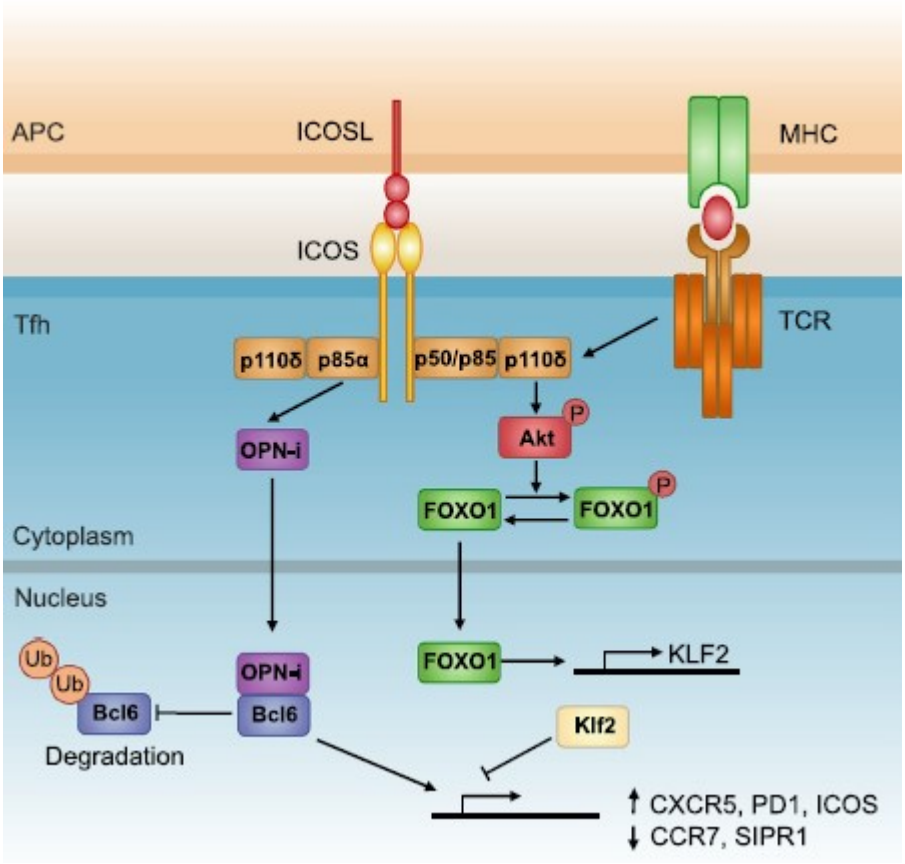

Figure 1.3 ICOS signaling pathways mediating Tfh differentiation

ICOS-mediated recruitment of the PI3K p85α subunit has a dual role in transcriptional regulation of genes involved in Tfh cell differentiation. On one hand, p85α forms a complex with intracellular osteopontin (OPN-i) which migrates into the nucleus and protects Bcl6 from ubiquitin-dependent degradation. On the other hand, activation of Akt through PI3K inhibits FOXO1 function through phosphorylation-mediated cytosolic retention. This timely inhibition of FOXO1 upregulates Bcl6 and downregulates KLF2, promoting expression of Tfh signature genes involved in Tfh migration and function.

ICOS-mediated inhibition of KLF2 expression is another key aspect in the maintenance of Tfh identity (96, 97). Indeed, ICOS blockade results in KLF2 upregulation and subsequent egress of Tfh cells from GCs due to increased expression of homing receptors CCR7 and sphingosine-1-phosphate receptor 1 (S1PR1) (96). Further, KLF2 upregulation triggers a reversion to non-Tfh phenotypes due to increased expression of non-Tfh transcription factors including B lymphocyte-induced maturation protein-1 (BLIMP-1), T-box expressed in T cells (T-bet), and GATA3 (97). Mechanistically, it is likely that ICOS regulates KLF2 through FOXO1 since the latter is a known positive regulator of KLF2 (93).

While ICOS is known to be indispensable for sustained Bcl6 expression, it is likely not required for early Bcl6 induction. ICOS-deficient T cells were shown to yield $Bcl6^+$ pre-Tfh cells upon immunization, although these were not able to fully differentiate into mature Tfh cells (96). Also, ICOSL-deficient dendritic cells loaded with OVA_{323} peptide were able to induce the differentiation of OT-II $CD4^+$ T cells into $Bcl6^+$ pre-Tfh cells (80). These data seemingly contradict previous work by Choi *et al.* showing that ICOS is required for the differentiation of $CXCR5^+Bcl6^+$ cells within 2-3 days upon viral infection (98). This discrepancy was addressed by A. Hutloff and colleagues who resolved that ICOS was dispensable for Bcl6 expression within the first two days following immune challenge (96). Indeed, the flow cytometry gating of CXCR5+Bcl6+ double positive cells likely precluded the study of early Bcl6 expression since CXCR5 is induced at a later stage. Therefore, ICOS seems dispensable for Bcl6 expression at least until CXCR5 is expressed. However, it is possible that the use of TCR transgenic mice and protein immunization versus viral infection models introduced differences in Tfh differentiation kinetics. On the other hand, CD28 was shown to be indispensable for the differentiation of Bcl6+ pre-Tfh cells (96). Thus, it appears that the main role of ICOS lies in maintaining the Tfh phenotype rather than early Bcl6 induction.

1.3.2 TCR-independent ICOS signaling at the T-B border

Upon DC-mediated activation, pre-Tfh cells begin to express low levels of Bcl6, CXCR5, PD-1 and ICOS (78, 99, 100). Due to combined expression of homing receptors CXCR5 and CCR7, pre-Tfh cells are guided towards the T-B border. It is at this location that pre-Tfh encounter their cognate B cells, a key event required for full commitment to the Tfh lineage (100). However, pre-Tfh cells will mostly encounter bystander B cells unable to provide the required signals for their further differentiation. Thus, pre-Tfh cells

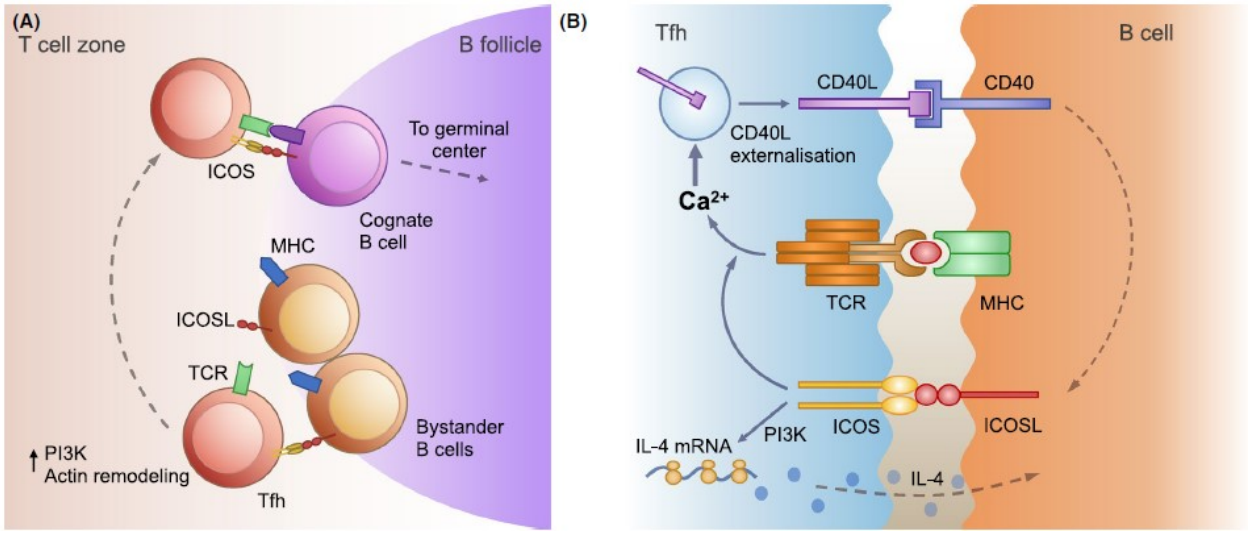

Figure 1.4 ICOS signaling in Tfh migration and T-B collaboration
(A) Bystander B cells expressing ICOSL promote T-cell motility at the T-B border allowing cognate T-B conjugation and GC entry. Ligation of ICOS without TCR engagement induces transient actin remodeling supporting persistent T cell movement, which increases the chance of Tfh cells finding cognate B cell clones. This process is dependent on the ICOS-PI3K pathway and presumably activation of small GTPases directly and/or indirectly by ICOS. (B) In the context of TCR engagement, ICOS ligation allows focused delivery of T cell “help” to cognate GC B cells within the GC. The ICOS-PI3K-mTOR pathway has been shown to increase translation of IL-4 mRNA by promoting polysome formation. In parallel, ICOS-mediated calcium signaling facilitates CD40L externalisation providing CD40 costimulation to GC B cells. In turn, CD40 signaling increases ICOSL expression level. This feed-forward loop may ensure acute localized delivery of CD40L and IL-4 which are key Tfh effector molecules, thereby promoting selection of high affinity GC B cell clones.

must be able to remain mobile at the T-B border to facilitate their pairing with cognate B cells. Interestingly, ICOS-ICOSL interactions between T and B cells were shown to promote the formation of pseudopods along with continued migration of pre-Tfh cells at the T-B border (Figure 1.4 A) (80). Moreover, MHC class II-deficient B cells were able to promote pre-Tfh motility, indicating that cognate interactions are not required for this ICOS-dependent mechanism (80). Importantly, Xu *et al.* demonstrated that the impact of ICOS on motility was PI3K-dependent since pharmacological inhibition of p110δ blocked T cell migration in lipid bilayers. Additionally, we have demonstrated that ICOS can activate small GTPases able to regulate actin dynamics independently of TCR engagement, providing another mechanism for bystander B cells to promote T cell migration (47).

1.3.3 ICOS signaling within the GC

Within the GC, B cells must acquire help from Tfh cells to differentiate into plasma cells. While high affinity interactions between TCRs and peptide MHC-complexes allow pairing of T and B cells, they are usually of short duration (~20 minutes) and likely would not allow enough time for T cells to express effector “helper” signals required by themselves (101). Tfh cells can overcome this issue by pre-emptively preparing mediators of B cell help. For example, CD40L proteins and IL-4 mRNA are presynthesized and kept within Tfh cells until required (101, 102). Interestingly, ICOS-ICOSL interactions were found to be one of the key signals controlling the release of these helper molecules (Figure 1.4 B).

Firstly, imaging studies have shown that ICOSL-expressing GC B cells pair with Tfh cells in a more “entangled” manner characterized by an increased surface area of contact (101). These ICOS-mediated interactions trigger spikes of intracellular calcium in Tfh cells. In turn, increased calcium flux causes the exocytosis of vesicles containing CD40L which results in CD40 stimulation on GC B cells (101). Interestingly, CD40

engagement causes increased ICOSL expression by GC B cells. Thus, an ICOS-dependent “feed-forward” mechanism is established and leads to the promotion of plasma cell differentiation.

Secondly, ICOS-PI3K signaling was shown to promote polysome formation (103). Downstream effects of PI3K-Akt and mammalian target of rapamycin (mTOR) signaling lead to 4E binding proteins (4E-BPs) inactivation by phosphorylation (104). Because 4E-BPs normally inhibit eukaryotic translation initiation factor 4E (eIF4E), a net effect of ICOS costimulation is enhanced translation. Specifically, *in vitro* experiments have shown that ICOS-PI3K signaling can increase IL-4 mRNA translation in T cells, thus providing an additional ICOS-dependent B cell help mechanism during GC reactions (103).

1.4 ICOS signaling in non-Tfh effector T cells

1.4.1 Th1, Th2, and Th17 cells

While ICOS signaling is often associated to GC reactions and Tfh cells, some data highlight the importance of ICOS in other effector T cells subsets. For example, ICOS was shown to promote the expression of Th1 and Th2 cytokines including IFN-γ, TNF-α, IL-4, IL-5, and IL-10 (3, 11, 19, 105). Early work has found increased ICOS expression in Th2 cells when compared to Th1 cells (20, 42). Further, interfering with ICOS-ICOSL interactions was shown to cause specific Th2 differentiation defects (106). Mechanistically, ICOS was found to promote the expression of the transcription factor c-Maf along with IL-4 and its receptor, key factors in Th2 differentiation (107, 108). Consequently, ablation of ICOS signaling by genetic deletion or antibody blockade was shown to cause Th2 response defects and impaired pathogen clearance along with reduced severity of Th2-mediated airway inflammation (109-111). Thus, ICOS signaling is a critical component of Th2 differentiation and function.

The impact of ICOS signaling on Th1 cells is not as clear. Firstly, Th1 cells can differentiate in the absence of ICOS signaling (112). Secondly, the effects of ICOS ablation on Th1 function vary depending on the experimental model chosen. For example, ICOS-deficient mice infected with Th1-inducing pathogens such as *Salmonella enterica* or *Listeria monocytogenes* showed reduced Th1 function and increased bacterial load (113, 114). On the other hand, ICOS-deficient mice infected with *Mycobacterium tuberculosis* or ICOSL-deficient mice infected with *Chlamydia muridarum* displayed enhanced Th1 responses (115, 116). However, the bacterial burden was shown to be increased in both of these settings. These discrepancies could be explained by the impact of ICOS deficiency in other T cell subsets.

In the case of *C. muridarum*, Th17 cells are required for proper anti-pathogen response. Interestingly, absence of ICOS-PI3K signaling was shown to cause impaired Th17 responses (64). Congruently, ICOS blockade performed in the context of a Th17-dependent disease model (experimental autoimmune encephalomyelitis, EAE) led to reduced severity (117). Mechanistically, ICOS was shown to promote IL-23-mediated expansion of Th17 cells (64, 118). In sum, ICOS signaling plays varied roles in T helper cell subsets which cannot be fully disentangled. Additional studies with more targeted approaches would be required to understand the specific impacts of ICOS functions in this regard.

1.4.2 $CD8^+$ T cells

Relatively little is known about the role of ICOS in $CD8^+$ cytotoxic T cells (CTLs) since most studies have focused on $CD4^+$ T cells. An early study showed that ICOS blockade causes impaired CTL responses against *L. monocytogenes* (114)**.** Additionally, ICOS-deficient mice displayed poor bacterial clearance in a *S. enterica* infection model that could be at least in part attributed to CTL defects (113). However, a previous study showed that ICOS-deficient mice could still produce influenza-specific $CD8^+$ T cells (119). Antibody-mediated ICOSL blockade in bacterial and viral infection models also resulted in compromised CTL responses with reduced granzyme expression and diversion from

cytotoxic to memory-like phenotypes (120). Thus, these findings suggest that ICOS can support CTL functions but could be dispensable for their early expansion.

The role of ICOS in CTLs was also explored in the field of anti-tumor immunity. Studies have shown that the induction of ICOSL expression by tumor cells enhanced CTL-dependent tumor rejection (121, 122). Further, ICOS appears to be more efficient at improving recall responses compared to initial T cell priming by the tumor. Indeed, adoptive transfer of anti-tumor CTLs caused rapid rejection of ICOSL-expressing tumors and improved CTL cytokine production when compared to naïve $CD8^+$ T cells exposed to similar tumors (122).

As previously mentioned, ICOS-PI3K signaling in $CD4^+$ T cells was linked to GVHD induction (62). $CD8^+$ T cells are also known to be mediators of GVHD (123). Interestingly, it was shown that ICOS can act in a PI3K-independent manner in CD8+ T cells to promote GVHD initiation (62). Thus, this suggest that other ICOS-dependent pathways such as calcium signaling could play a role in $CD8^+$ T cells in specific settings.

1.5 The role of ICOS in Treg cells

1.5.1 Overview of Treg cells

Regulatory T cells are a subset of $CD4^+$ T cells that can modulate the immune system to prevent autoimmunity by exerting suppressive effects on effector T cells. In the 1990s, expression of the IL-2 receptor α chain (IL2rα, CD25) by $CD4^+$ T cells was found to be a reliable marker for this subset in mice (124). Similar regulatory T cells were then found in humans by several groups in the early 2000s (125-131). A few years later, $CD4^+CD25^+$ T cells in mice and humans were found to share the expression of transcription factor forkhead box P3 (Foxp3) which became the main defining marker of regulatory T cells from that point on (132-134). Further, *Foxp3* genetic mutations were found to cause severe autoimmunity that could be attributed to Treg dysfunction. For

example, Scurfy mice carrying a X-linked frameshift *Foxp3* mutation spontaneously develop a fatal lymphoproliferative disease characterized by inflammatory multi-organ failure (135). Thus, $CD25^{+}Foxp3^{+}$ Treg cells were established as gatekeepers preventing self-reactivity in mice. In humans, *Foxp3* loss-of-function mutations were associated with immune dysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome caused by Treg deficiency or functional impairments (136). However, the use of CD25 and Foxp3 as Treg markers was found to be insufficient in humans since those proteins are not exclusive to human Tregs. Additional studies identified other potential human Treg markers such as the methylation status of the Treg-specific demethylated region (TSDR) or absence of the IL-7 receptor α-chain (CD127) (137, 138).

Tregs were found to arise primarily from the thymus as a distinct T cell subset, although studies have shown that conventional T cells can gain Foxp3 expression in peripheral sites to become peripheral Tregs (pTregs) (139, 140). Natural/thymic Tregs (tTregs) are positively selected in the thymus before their initial commitment to the Treg lineage. Interestingly, the affinities of tTreg TCRs are skewed towards self-peptide/MHC which would normally halt their progression during the negative selection stage. However, studies have shown that T cells with moderate TCR affinity towards self antigens are driven to the Treg lineage while T cells with high affinity towards self-peptides are removed (141). In contrast, pTreg differentiation is likely triggered by non-self antigens such as those provided by food and microbiota (142). This suggests different purposes for tTregs and pTregs. While tTregs were established as regulators of autoimmunity, pTregs could instead serve to maintain tolerance towards commensal or environmental antigens.

1.5.2 Treg functional mechanisms

The ability of Tregs to suppress other immune cells has long been recognized. However, the process through which they fulfill this function remains only partly understood. Generally, Tregs are thought to mediate suppression either through cell-cell

contact or by secreting cytokines and enzymes, although other mechanisms including cytokine deprivation and metabolic disruption have been described (143).

Firstly, the coinhibitory molecule CTLA-4 is expressed by Tregs and has long been suggested as a central component of Treg-mediated suppression (144, 145). Indeed, CTLA-4-deficient mice displayed spontaneous autoimmunity that can be treated by Treg transfer, and CTLA-4 blockade in a murine colitis model resulted in increased disease severity due to Treg defects (144-146). Functionally, Tregs were shown to reduce the expression of CD80 and CD86 by APCs in a CTLA-4-dependent manner (147-149). The exact mechanism was elucidated by Qureshi *et al.* who demonstrated that CTLA-4 on Tregs allows the capture of CD80/CD86 through a process called trans-endocytosis, thereby impairing CD28 costimulation (150). However, previous studies had shown that CTLA-4-deficient Tregs still possessed suppressive abilities which suggested compensatory or complementary suppression mechanisms (151, 152).

The second suppression mechanism employed by Tregs is immunosuppressive cytokine production. While research in this field is ongoing, IL-10 and TGF-β have been identified as potential mediators of Treg suppression. Blocking IL-10 or using IL-10-deficient Tregs was shown to negatively impact the ability of Tregs to supress T cell transfer-induced colitis (153). Further, IL-10 was found to be important for Treg function *in vivo* in an EAE autoimmune disease model (154). However, some data suggests that IL-10 production by Tregs is not always required. For example, allergic airway hyper-reactivity was still suppressed after the transfer of IL-10-deficient Tregs (155). Interestingly, overall IL-10 production was still high in this experiment which suggests that other IL-10 sources can compensate. The requirement of Treg-derived TGF-β also appears to be context dependent. Indeed, studies have shown that Tregs do not require TGF-β to suppress T cell proliferation *in vitro* (156, 157). However, *in vivo* experiments have demonstrated that Treg-derived TGF-β is required to prevent colitis (144, 158). Aside from potentially influencing suppressive ability, TGF-β was found to be required to maintain peripheral Treg numbers (159). This could explain discrepancies between *in vitro* and *in vivo* experiments. Other Treg-derived cytokines such as IL-35 have also been shown to affect suppressive ability, although the mechanisms remain to be clarified (160).

Lastly, Tregs were found to use other suppression mechanisms in certain settings. For example, Tregs were shown to express granzyme B which they can use to kill B cells, NK cells and CTLs by cytolysis (161-163). This cytolytic ability appears to differ from that of CTLs since it is partially perforin independent. Since Tregs express high levels of CD25, studies have suggested that they could deprive other immune cells of IL-2 by consuming it in large amounts (164, 165). Although the relative importance of this mechanism is still subject to debate, a study has shown that Treg-mediated IL-2 deprivation can lead to effector T cell apoptosis *in vitro* and *in vivo* (166). However, other groups did not observe significant induction of cell death by Tregs (157, 167, 168). Thus, it appears that Tregs employ multiple pathways to suppress immune cells which can differ depending on the context.

1.5.3 ICOS signaling in Treg cells

While ICOS has mostly been studied in the context of conventional T cells, several lines of evidence suggest that ICOS signaling is also important for Treg cells. Studies have shown that ICOS-deficient mice have reduced numbers of $Foxp3^+$ Tregs (169, 170). Further, ICOS blockade performed in T-B co-culture experiments have resulted in lowered Foxp3 expression (171). Mechanistically, ICOS deficiency was shown to cause increased methylation of Foxp3 conserved non-coding DNA sequence 2 (CNS2), thereby destabilizing Foxp3 and possibly causing reduced Treg numbers (172). However, this mechanism might be more relevant in the periphery since ICOS-deficient mice displayed normal amounts of $Foxp3^+$ cells in the thymus (170).

Aside from promoting Foxp3 stability, ICOS was linked to increased Treg survival. Indeed, $ICOS^+$ Tregs stimulated with α-CD3 *in vitro* survived longer than their $ICOS^-$ counterparts (173). Regulatory T cells are known to express ICOS at varying levels depending on the subset. Specifically, studies have shown that $CCR7^{lo}CD44^{hi}CD62L^{lo}$ effector Tregs (eTregs) express ICOS to a higher level than resting/central Tregs (cTregs) (174, 175). Interestingly, ICOS blockade resulted in a preferential loss of eTregs which indicates that ICOS signaling could be required to prevent eTreg apoptosis (55, 174). The

survival of Treg cells can also be influenced by IL-2 signaling. Indeed, IL-2 blockade was shown to cause a specific loss of cTregs without affecting eTregs (174). The expression of CD25 was found to be lowered in eTregs, possibly due to a lack of IL-2 sources in their environment (174). Taken together, these data suggest that eTregs switch from IL-2 to ICOS signaling to promote their survival.

The suppressive ability of Tregs was also linked to ICOS in some settings. For example, impaired ICOS signaling caused by blockade or genetic deletion abrogated the development of respiratory tolerance in an airway inflammation model due to low Treg numbers and lack of Treg-derived IL-10 (169). Further, unlike WT Tregs, transfer of ICOS-deficient Tregs did not ameliorate airway inflammation. Also, abrogation of ICOS signaling in β islet-specific TCR transgenic (BDC2.5-NOD) mice resulted in the exacerbation of a type I diabetes (T1D)-like disease linked to Treg defects (56). The impact of ICOS signaling in various autoimmune diseases will be further discussed in chapter 1.7. The exact mechanism through which ICOS can promote Treg function has yet to be elucidated. Several studies have highlighted positive correlations between ICOS and IL-10 levels in Tregs, but some have observed unchanged IL-10 production in ICOS-deficient mice (172, 176-178). Thus, additional research will be required to understand ICOS-dependent mechanisms impacting Treg function.

Among ICOS-dependent pathways, PI3K signaling has been implicated in Treg function. Firstly, the PI3K catalytic subunit p110δ was shown to be critical for Treg cell survival and function (179). Secondly, steady-state Tregs maintain high levels of phosphorylated 4E-BP1 and S6, signs of active PI3K-mTORC1 signaling (180). Congruently, Treg-specific ablation of Raptor (part of mTORC1) resulted in Treg functional defects and fatal multi-organ autoimmune inflammation (180). Interestingly, Raptor-deficient Tregs presented decreased ICOS expression, suggesting a positive feedback loop involving ICOS-PI3K-mTORC1 signaling. Third, Treg-specific ablation of PTEN (PI3K antagonist) led to Treg instability and the development of a lymphoproliferative disease characterized by dysregulated Th1 and Tfh responses (181, 182). Thus, Treg function requires precise control of PI3K activity which can be mediated through ICOS signaling.

1.6 The role of ICOS in Tfr cells

1.6.1 Overview of Tfr cell differentiation

In 2011, three groups described a $CD4^+Foxp3^+$ regulatory T cell subset with characteristics reminiscent of Tfh cells such as expression of CXCR5, PD-1, Bcl6, and ICOS (183-185). Henceforth named T follicular regulatory cells, this subset was found to localize in and around GCs and was therefore hypothesized to play regulatory roles during GC reactions (186). No Tfr lineage-defining factor has been identified to date, although they require concomitant expression of Foxp3 and Bcl6 (187, 188). Loss of Foxp3 expression by Tfrs leads to an altered profile with increased Tfh features while forced Foxp3 expression can turn Tfh cells into Tfr-like cells (187). These data suggest that Tfh cells could act as Tfr precursors *in vivo*. Indeed, strong IL-2 signaling was found to inhibit Tfr differentiation which is a mechanism more comparable to Tfh cells than Tregs (189). However, most research has shown that Tfr cells differentiate from thymic Tregs following interactions with dendritic cells (183-185, 190). Further, Tfr cells were found to display a TCR repertoire generally skewed towards self-antigens, reinforcing their pedigree as regulatory T cells (191). Of note, induced Tregs were shown to possess the ability to differentiate into Tfrs specific for the immunizing antigen under specific circumstances (192).

Following initial priming, Tfr cells must express CXCR5 to migrate towards GCs (183-185). Interestingly, the transcription factor NFAT2 was found to be uniquely required for Tfr cells to upregulate CXCR5 (193). Also, recent work has identified Tfh-derived sclerostin domain-containing protein 1 (SOSTDC1) as an additional Tfr differentiation cue which was shown to work by inhibiting the β-catenin pathway in Tfrs (194). During their differentiation, some Tfr cells were shown to downregulate CD25 (189, 195, 196). This finding has been a subject of debate in the literature with early studies describing Tfrs as solely $CD25^+$ and later studies suggesting that $CD25^-$ Tfrs could be regarded as “ex-Tfr” cells (183-185, 187). The availability of IL-2 at different stages of infection and in different organs was proposed as a regulator of CD25 expression levels in Tfrs (189, 195). Also, recent work has shown that $CD25^-$ Tfrs possess reduced but not absent regulatory

features and preferentially localize within the GC, suggesting that CD25 downregulation could be a necessary adaption for Tfrs to access the GC (195).

1.6.2 Tfr depletion models

The study of Tfr cells has been complicated by the lack of unique markers required for targeted experiments. Some early studies reported attempts to adoptively transfer Tfr cells into lymphopenic mice to evaluate their suppressive abilities (185, 197). Results from these *in vivo* experiments supported *in vitro* findings which showed that Tfr cells could suppress T cell proliferation and antibody production (190, 197). However, this approach does not achieve a physiological reconstitution of Tfr populations and is limited by the loss of transferred Tfr cells over time. In parallel, another group performed Tfr depletion using a *Sh2d1a* $(SAP)^{-/-}$:$Foxp3^{DTR}$ mixed bone marrow chimera which resulted in increased Tfh and GC B cell responses (183). More recent *in vivo* Tfr depletion models using Foxp3-cre systems now permit more targeted approaches less prone to off-target effects. The most common method relies on Foxp3-specific deletion of Bcl6 (Bcl6FC), a transcription factor required for follicular T cells (188, 189, 198-200). The overall impact of Tfr loss on GC reactions in Bcl6FC mice was found to be relatively modest with little to no change in Tfh and GC B cell populations (188, 199, 200). Despite this, the Bcl6FC system revealed two main functions for Tfr cells: prevention of autoantibody generation and modulation of antigen-specific antibody responses. Some limitations of the Bcl6FC model have been observed, including incomplete Tfr depletion and possible off-target effects on other regulatory T cell subsets (188, 199-201). To remedy this, Clement *et al.* developed a Tfr cell-deleter model where depletion requires co-expression of Foxp3 and CXCR5 (*Cxcr5*$^{IRES\text{-}LoxP\text{-}STOP\text{-}LoxP\text{-}DTR}$ *Foxp3*^{cre}) (202). This approach showed that Tfr cells could regulate early but not late antigen specific GC responses and prevent autoreactive IgG and IgE production. Thus, the prevailing paradigm now favors more nuanced roles for Tfr cells rather than broad suppression of GC responses.

1.6.3 ICOS signaling in Tfr cells

Initial studies describing Tfr cells reported high ICOS expression at the cell surface (183, 184). However, little is known about how ICOS might influence Tfr differentiation or function. Some reports have shown that ICOS is required for the formation of Tfr cells in the context of protein immunization, chronic GVHD, and infection (197, 203, 204). Since Tfr and Tfh cells share several features, it is likely that ICOS-dependent pathways known to be important for Tfh cells could act in Tfr cells as well. While no direct links between ICOS signaling mechanisms and Tfr differentiation or function have been described, some studies have shown evidence of PI3K and calcium signaling influencing Tfr cells. First, elevated PI3K-mTOR signaling found in Roquin-deficient Tregs correlated with the downregulation of CD25 and conversion of Tregs to Tfr cells (205). Second, the formation of complexes between PI3K regulatory subunit p85α and OPN-i was found to protect Bcl6 from degradation and supported Tfr differentiation in a cell-intrinsic manner (95). Third, the calcium pathway could be involved in CXCR5 upregulation by Tregs since the latter was shown to require calcium-dependent NFAT2 activity (193). Congruently, T cell-specific NFAT2 abrogation was found to cause a selective defect in Tfr differentiation coupled with increased severity of a lupus-like disease caused by chromatin immunization (193). Direct interference with calcium signaling caused by the abrogation of stromal interaction molecules 1 & 2 (STIM1/2) also led to reduced Tfr numbers and spontaneous autoantibody production (206). Thus, ICOS could support Tfr differentiation by propagating signals through PI3K and calcium pathways. Further, other ICOS-dependent mechanisms known to promote Tfh differentiation such as KLF2 downregulation could be required for Tfr cell differentiation (96).

1.7 ICOS signaling in autoimmune diseases

1.7.1 Overview of autoimmune diseases

The double-edged nature of the immune system and concepts of autoimmunity have been a key subject of study over the last 60 years. The first notion of immunological tolerance dates back to work by Nobel laureate Macfarlane Burnet in the 1940s. In following years, several studies reinforced the theory that the immune system possesses inherent mechanisms preventing it from targeting self antigens (207). Epidemiological studies have found that approximately 3-5% of the general population are affected by autoimmune diseases (208, 209). While there are disease-to-disease variations in affected age groups and geographical prevalence, women were consistently found to be more likely to develop an autoimmune disease for reasons which are still unclear. The pathogenicity of some diseases can be traced back to genetic factors such as mutations of autoimmune regulator (AIRE) in autoimmune polyendocrine syndrome type I (APS1) and Foxp3 mutations in IPEX syndrome (210, 211). However, most autoimmune diseases are thought to originate from combinations of genetic and environmental elements. Several genetic polymorphisms were identified as risks factors for developing autoimmunity, but the contribution of individual mutations is relatively low for most diseases (212). Further, monozygotic twins were found to share autoimmune disorders at a rate of only 12 to 67%, suggesting external causes of autoimmunity (213).

Amongst potential triggers of autoimmune diseases, infections have been the most studied (214). For example, Epstein-Barr virus (EBV) has been associated with systemic lupus erythematosus (SLE), Sjogren's syndrome (SS), rheumatoid arthritis (RA), multiple sclerosis (MS), and primary biliary cirrhosis (PBC) (215-217). Molecular mimicry and epitope spreading have been cited as possible mechanisms linking viral or bacterial infections to the pathogenesis of autoimmune diseases (218-220). More recently, research has focused on impacts of the microbiota in autoimmunity. Studies have provided evidence that T1D could be initiated due to dysregulated responses to commensal bacteria (221). Additionally, increased colonization by segmented

filamentous bacteria (SFBs) has been associated with autoantibody production (222). Some non-infectious environmental factors have been linked to autoimmune diseases. For instance, smoking has been shown to increase the risk of developing RA, SLE, SS and PBC (223-225). In sum, mechanisms leading to the development of autoimmune disease remain partially understood. This is due in part to the difficulty in noticing abnormal pre-symptomatic immune reactions. While animal models have brought some insights in this regard, more research is required to understand and perhaps prevent the instigation of autoimmunity.

Following the initiation phase, most autoimmune disorders are characterized by a propagation phase. Pathogenic imbalances of effector and regulatory T cells are often observed at this stage (226). Further, autoantibodies are found in multiple diseases during this phase and can be used for diagnosis (227). These autoantibodies can also cause tissue inflammation and destruction through processes such as complement activation and antibody-dependent cell-mediated cytotoxicity (ADCC) (228). In this inflammatory setting, cells start to produce additional inflammatory cytokines and dying cells can release new antigens, thereby causing amplified autoimmune reactions through epitope spreading (229). If left unchecked, this process can sometimes evolve into a relapsing/remitting cycle where pathogenic and regulatory cells struggle for balance (230). However, failure of regulatory mechanisms eventually leads to widespread and often irreversible tissue damage.

Most therapeutic approaches for autoimmune diseases have focused on the use of immunosuppressive drugs or biological agents which can inhibit inflammatory pathways. For example, anti-TNF-α drugs have been successfully used in the treatment of RA and SLE (231). However, anti-TNF therapies are contraindicated for MS as they have been found to increase disease severity and trigger a CNS demyelinating disease in patients with other autoimmune conditions (232, 233). Costimulation blockade using CTLA-4-Ig was shown to induce autoreactive T cell anergy and has found moderate success in the treatment of RA and T1D (234, 235). Adoptive transfer of Tregs was attempted but has met little success due in part to difficulties in isolating and expanding pure Treg populations without affecting their suppressive abilities (236, 237). Other

therapies including manipulation of IL-2 signaling and self-antigen desensitization have also undergone trials with mixed results (238, 239). Thus, current therapeutic options have met some success but are fraught with conflicting results, underlining the need for continued research in mechanisms regulating autoimmune disorders.

The next sections of this thesis will focus on specific autoimmune diseases in which the role of ICOS has been studied. Emphasis will be placed on RA since clinical evidence suggests that ICOS could be involved in its pathogenesis.

1.7.2 Rheumatoid arthritis general concepts

Rheumatoid arthritis is one of the most common autoimmune diseases and is estimated to affect 0.5-1% of the population (240). Women are 2 to 3 times more likely to develop RA, and the highest incidence is observed in the sixth decade (241). The main symptoms of RA are chronic joint inflammation and progressive destruction caused by immune cells infiltrating synovial tissues (242). This disease places considerable burden on patients and is estimated to increase the risk of premature mortality by 50% (243). The pathogenesis of RA is still incompletely understood, but it has been shown to involve components of innate and adaptive immunity (244).

The early stages of RA are often characterized by the appearance of anti-citrullinated peptide antibodies (ACPAs). These antibodies are made up of various isotypes (IgG, IgA, IgM) and can target a range of endogenous proteins including type II collagen and histones, but also foreign antigens such as EBV nuclear antigen 1 (EBNA-1) (245). This targeting is thought to be the result of protein citrullination, a posttranslational modification which can alter peptide immunogenicity (246). The appearance of ACPAs has been linked to a host of genetic factors including human leukocyte antigen (HLA) – DR1 and DR4 (247). Congruently, monozygotic twins were shown to co-develop RA at an incidence rate higher than that of the general population (248). Environmental risk factors such as smoking and infectious agents have also been linked to increased ACPA production (249-252). The involvement of ACPAs in the

pathogenesis of RA has been shown to function by binding of Fc receptors on myeloid cells and complement activation (253, 254). Of note, ACPA-negative forms of RA have been reported and were found to display novel and potentially pathogenic autoantibodies (255). Another common finding in RA patients is the presence of rheumatoid factor (RF), autoantibodies which can target and form complexes with the Fc portion of IgG (256). These complexes are found to accumulate in synovial tissues and cause completement activation, thus contributing to disease progression.

Although T and B cells have been observed to infiltrate synovial tissues in RA patients, the mechanisms responsible for their activation remain ill defined (257). One possibility is that T cells could react to citrullinated neoantigens in circulation. Also, dendritic cells were shown to accumulate in the joints of RA patients and are thought to serve as APCs in this setting (258). Once components of the innate and adaptive immune system have been activated, inflammatory cytokines including IL-1, TNF-α, IL-6, and IL-17A are produced in synovial tissues (242, 244). In turn, this promotes increased leukocyte activation but also cartilage damage due to chondrocyte apoptosis, as well as bone erosion caused by pro-osteoclastogenic effects of inflammatory cytokines (259). Of note, osteoclasts were also shown to be directly activated by ACPA binding (260). Thus, the pathogenesis of RA involves many aspects of the immune system which renders its study complicated but also offers multiple therapeutic opportunities.

Therapeutic approaches for RA involve the use of disease-modifying antirheumatic drugs (DMARDs) in combination with nonsteroidal anti-inflammatory drugs (NSAIDs) and corticosteroids. While the latter are generally intended as symptomatic treatments, DMARDs have the potential to halt the progression of RA. The initial DMARD given to RA patients is methotrexate (MTX), a folic acid analog with immunosuppressive properties. While the mechanism of action of MTX is not fully understood, it was found to effectively reduce RA severity in up to 70% of patients (261). However, discontinuation of MTX due to side effects or loss of efficacy over time has underlined the need for additional therapeutic options. Other DMARDs such as leflunomide, sulfasalazine and hydroxychloroquine have been used in patients who do not respond to MTX monotherapy. Akin to MTX, their mechanisms of action remain ill-defined and multiple

side effects have been reported (242). Thus, a second line of drugs referred to as biological DMARDs (BDMARDs) has been developed with the intent of taking more targeted approaches. As previously mentioned, inflammatory cytokines play key roles in the pathogenesis of RA and were therefore chosen as BDMARD targets. Several TNF-α, IL-1, and IL-6 inhibitors have been successfully used alone or in combination with MTX, but these treatments remain expensive and have multiple contraindications (262). Lastly, drugs causing B cell depletion (α-CD20) or T cell anergy (CTLA-4-Ig) have shown promise but lack specificity and therefore cause unintended side effects (242). Thus, recent advances in RA treatments have brought better prognoses for more patients, but additional studies are required to develop targeted therapies which can minimize side effects.

1.7.3 Murine models of rheumatoid arthritis

Studying mechanisms underpinning the pathogenesis of RA in humans is difficult since patients are usually diagnosed in late disease stages. Thus, several animal models replicating features of human RA have been developed.

Collagen-induced arthritis (CIA) is considered to be the gold standard of murine models of RA. This model was first developed in rats by Trentham *et al*. but was later adapted for use in mice (263, 264). Type II collagen (CII) is primarily found in the joints of adult rodents (265). Thus, by immunizing CIA-susceptible DBA/1 mice with an emulsion of bovine CII and an adjuvant, an autoimmune reaction closely resembling human RA is triggered. After immunization, autoantibodies targeting CII can be detected in CIA mice. These antibodies are mostly of the IgG2a/b isotypes and were shown to be highly arthritogenic (266). Interestingly, C57BL/6 mice were found to be deficient in anti-CII antibodies after immunization, potentially explaining their resistance to CIA induction (266). Susceptibility to CIA was also linked to specific MHC class II molecules including I-A^q which binds the same CII peptide region in mice as HLA-DR4 does in humans (267). Similarities with human RA such as the presence of RF and ACPAs were noted in CIA

mice (268). Further, polyarticular pathology characterized by immune cell infiltration of synovial tissues and bone destruction is a shared feature of human RA and CIA (268).

Several studies have supported the view that anti-CII autoantibodies able to fix complement are the main source of localized joint inflammation in CIA (269-272). This is well illustrated by a complementary RA model named collagen-antibody induced arthritis (CAIA). Indeed, injection of monoclonal anti-CII antibodies was found to be sufficient to induce arthritis in mice and has the advantage of functioning in CIA-resistant mice (273). However, CAIA is transient in nature which highlights the requirement of other components of the immune system for arthritis maintenance. Deficiency of recombination-activating gene 1 (RAG-1) in DBA/1 mice led to reduced CIA severity, supporting a role for components of the adaptive immune response in CIA maintenance (274). The weakened, but still present, arthritis observed in these T and B cell-deficient mice was attributed to the ability of CII to activate matrix metalloproteinases (274). Treatment with anti-CD4 antibodies led to reduced severity of established CIA and showed synergistic effects when used in combination with anti-TNFα/β antibodies (275). Also, Th1 and Th17 responses have been shown to be required for CIA induction and maintenance (276-278). Lastly, mice deficient in CD28, SLAM-associated protein (SAP) or CXCR5 could not produce anti-collagen antibodies and were all resistant to CIA induction (279-281). Thus, CIA involves components of humoral and cellular immunity, and recapitulates many aspects of human RA.

The K/BxN arthritis model was first described by the Mathis/Benoist group in 1996 (282). This model consists of a cross between TCR transgenic KRN mice with nonobese diabetic (NOD) mice. The K/BxN TCR is known to recognize glucose-6-phosphate isomerase (G6PI), a ubiquitously expressed glycolytic enzyme (283). Arthritis occurs spontaneously in K/BxN mice due to sustained production of arthritogenic anti-G6PI IgG1 antibodies (284). Interestingly, it was found that transfer of K/BxN serum to other mouse strains could cause a form of serum transfer arthritis (STA) (285). This form of arthritis was found to depend on the presence of neutrophils and macrophages, but not T and B cells (286-288). This method is useful since K/BxN STA can be used in mouse strains which are resistant to CIA, and has an induction rate of nearly 100% (289). Also, the

onset of K/BxN STA occurs within 2 days of serum transfer whereas CIA induction requires 3 to 5 weeks (289, 290). However, like CAIA, K/BxN STA is transient and only involves passive transfer of antibodies and therefore cannot simulate the priming phase of human RA. Further, while anti-CII antibodies were detected in human RA patients and CIA mice, anti-G6PI antibodies remain mostly unique to K/BxN mice (291). Nevertheless, this model remains a useful tool to study the effector phase of RA in mouse strains which are resistant to CIA.

Several genetic models of human RA have been developed in mice to identify genes which contribute to the pathogenesis of RA. These models are used less often due to their lack of adaptability and potential confounding factors when introducing additional genetic tools. BALB/c mice deficient in IL-1 receptor antagonist (IL-1RA$^{-/-}$) were found to spontaneously develop a T cell-dependent autoimmune arthritis with features of human RA such as the presence of RF and anti-CII antibodies (292, 293). Interestingly, this research has led to the approval of recombinant human IL-1RA to treat RA (294). A point mutation in the gene encoding ZAP-70 was also shown to cause a form of spontaneous T-cell mediated autoimmune arthritis (SKG mice) (295). Lastly, TNFΔ^{ARE} mice display an elevated level of TNF-α and spontaneously develop arthritis along with inflammation of the small intestine and growth defects (296). This model was particularly useful to study the effects of TNF-α inhibitors which are now used as BDMARDS to treat human RA.

1.7.4 Impact of ICOS in rheumatoid arthritis

Since ICOS is involved in class-switched antibody production and effector T cell function, it is likely to play a role in the pathogenesis of RA. Indeed, a study has shown that antibody mediated ICOS blockade reduced the severity of CIA in mice (297). Additionally, ICOS blockade resulted in significantly reduced titers of anti-CII antibodies and inflammatory cytokines (297). However, blockade was performed during the priming phase of CIA, and therefore these experiments cannot discern whether ICOS is required for CIA maintenance. Further, the role of signaling pathways downstream of ICOS were not studied. The impact of ICOS-PI3K signaling in RA is worth testing since PI3K inhibition

is known to reduce disease severity in murine models of RA (298). Specifically, p110γ inhibition resulted in the amelioration of CIA, CAIA, and K/BxN STA (299, 300). Also, p110δ inhibition or genetic deletion was found to reduce arthritis symptoms in K/BxN STA (299). Finally, combined abrogation of p110γ/δ led to an even greater amelioration of arthritis in this model.

In humans, polymorphisms in the *Icos* gene were found to be associated with RA (301). Interestingly, the amount of ICOS-positive T cells in the blood and synovial fluid of RA patients was found to be elevated when compared with healthy controls (302). Further, ICOSL was detected in the joints of RA patients (302). Taken together, these findings strongly suggest that ICOS signaling is involved in human RA and that it could be a therapeutic target.

1.7.5 ICOS signaling in systemic lupus erythematosus

Systemic lupus erythematosus is an autoimmune disease characterized by multiorgan inflammation caused by the deposition of immune complexes and subsequent activation of components of innate and adaptive immune components (303). Patients can present with symptoms involving almost any system which leads to a substantial degree of misdiagnosis. Several autoantibodies have been associated with SLE including primarily a host of anti-nuclear autoantibodies (ANAs) (304). Some degree of genetic predisposition for SLE has been found, but this disease is mostly thought to be caused by a combination of genetic, hormonal, and environmental factors. Like many autoimmune disorders, SLE primarily affects women and has a 9:1 female-to-male ratio (305). Therapeutic options for SLE are similar to those used in RA and include NSAIDs along with several immunosuppressive drugs (303). One of the most common murine models used to study SLE are lupus-prone MRL/*lpr* mice (306). These mice spontaneously develop a SLE-like pathology including autoantibody production, lymphadenopathy, and renal disease due to mutations in the *Fas* gene and other factors in the MRL genetic background. Other mouse strains such as NZB/W F1 and NZM and mice homozygous for the *sanroque* mutation have also been found to spontaneously

develop a lupus-like disease (25, 307, 308). Lastly, inducible SLE models such as pristane-induced lupus have facilitated the study of SLE in non-autoimmune strains (309).

Several studies have highlighted potential roles of ICOS in the pathogenesis of SLE. First, the lupus-like disease found in *sanroque* mice is thought to originate from dysregulated expression of immune-related genes including *Icos* (25, 310). Second, deletion of the *Icos* gene in MRL/*lpr* mice was shown to diminish autoantibody production and impaired inflammatory cytokine production (311, 312). However, ICOS abrogation did not significantly alter the degree of lymphadenopathy and only modestly reduced the severity of glomerulonephritis in these mice (311). This suggests that antibody-independent pathways can mediate organ inflammation in this model. However, ICOS abrogation may also have impaired regulatory T cell subsets. Third, some studies have shown that impaired ICOSL expression by $CD11c^+$ cells or B cells can impact disease progression in murine models of SLE. Specifically, CD11c-specific ICOSL deficiency in MRL/*lpr* mice did not alter autoantibody production but significantly ameliorated kidney and lung inflammation (313). Further, this study suggests that ICOSL expression by $CD11c^+$ cells in inflamed organs is required to protect invading T cells from apoptosis by stimulating the ICOS-PI3K pathway. On the other hand, B cell-specific deficiency of the metalloprotease ADAM10 in lupus-prone mice was shown to cause decreased ICOSL levels, increased ICOS levels along with impaired GC reactions and reduced autoantibody production (314). Thus, ICOS signaling appears to be crucial for autoantibody production in mouse models of SLE but could be partially dispensable for the induction of inflammation.

In humans, ICOS expression by peripheral blood $CD4^+$ T cells was found to be significantly higher in SLE patients when compared to healthy controls (315, 316). This increase of ICOS levels was mirrored by decreased ICOSL expression by B cells, and clusters of B cells were found near ICOS+ T cells in the kidneys of SLE patients (316). Further, ICOS stimulation of T cells isolated from SLE patients caused increased IFN-γ production *in vitro* and enhanced anti-dsDNA IgG production by B cells in a T-B coculture experiment (315). A phase I clinical trial evaluating the impact of antibody-mediated ICOSL blockade in SLE patients found some differences in antigen-specific antibody

production but no discernable changes in immune cell populations or disease severity. Taken together, these results suggest that ICOS could be involved in certain aspects of SLE pathogenesis but may be dispensable for disease progression (317).

1.7.6 Role of ICOS in type I diabetes

Type I diabetes is an autoimmune disorder that results in the destruction of insulin-producing β cells found in pancreatic islets (318). Patients are typically diagnosed with T1D between childhood and young adulthood, and usually present with symptoms of polydipsia, polyphagia and polyuria which are all underpinned by hyperglycaemia (319). Autoantibodies targeting β-cell antigens are found in around 90% of T1D patients and are often present before the onset of symptoms (320, 321). While some T1D patients retain the ability to produce low levels of insulin, most will require insulin supplementation to prevent damaging effects of hyperglycaemia (322). The foremost T1D murine model is the NOD mouse (323). This strain is characterized by a genetic baggage conferring a propensity for autoimmunity, and spontaneously develops a T cell-mediated T1D-like disease. Most female NOD mice develop diabetes by 40 weeks, but incidence is much lower in male mice (324). NOD mice expressing a TCR transgene named BDC2.5 are also commonly used to study the fate of diabetogenic T cells (325). This TCR was found to recognize peptides from chromogranin A, a protein expressed by β cells (326). Diabetes incidence is lower in BDC2.5-NOD mice than in NOD mice due to protective impacts of Treg cells in prediabetic lesions (327).

Studies on the role of ICOS in the pathogenesis of T1D have provided conflicting results. Genetic deletion of ICOS or ICOSL was shown to reduce diabetes incidence in NOD mice (328-330). However, abrogation of ICOS signaling in NOD mice also caused a neuro-muscular autoimmune disorder characterized by hind leg paralysis (329, 330). This deviation of autoimmunity was found to be $CD4^+$ T cell-dependent and suggests that ICOS signaling is required to maintain the balance between autoreactive effector T cells and Tregs (329, 330). Contrasting the results from NOD mice, abrogation of ICOS signaling by blockade or genetic deletion in BDC2.5-NOD mice was shown to accelerate

the development of diabetes (56, 327, 329). A study by Kornete *et al.* has shown that Tregs found in pancreatic LNs of pre-diabetic mice can upregulate the homing receptor CXCR3 in an ICOS-dependent manner (331). This mechanism was found to be triggered by T cell-mediated pancreatic inflammation and promotes CXCR3-dependent chemotaxis of $ICOS^+$ Tregs to pancreatic islets. Of note, the suppressive ability of pancreatic $ICOS^+$ Tregs was found to be greater than that of $ICOS^-$ Tregs (56). Thus, these results suggest that the role of ICOS in murine models of T1D is to support Treg function.

1.8 Rationale and hypothesis

Costimulatory signals provided by ICOS were shown to be required for Tfh differentiation, GC reactions, and class switched antibody production (16). Indeed, ICOS deficiency in mice or ICOS null mutations in humans results in defective humoral immunity (11-13, 332). Additionally, Th1, Th2, Th17, and $CD8^+$ T cells were all found to require ICOS for their effector functions (333). However, some findings also indicate that Treg and Tfr cells may need ICOS for their survival and function (16, 334). In sum, ICOS signaling plays a dual role in promoting effector and regulatory T cells.

This concept is particularly interesting when considering the pathogenesis of autoimmune diseases. On one hand, ICOS can support the production of autoantibodies and inflammatory cytokines. On the other hand, ICOS signaling may help regulatory T cells to mitigate disease severity by enhancing their suppressive abilities. Thus, understanding the relative contribution of ICOS and its downstream signaling pathways during disease initiation and maintenance is of critical importance for the development of novel therapeutic options. Preliminary data suggests that ICOS could be involved in human RA (301, 302). In a murine RA model, ICOS blockade inhibits the production of autoantibodies and prevents arthritis induction (297). However, it is unclear if ICOS abrogation would ameliorate established arthritis. Further, PI3K inhibition was shown to reduce arthritis severity in several murine RA models (299, 300). Since ICOS is a potent activator of PI3K, this suggests that targeting the ICOS-PI3K pathway could be of therapeutic value. Of note, ICOS-PI3K signaling was shown to promote lupus maintenance in MRL/*lpr* mice by enhancing the survival of autoimmune T cells (313).

Therefore, we hypothesize that ICOS-PI3K signaling can promote initiation and maintenance of arthritis in a murine model of RA.

The recent discovery of Tfr cells has provided insights into immune mechanisms responsible for the prevention of autoimmunity. Specifically, Tfr-deficient mice were found to produce autoantibodies due to dysregulated GC responses (199). Also, increased Tfh: Tfr ratios due to impaired Tfr generation is a common feature of human autoimmune

disorders (335, 336). Some data support the view that ICOS is required for Tfr differentiation (197, 203, 204). However, the mechanisms involved have not been elucidated. Studies have shown that Tfr cells are characterized by a mixture of Treg and Tfh features. Thus, it is possible that ICOS-dependent mechanisms known to promote Tfh differentiation such as KLF2 downregulation could also support Tfr generation (96). However, Tfr cells were found to mostly originate from Tregs which utilize signaling pathways known to antagonize the Tfh transcriptional program such as IL-2 and BLIMP-1 (183-185, 190). This suggests that Tfr cells may require distinctive differentiation cues. Interestingly, a study has shown that Tfr cells uniquely require NFAT2 activation to upregulate CXCR5 (193).

Thus, we hypothesize that ICOS prevents autoantibody generation by supporting Tfr differentiation through unique pathways and/or known Tfh differentiation pathways.

1.9 Aims and objectives

1.9.1 Aim 1

The focus of Aim1 is to study the role of ICOS-PI3K signaling in the induction and maintenance of CIA, a murine model of RA. This can be broken down into the following objectives.

1. Investigate whether ICOS-PI3K signaling is required for CIA induction.
2. Establish an inducible ICOS genetic deletion model to test the requirement of continuous ICOS signaling for CIA maintenance.
3. Determine which mechanisms downstream of ICOS contribute to CIA induction and maintenance.

1.9.2 Aim 2

Aim 2 is to investigate whether ICOS can contribute to Tfr cell differentiation. This can be subdivided into the following objectives.

1. Study the impact of regulatory T cell-specific ICOS abrogation on Tfr populations in protein immunization and viral infection settings.
2. Evaluate whether Foxp3-specific ICOS deletion leads to autoimmunity.
3. Establish which ICOS-dependent mechanism(s) can contribute to Tfr differentiation.

Chapter 2: ICOS signaling controls induction and maintenance of collagen-induced arthritis

Chapter 2 presents a modified version of original work published in the *Journal of Immunology* **200**(9):3067-3076. doi: 10.4049/jimmunol.1701305. Epub 2018 Mar 26. PMID: 29581356.

ICOS signaling controls induction and maintenance of collagen-induced arthritis

Vincent Panneton *,†, Sahar Bagherzadeh Yazdchi *,‡, Mariko Witalis *,§, Jinsam Chang *,§, Woong-Kyung Suh *,†,‡,§,¶

* IRCM (Institut de Recherches Cliniques de Montréal), Montreal, Quebec, Canada

† Department of Microbiology, Infectiology, and Immunology, University of Montreal, Quebec, Canada

‡ Department of Microbiology and Immunology, McGill University, Montreal, Quebec, Canada

§ Molecular Biology Program, University of Montreal, Quebec, Canada

¶ Corresponding author:

Tel) 514-987-5720

Fax) 514-987-5768

Email) woong-kyung.suh@ircm.qc.ca

Running title: Role of ICOS signaling in CIA initiation and maintenance

2.1 Abstract

The inducible T-cell costimulator (ICOS) is a key costimulatory receptor facilitating differentiation and function of follicular helper T cell and inflammatory T cells. Rheumatoid arthritis patients were shown to have elevated levels of $ICOS^+$ T cells in the synovial fluid suggesting a potential role of ICOS-mediated T cell costimulation in autoimmune joint inflammation. In this study, using ICOS knockout and knockin mouse models, we found that ICOS signaling is required for the induction and maintenance of collagen-induced arthritis (CIA), a murine model of rheumatoid arthritis. For the initiation of CIA, the tyrosine 181 (Tyr^{181})-based SH2-binding motif of ICOS that is known to activate phosphoinositide 3-kinase was critical for antibody production and expansion of inflammatory T cells. Further, we found that Tyr^{181}-dependent ICOS signaling is important for maintenance of CIA in an antibody independent manner. Importantly, we found that a small molecule inhibitor of glycolysis, 3-bromopyruvate, ameliorates established CIA suggesting an overlap between ICOS signaling, phosphoinositide 3-kinase signaling, and glucose metabolism. Thus, we identified ICOS as a key costimulatory pathway that controls induction and maintenance of CIA and provide evidence that T cell glycolytic pathways can be potential therapeutic targets for rheumatoid arthritis.

2.2 Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by immune cell infiltration of the synovial tissues leading to inflammation with potential destruction of cartilage and bone. The pathology of RA involves components of innate and adaptive immunity (244). While the pathogenesis of RA remains poorly understood, production of autoantibodies along with infiltration of synovial fluid and tissues by inflammatory cells have been identified as key events (257, 337, 338). Collagen-induced arthritis (CIA) is a murine model that closely mimics RA (263). Susceptible mouse strains such as DBA/1 develop humoral and cellular immune reactions to bovine type II collagen (bCII) following immunization which leads to chronic joint inflammation (339). Studies

have shown that T cell costimulation is involved in CIA. For example, mice deficient in CD28 are resistant to CIA and blockade of the inducible T-cell costimulator (ICOS) using a monoclonal antibody ameliorated CIA (279, 340). Interestingly, RA patients were shown to have elevated levels of $ICOS^+$ T cells along with ICOS ligand (ICOSL) in their synovial tissues suggesting potential therapeutic value of ICOS-mediated T cell costimulatory pathways (341).

ICOS is a member of the CD28 superfamily mainly expressed at the surface of activated T cells (2, 342). The major role of ICOS is to support differentiation and function of $CD4^+$ follicular helper T (Tfh) cells during germinal center (GC) reactions (101, 343-345). Consequently, lack of ICOS or ICOSL in humans and mice causes severe defects in class-switched antibody production (11-13, 31, 332). Alternatively, ICOS overexpression caused by the *sanroque* mutation leads to a lupus-like disease with an increase of Tfh cells and spontaneous GC reactions in mice (346, 347). Additionally, ICOS signaling plays important roles in the production of an array of Th1, Th2 and Th17 inflammatory cytokines depending on the context of immune stimuli (176, 348-350).

Despite evidence suggesting that ICOS could be implicated in CIA and RA, little is known about the specific downstream signaling components involved. We have previously described an evolutionarily-conserved SH2 binding motif (Y^{181}MFM) in the cytoplasmic tail of ICOS which is critical for the activation of the PI3K-Akt-mTOR signaling cascade (351, 352). Thus, mice carrying the $ICOS^{Y181F}$ mutation cannot signal through this pathway and display reduced numbers of Tfh cells and impaired GC reactions (351). However, the $ICOS^{Y181F}$ mutation does not completely phenocopy ICOS deficiency. For example, the anti-chlamydial Th17 response and graft-versus-host disease severity were only partially reduced in $ICOS^{Y181F}$ mice compared to $ICOS^{-/-}$ mice (350, 353). These findings suggest the existence of PI3K-independent signaling mechanisms of ICOS. In fact, there are two more signaling motifs identified in the cytoplasmic tail of ICOS. The second motif, $KKKY^{170}$, in the cytoplasmic tail of ICOS can potentiate intracellular calcium release from the endoplasmic reticulum triggered by T-cell receptor (TCR) engagement (47). The third TNFR-associated factor-like IProx motif links ICOS to TANK-binding

kinase 1 (TBK1) and serves as an important mechanism promoting the late stage of Tfh cell differentiation (45). However, its downstream signaling mechanisms remain unclear.

In this study, we evaluated the impact of ICOS signaling on CIA initiation and maintenance using ICOS mutant mice. We found that ICOS is required for both the induction and maintenance of CIA. Although these two processes are heavily dependent on Tyr^{181}-mediated ICOS signaling, the downstream effector pathways are distinct. Importantly, we uncovered a potential overlap between ICOS signaling, T cell glycolysis, and joint inflammation.

2.3 Material and methods

2.3.1 Mice

ICOS germline knockout (-/-) and $ICOS^{Y181F}$ knockin mice have been previously described (13, 351). For CIA experiments, these two lines have been backcrossed onto DBA/1J background for 12 generations. DBA/1J mice (Jax 000670), UBC-CreERT2 mice (Jax 008085), and FLPe FRT deleter line (Jax 005703) were purchased from the Jackson Laboratory. All the mice were housed in the IRCM Animal Facility under specific pathogen-free conditions. Animal experiments were performed in accordance with animal use protocols approved by the IRCM Animal Care Committee.

2.3.2 Generation of ICOS conditional knockout mice

ICOS conditional knockout (cKO) mouse strain was generated using C57BL/6 embryonic stem cell line Bruce-4 at the IRCM transgenic core facility. We generated 5.4 kb 5' and 4.5 kb 3' homology arms by assembling DNA fragments (1.2-2.3 kb) amplified through high-fidelity PCR (Platinum Pfx DNA polymerase, Thermo Fisher) using C57BL/6 RPCI-23 mouse BAC clone DNA as template. Cloned DNA fragments were verified by

sequencing and aligning against the C57BL/6 genome sequence available from NCBI. We designed a targeting vector in which exon 2 (encoding extracellular Ig domain) and exon 3 (encoding transmembrane segment) of ICOS gene were flanked by loxP elements and a neomycin resistance (Neo) cassette was flanked by FRT elements. After electroporation of the targeting vector, G418-resistant ES clones were screened for successful homologous recombination in the *Icos* locus by Southern blot. Four ES clones with conditional ICOS allele were injected into C57BL/6 blastocysts and three of them gave germline transmission. Heterozygous mice containing ICOS mutant allele were bred with FLPe FRT deleter mice to remove the Neo cassette. Resulting ICOS-floxed, Neo-deleted heterozygous mice were further backcrossed for 2 generations onto C57BL/6 background and used for experiments.

2.3.3 Collagen-induced arthritis

CIA was induced in mice with a DBA/1J background (for germline KO experiments) or in a 50:50 mix of DBA/1J:C57BL/6J background (for conditional KO experiments). Eight- to twelve-week-old mice were immunized intradermally at the base of the tail with 50μg of bovine type II collagen (2mg/mL, Chondrex Inc.) emulsified in an equal volume of Complete Freund's Adjuvant (CFA, 4mg/mL of *M.tuberculosis*, Chondrex Inc.). Clinical arthritis was assessed for each paw in a blinded manner based on the following criteria: 0 = healthy, 1= mild swelling of one joint, 2 = mild swelling of multiple joints, 3 = severe swelling of multiple joints, 4 = severe swelling and ankylosis. The scores were added up for a maximum of 16 per mouse. For conditional KO experiments, mice were fed tamoxifen dissolved in corn oil (Sigma-Aldrich) daily at a dose of 200μg/g of body weight by oral gavage for 5 days. For glycolysis inhibition experiments, mice were injected intraperitoneally with 3-bromopyruvate (Sigma-Aldrich) at a dose of 5mg/kg/day for 3 days.

2.3.4 Histology

Paws were dissected and fixed in 10% neutral buffered formalin for 12h at 4°C. Paws were washed in 1x PBS and decalcified in 0.5M EDTA replaced every 3 days for 2 weeks at 4°C. Decalcified paws were embedded in paraffin and 5 μm sections were stained with hematoxylin & eosin (H&E) for assessment of immune infiltration and destruction of joints.

2.3.5 Antibody ELISA

Serum was obtained by cheek bleeding at different disease stages. Plates were coated with 5μg/mL ELISA grade collagen (Chondrex Inc.) overnight at 4°C. A two-fold serial dilution of serum was performed starting at 1:100. Antibodies were detected using anti-IgG2a-AP or anti-IgG2b-AP along with p-nitrophenyl phosphate substrate (Southern Biotech). The reaction was stopped using 1.5N NaOH and plates were read at 405nm.

2.3.6 Proliferation assay and cytokine ELISA

Cells were isolated by mechanical disruption from draining lymph nodes of immunized mice and resuspended at $1x10^6$/mL in RPMI medium (Life Technologies) supplemented with 10% FBS (Wisent), 10mM HEPES (ThermoFisher), 100U/mL penicillin-streptomycin (ThermoFisher) and 55μM β-mercaptoethanol (ThermoFisher). Cells were restimulated with 100μg/mL heat denatured bovine type II collagen (Chondrex Inc.) or 1μg/mL anti-CD3 (clone 145-2C11, BioXcell) for 2 days at 37 °C in 96-well plates ($1x10^5$/well). Cytokine concentrations in culture supernatant were measured using cytokine Ready-SET-Go! ELISA kits according to manufacturer's instructions (ThermoFisher). For proliferation assays, cells were pulsed with 1μCi / well ^{3}H-thymidine (PerkinElmer) for 8 hours at 37°C, lysed and harvested using a Filtermate harvester (Packard) and then analyzed using a TopCount NXT scintillation counter (Packard).

2.3.7 Glucose metabolism assay

Lymph node cells from naïve WT DBA/1 mice were stimulated with 1μg/mL anti-CD3 for 2 days at 37 °C in 96-well plates ($1x10^5$/well) the same manner as proliferation assay. During the last 4hr of incubation, cells were exposed to 3-BrPA (Sigma-Aldrich; 3 or 6 μM) and D-[3-^{3}H]-glucose (PerkinElmer; 1μCi/well). Cells were then lysed, harvested, and incorporated radioactivity was measured as described in proliferation assay.

2.3.8 Flow cytometry analysis of ICOS conditional KO

To quantify the efficacy of conditional ICOS deletion, single cell suspensions were prepared from draining lymph nodes of ICOS conditional KO mice treated or not with tamoxifen for 5 days. Cells were incubated for 24h at 37°C in complete RPMI medium supplemented with 1μg/mL anti-CD3 (clone 145-2C11, BioXcell). Viability was determined by Trypan blue staining (ThermoFisher). Cells were blocked with anti-mouse CD16/CD32, then stained with anti-CD4 FITC (GK1.5) or anti-CD8a-APC (53-6.7) along with anti-ICOS-PeCy7 (7E.17G9) using 0.3μL/stain (ThermoFisher). Data were collected using BD LSRFortessa (BD Biosciences) and analyzed by FlowJo v10 (FlowJo LLC).

2.3.9 Statistical analysis

All data are presented as mean and standard error of mean (SEM) unless specified otherwise. Differences between experimental groups were tested with two-tailed Student's *t*-tests or one-way ANOVA with Tukey's multiple comparisons test in Prism 7 (GraphPad software). Statistical significance was judged based on *p* values and denoted as follows: * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

2.4 Results

2.4.1 ICOS is required for the initiation of collagen-induced arthritis

To assess the role of ICOS in the induction of CIA, we immunized mice with an emulsion of bCII and CFA at the base of the tail. WT DBA/1J mice developed signs of arthritis 5 weeks after immunization (Fig. 2.1 A). This is characterized by paw swelling and erythema along with histological features such as immune cell infiltration of the joint space and erosion of cartilage (Fig. 2.1 B). However, $ICOS^{-/-}$ and $ICOS^{Y181F}$ DBA/1J mice did not develop arthritis for the entire duration of the experiment (Fig. 2.1 A). Next, we analyzed anti-bCII IgG2a and IgG2b serum levels 2 weeks post-immunization as these isotypes are known to activate complement and are required for the induction of arthritis (266). Consistent with disease scores, antibody titers were at least 3-fold lower in $ICOS^{Y181F}$ and $ICOS^{-/-}$ mice compared to WT (Fig. 2.1 C). To evaluate the role of ICOS in antigen-specific T cell responses, we immunized mice and harvested draining lymph nodes (dLN) 2 weeks later. Next, we restimulated a suspension of total dLN cells with bCII for 2 days and measured T cell proliferation and cytokine production. Cells from $ICOS^{Y181F}$ or $ICOS^{-/-}$ mice displayed a 3-fold reduction of proliferation in response to the immunizing antigen when compared to cells from WT mice (Fig. 2.1 D). Levels of IFN-γ, IL-17, TNF-α and IL-4 were measured in culture media from proliferation assays by ELISA (Fig. 2.1 E). No significant differences were observed for IFN-γ and IL-17. TNF-α was 2-fold lower than WT in $ICOS^{-/-}$ samples. No IL-4 production was detectable in any samples (data not shown). Taken together, these results indicate that ICOS and its ability to signal through PI3K are required for CIA initiation primarily through induction of antibody responses and expansion of inflammatory T cells.

2.4.2 Generation of ICOS conditional knockout mice

Although it became clear that ICOS is required for the initiation of CIA in mice, more clinically relevant questions remain unanswered. Is ICOS required to sustain joint

inflammation beyond its role in the initiation phase? If so, what are the dominant signaling components involved? In order to address these questions, we decided to generate ICOS conditional KO mice. We generated a targeting vector based on C57BL/6 *Icos* genomic sequence and utilized a C57BL/6-derived ES cell line Bruce-4 to expedite downstream applications without extensive backcrossing. As shown in Fig. 2.2 A, we designed the vector to eliminate exons encoding extracellular Ig domain (exon 2) and transmembrane segment (exon 3) by the Cre-loxP system and to remove Neo cassette by the FLPe-FRT system. Through Southern blot analysis of ES cell clones (~400), we identified 4 clones harboring ICOS-cKO-NEO allele in the *Icos* locus (Fig. 2.2 B). After germline transmission (Fig. 2.2 C), the Neo cassette was deleted *in vivo* by breeding with FLPe FRT-deleter line. We confirmed that T cells harboring the ICOS-cKO allele express normal levels of ICOS upon stimulation and that ICOS expression is completed abrogated in CD4-Cre^{+} T cells (Supplementary Fig. 2.1) or when Cre enzyme activity is turned on by tamoxifen treatment in UBC-CreERT2 transgenic mice (Fig. 2.2 D).

2.4.3 ICOS is required for the maintenance of collagen-induced arthritis

To determine if ICOS is required in the maintenance of established CIA, we used our conditional KO system in which ICOS deletion is induced by tamoxifen (Fig. 2.3 A). Although it has been shown that the C57BL/6 genetic background confers resistance to CIA (354), mice bearing ~50% of DBA/1 background are susceptible to CIA (280). We were able to induce CIA in this mixed genetic background with disease kinetics similar to WT DBA/1 mice (Fig. 2.3 B). At the peak of arthritis symptoms, we induced the deletion of ICOS and observed a marked amelioration of disease severity (Fig. 2.3 B). A similar pattern was observed when we pooled data from paws that were highly inflamed (score 3-4) indicating a critical role for ICOS in the maintenance of severe joint inflammation (Supplementary Fig. 2.2). The slight reduction in disease scores seen in control mice is a feature of normal CIA progression (355). We also evaluated serum anti-bCII antibody titers at different disease stages but found no significant correlation with disease scores (Fig. 2.3 C). Histological analysis reveals a striking recovery after ICOS deletion, although

some mice with severe disease were left with residual cartilage damage and joint inflammation (Fig. 2.3 D).

Next, we modified the conditional knockout system to obtain $ICOS^{fl/Y181F}$ mice (Fig. 2.4 A). While $ICOS^{Y181F}$ mice are resistant to CIA, $ICOS^{fl/Y181F}$ mice develop arthritis symptoms 5 weeks post-immunization indicating that the Y181F mutation does not act as a dominant-negative (Fig. 2.4 B). We performed tamoxifen gavage at the peak of arthritis symptoms which effectively leaves mice with only the mutant allele ($ICOS^{Y181F/-}$). This caused a substantial reduction in disease scores (Fig. 2.4 B). As before, no correlation between serum anti-bCII titers and disease scores was observed (Fig. 2.4 C). Histological analysis shows partial recovery with persistent cartilage erosion (Fig. 2.4 D). Thus, ICOS is required for the maintenance of CIA and Tyr181-dependent ICOS signaling mechanisms are needed to sustain the maximal disease process.

2.4.4 Glycolysis inhibitor ameliorates collagen-induced arthritis

We have previously shown that ICOS can activate mTOR activities through the PI3K-Akt signaling axis (352). Further, a recent study has shown that ICOS costimulation can augment glycolysis in activated T cells through an mTOR-dependent mechanism (94). In addition, PI3K-Akt signaling has been implicated in augmentation of glucose uptake in activated T cells (356). Interestingly, increased glucose metabolism in the inflamed joint has been observed in RA patients (357, 358), and inhibition of glycolysis ameliorated arthritis in K/BxN transgenic mice (359). We therefore hypothesized that a glycolysis inhibitor could be used to treat CIA. To test this idea, we immunized WT DBA/1 mice and injected 3-bromopyruvate (3-BrPA) intraperitoneally for 3 days at the peak of disease (Fig. 2.5 A). This caused a partial but persistent reduction in disease score reminiscent of the improvement seen in $ICOS^{Y181F}$ conditional KO experiments (Fig. 2.4 B). Histological analysis revealed reduced immune cell infiltration and normal cartilage (Fig. 2.5 B). These results suggest that the amelioration of CIA observed in conditional KO experiments could be due to reduced glycolysis in ICOS-deleted T cells.

We further examined the impact of 3-BrPA on T cell proliferation and function *in vitro* (Fig. 2.5 C and D). First of all, T cell viability was heavily compromised when draining LN cells from immunized mice were restimulated in the presence of 3-BrPA. Even at a low dose of 3-BrPA (3 μM) where T cell viability is minimally affected, proliferation and cytokine production was greatly reduced (Fig. 2.5 C). Consistent with the notion that activated T cells utilise glucose as a source of biosynthetic building blocks (360), we found that glucose-derived tritium tracers were readily incorporated into macromolecules (Fig. 2.5 D). Importantly, this process is inhibited by 3-BrPA. Therefore, 3-BrPA effectively inhibits T cell proliferation and cytokine production, at least in part, through inhibition of intracellular glucose metabolism. However, we failed to determine if there is a synergy between 3-BrPA and ICOS mutations because 3-BrPA (3 μM) left no room to detect additional impact of ICOS-Y181F mutation (Supplementary Fig. 2.3).

2.5 Discussion

Our study reveals a critical role of ICOS in the initiation and maintenance of CIA. We show that ICOS is required for the generation of anti-bCII antibodies in the early disease phase. Also, ICOS deficiency greatly reduced inflammatory T cell responses during disease progression. $ICOS^{Y181F}$ closely resembled $ICOS^{-/-}$ mice during CIA initiation, highlighting the prominent roles of the Tyr^{181}-dependent ICOS signaling in Tfh generation and GC-driven antibody generation at this disease stage. We generated a conditional ICOS KO model and showed that ICOS signaling is also required for CIA maintenance. Signaling mechanisms dependent on Tyr^{181} appear to play an important role in the maintenance stage as well. We also found that inhibition of glycolysis ameliorates CIA providing a potential link between ICOS costimulation, T cell glycolysis, and sustained joint inflammation.

Induction of CIA depends on the production of autoantibodies (266). This is also seen in collagen antibody-induced arthritis (CAIA) where injection of arthritogenic antibodies in mice can directly cause joint inflammation (273). ICOS is known to be critically involved in Tfh generation and function, and therefore in GC reactions and

production of class-switched high-affinity antibodies (11-13). Importantly, we have shown that ICOS-PI3K signaling plays a crucial role in Tfh cell generation (351). Accordingly, we observed similar defects in antibody production in immunized mice lacking ICOS or selectively defective in ICOS-PI3K signaling (Fig. 2.1 C). The requirement for humoral responses in autoimmune diseases has been described in several studies. Mice deficient in CD28, CXCR5 or SLAM-associated protein (SAP) are all resistant to CIA and show low levels of anti-collagen antibodies after immunization (279-281). ICOS is also known to be important for T cell proliferation and function (11-13, 361). We showed that T cells from immunized $ICOS^{Y181F}$ and $ICOS^{-/-}$ mice proliferate less when restimulated *in vitro* using the immunizing antigen (Fig. 2.1 D). Therefore, antibody production defects and impaired T cell expansion likely explain the resistance to CIA initiation in $ICOS^{-/-}$ and $ICOS^{Y181F}$ mice.

Models such as CAIA have shown that antibodies alone are insufficient to maintain disease progression. Consistently, we showed that anti-bCII antibody levels became irrelevant to CIA maintenance in conditional knockout experiments (Figs. 2.3 C and 2.4 C). Also, mice left with $ICOS^{Y181F}$ after tamoxifen gavage did not fully recover whereas mice that completely lost ICOS expression became free of inflammation in most cases. This suggests that signaling mechanisms depending on Tyr181 are important for CIA maintenance, but there are ICOS-mediated Tyr181-independent mechanisms still operating to contribute to sustained inflammation. This is also supported by differences seen in the production of TNF-α by restimulated dLN cells from immunized mice (Fig. 2.1 E). When compared to WT samples, cells from $ICOS^{-/-}$ mice showed a two-fold reduction of TNF-α whereas $ICOS^{Y181F}$ cells did not show a significant difference. TNF-α is known to be important for the progression of CIA and is involved in human RA (362, 363). We previously reported that ICOS can potentiate TCR-mediated intracellular calcium flux (47). In T cells, calcium signaling is known to regulate a wide array of processes ranging from differentiation to effector functions (364). In fact, inhibiting calcium signaling with cyclosporine has been one of the most widely used immunosuppressive therapies (365, 366). A recent study showed that a T-cell specific deletion of the store-operated calcium entry components STIM1 and STIM2 protected mice from experimental autoimmune encephalomyelitis (367). Therefore, it is possible that calcium signaling potentiation is one

of the main mechanisms evoked by ICOS in CIA maintenance. It remains possible that the recently described ICOS-TBK1 axis could also play a role in CIA maintenance, although it is more likely involved in CIA initiation considering that it promotes Tfh maturation (45).

Studies have shown that PI3K inhibitors can ameliorate CIA, but the mechanisms involved remain ill-defined (368, 369). The substantial recovery seen in mice left with $ICOS^{Y181F}$ after tamoxifen treatment prompted us to look for PI3K-dependent roles of ICOS other than antibody production. A recent study reported that ICOS-ICOSL interaction causes an increase of PI3K-Akt signaling which protected inflammatory T cells from apoptosis in lupus-prone mice (370). This could also hold true for joint-infiltrating T cells in CIA. Also, ICOS signaling can affect glycolysis through the PI3K-Akt-mTOR pathway (94, 352). Congruent to this, we found that a small molecule glycolysis inhibitor 3-BrPA can ameliorate established CIA. This confirms similar findings previously reported in the K/BxN arthritis model (359). Glycolysis promotes the proliferation of Th17 cells which produce IL-17, a key inflammatory cytokine in RA and CIA (371-373).

Therapeutic targeting of glycolysis has been tested in cancer research showing safety and efficacy. For example, administration of 3-BrPA was successfully tested as a cancer treatment in mice (374, 375). It has also shown efficacy in at least one human cancer patient and will soon undergo phase I clinical trials (376). Since glycolysis has been identified as a possible factor in the progression of RA, it could be interesting to see if 3-BrPA can be used to treat RA as well as cancer (357, 358).

In conclusion, we demonstrated that ICOS provides important costimulatory signals for the initiation and maintenance of CIA. For the initiation phase, ICOS signaling appears to be critical for antibody production but its role in the maintenance phase may involve inflammatory cytokines such as TNF-α. We identified a potential overlap between ICOS signaling and T cell glucose metabolism which can be targeted by readily accessible chemical inhibitors.

2.6 Acknowledgements

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2.7 Footnotes

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Correspondence should be addressed to: Dr. Woong-Kyung Suh, IRCM (Institut de Recherches Cliniques de Montréal), 110 avenue des Pins Ouest, Montreal, Quebec, Canada. Tel) 514-987-5720, Fax) 514-987-5768, Email) woong-kyung.suh@ircm.qc.ca.

Abbreviations used in this article: ICOS, Inducible Costimulator; ICOSL, Inducible Costimulator ligand; PI3K, phosphoinositide 3-kinase; CIA, collagen-induced arthritis; RA, rheumatoid arthritis; bCII, bovine type II collagen; 3-BrPA, 3-bromopyruvate.

2.8 Disclosures

The authors have no financial conflicts of interest.

2.9 Figures and figure legends

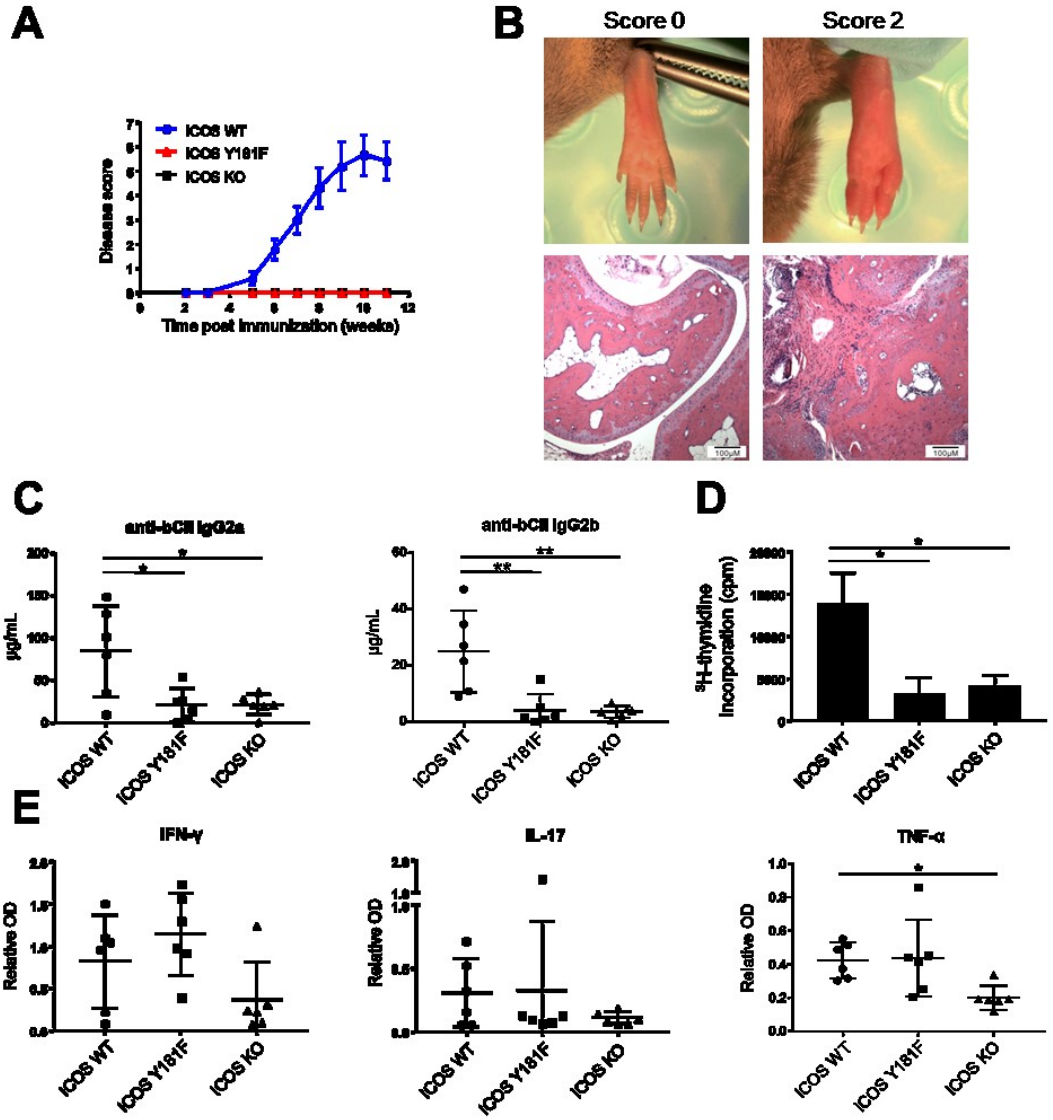


Figure 2.1 ICOS is required for the initiation of collagen-induced arthritis. **(A)** Eight- to twelve-week-old DBA/1 mice were immunized with bCII + CFA and disease scores were given by visual inspection according to the criteria described in *Materials and Methods*. Data represents two independent experiments with similar results. Mean scores ± SEM of 8 mice per group. **(B)** Representative pictures of normal and inflamed hind paw (disease score 2) along with H&E staining showing normal joint space (left) and immune cell infiltration in arthritic paw (right). **(C)** Serum from mice immunized as in (A) was taken two weeks post-immunization and anti-bCII IgG2a or IgG2b titers were measured by ELISA. Data represents two independent experiments with similar results, n=6 for each group. **(D)** Draining lymph node cells from WT DBA/1 mice were extracted two weeks post-immunization and restimulated *in vitro* with 100 μg/mL denatured bCII for 48h. ^{3}H-thymidine was added to measure incorporation during the last 8h. Data represents two independent experiments with similar results, n=6 for each group. **(E)** Cytokines (IFN-γ, TNF-α, IL-17) were detected in culture supernatant from proliferation assays by ELISA. n=6 per group for IFN-γ and TNF-α. n=6 ICOS WT, 8 ICOS Y181F and 8 ICOS KO for IL-17. *P < 0.05, ** P < 0.01, by one-way ANOVA with Tukey's multiple comparisons test.

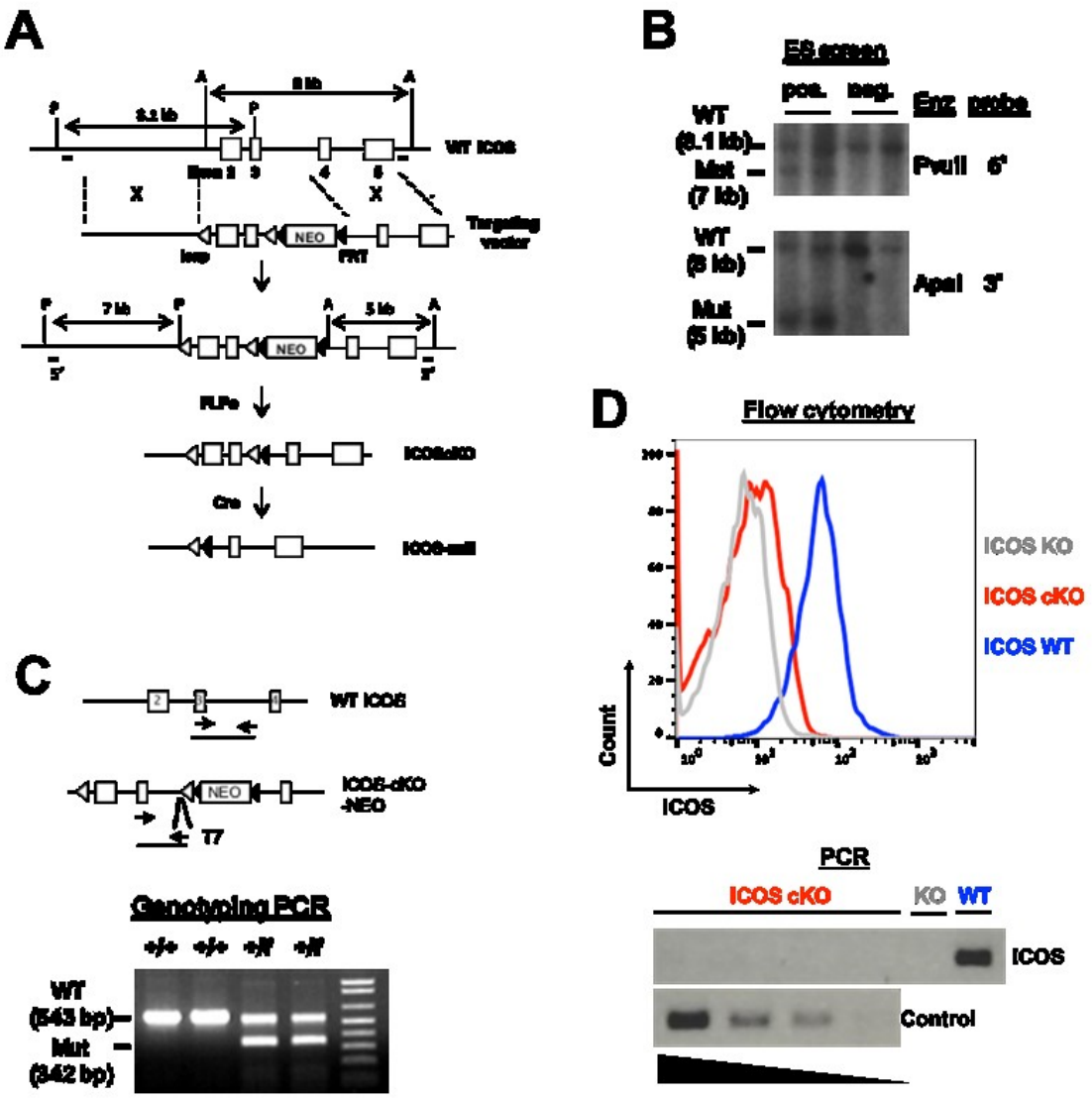


Figure 2.2 Generation of ICOS conditional KO mice. (**A**) Targeting strategy. Exon 2 and Exon 3 of ICOS gene was flanked with loxP elements and the Neo cassette was flanked by FRT elements. The flanked DNA segments were designed to be removed by Cre and FLPe enzyme, respectively. (**B**) ES cells with targeted ICOS allele were screened by Southern blot using 5' and 3' probes as indicated in (A). (**C**) PCR genotyping of ICOS alleles in heterozygous mice. Primers used are represented with arrows. (**D**) Confirmation of tamoxifen-induced ICOS deletion by flow cytometry and PCR. Lymph node cells from UBC-CreERT2^{+}; ICOS$^{fl/-}$ mice treated with tamoxifen were restimulated with 1μg/mL anti-CD3 for 24h to induce ICOS expression. Data represents two independent experiments and shows ICOS expression in CD4^{+} T cells. For PCR, genomic DNA isolated from CD4^{+} T cell blasts derived from tamoxifen-treated UBC-CreERT2^{+}; ICOS$^{fl/-}$, ICOS WT, or ICOS KO mice were amplified by primers specific for exon 2-3 of ICOS gene (ICOS) or control primers detecting Foxp3 gene (Control).

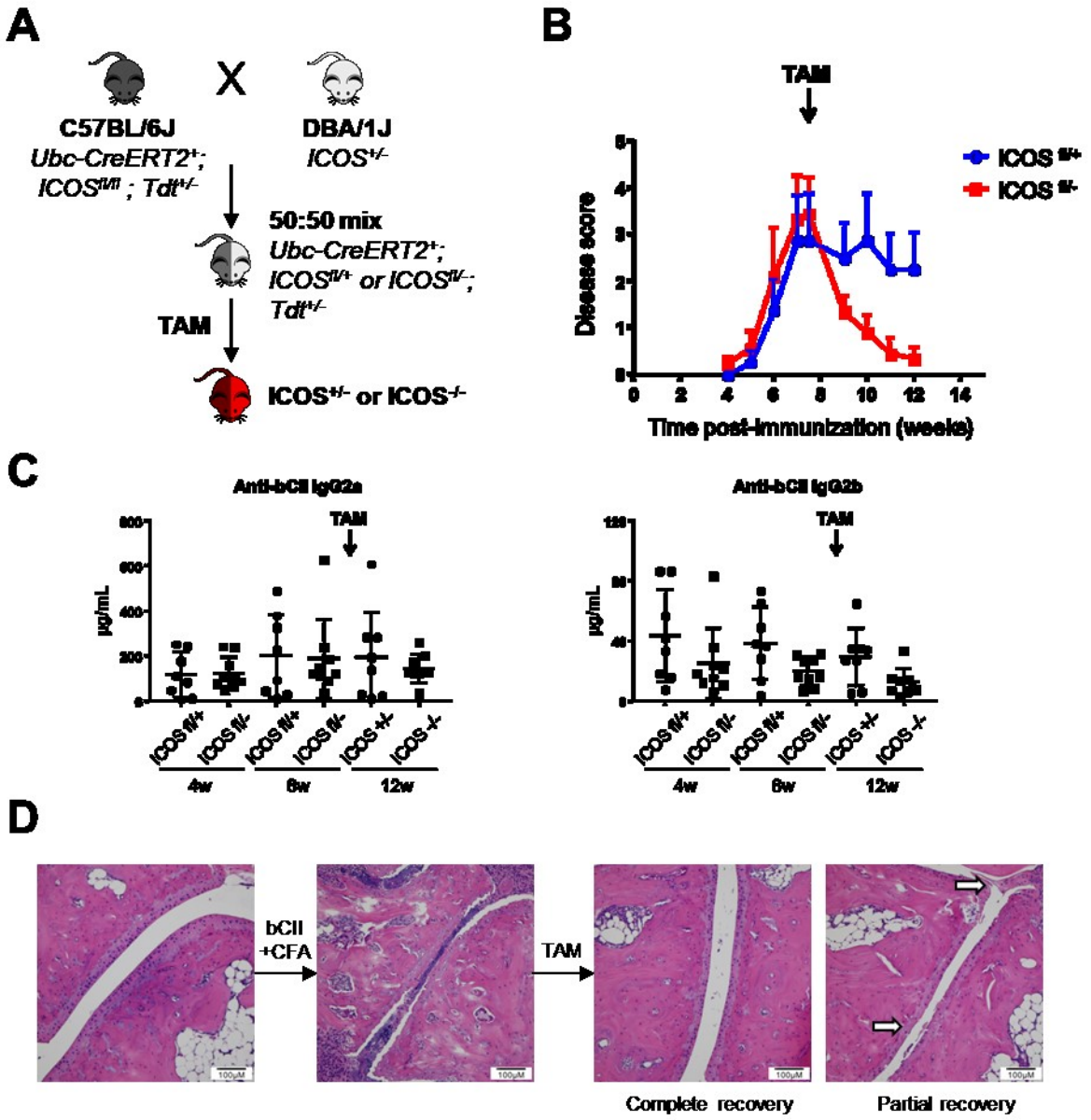


Figure 2.3 ICOS is essential for the maintenance of collagen-induced arthritis. **(A)** Experimental design of ICOS conditional KO experiments. UBC-CreERT2^{+} ICOS$^{fl/+}$ or ICOS$^{fl/-}$ mice are treated with tamoxifen for five consecutive days resulting in deletion of the floxed allele of ICOS. **(B)** Eight- to twelve-week-old mice described in (A) were immunized with bCII + CFA, then treated for five days with tamoxifen at the peak of disease. Disease scores were given by visual inspection according to the criteria described in Materials and Methods. Data pooled from two independent experiments, n= 8 ICOS$^{fl/+}$ and 9 ICOS$^{fl/-}$. **(C)** Serum was obtained from mice in (B) at 4, 6 and 12 weeks post-immunization and anti-bCII IgG2a or IgG2b titers were measured by ELISA. **(D)** Representative H&E stained histology images from mice in (B) at different disease stages. From left to right: before immunization, 7 weeks post-immunization, 12 weeks post immunization. Post-tamoxifen treatment pictures depict the spectrum of recovery in ICOS$^{fl/-}$ mice.

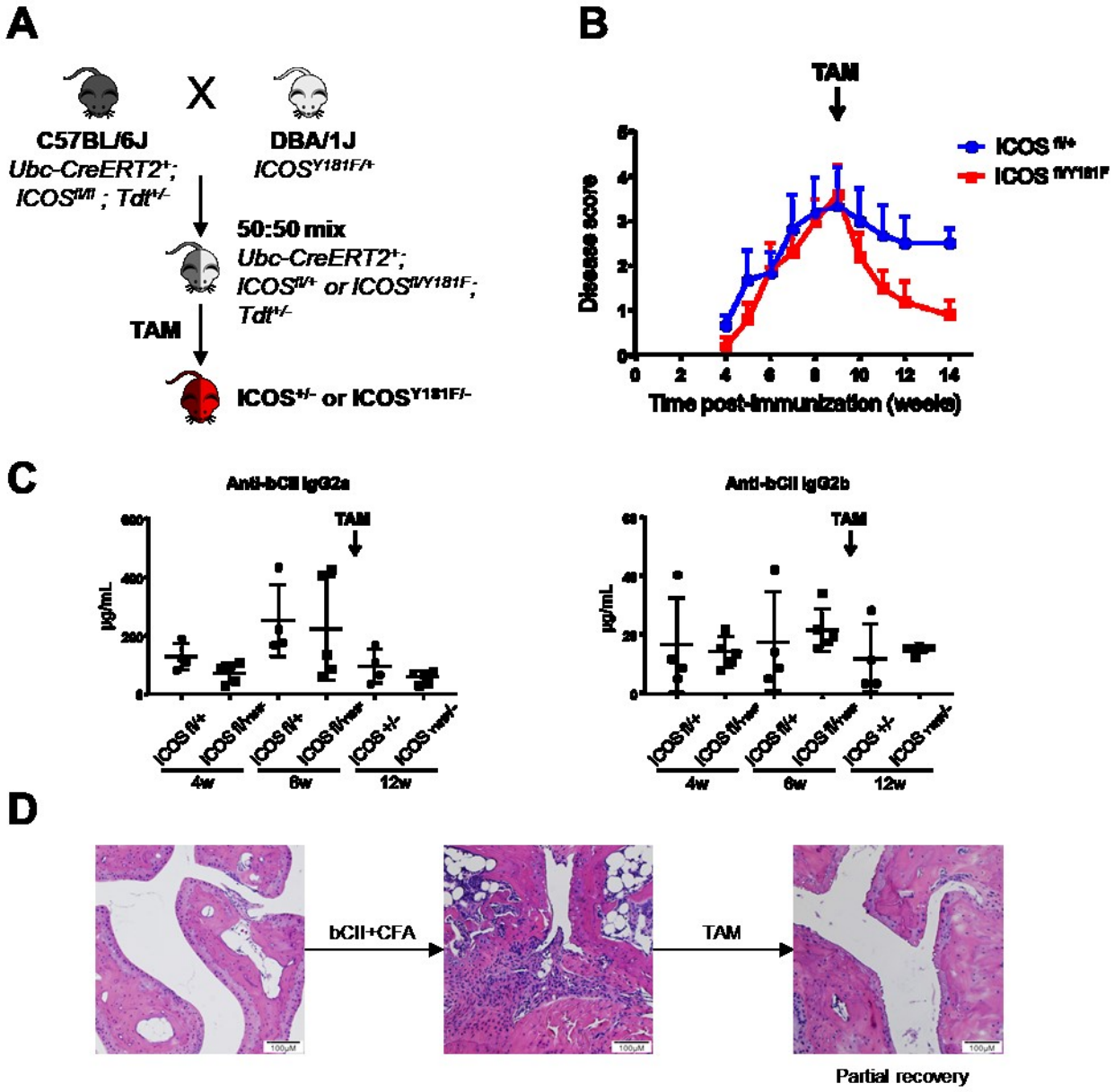


Figure 2.4 ICOS-PI3K signaling is a major contributor to maintenance of collagen-induced arthritis. **(A)** Experimental design of conditional ICOS-PI3K experiments. UBC-CreERT2^{+} ICOS$^{fl/+}$ or ICOS$^{fl/Y181F}$ mice are treated with tamoxifen for five consecutive days resulting in deletion of the floxed allele of ICOS. **(B)** Eight- to twelve-week-old mice described in (A) were immunized with bCII + CFA, then treated for five days with tamoxifen at the peak of disease. Disease scores were given by visual inspection according to the criteria described in Materials and Methods. Data pooled from two independent experiments, n= 6 ICOS$^{fl/+}$ and 10 ICOS$^{fl/Y181F}$. **(C)** Serum was obtained from mice in (B) at 4, 6 and 12 weeks post-immunization and anti-bCII IgG2a or IgG2b titers were measured by ELISA. **(D)** Representative H&E stained histology images from mice in (B) at different disease stages. From left to right: before immunization, 8 weeks post-immunization, 14 weeks post immunization.

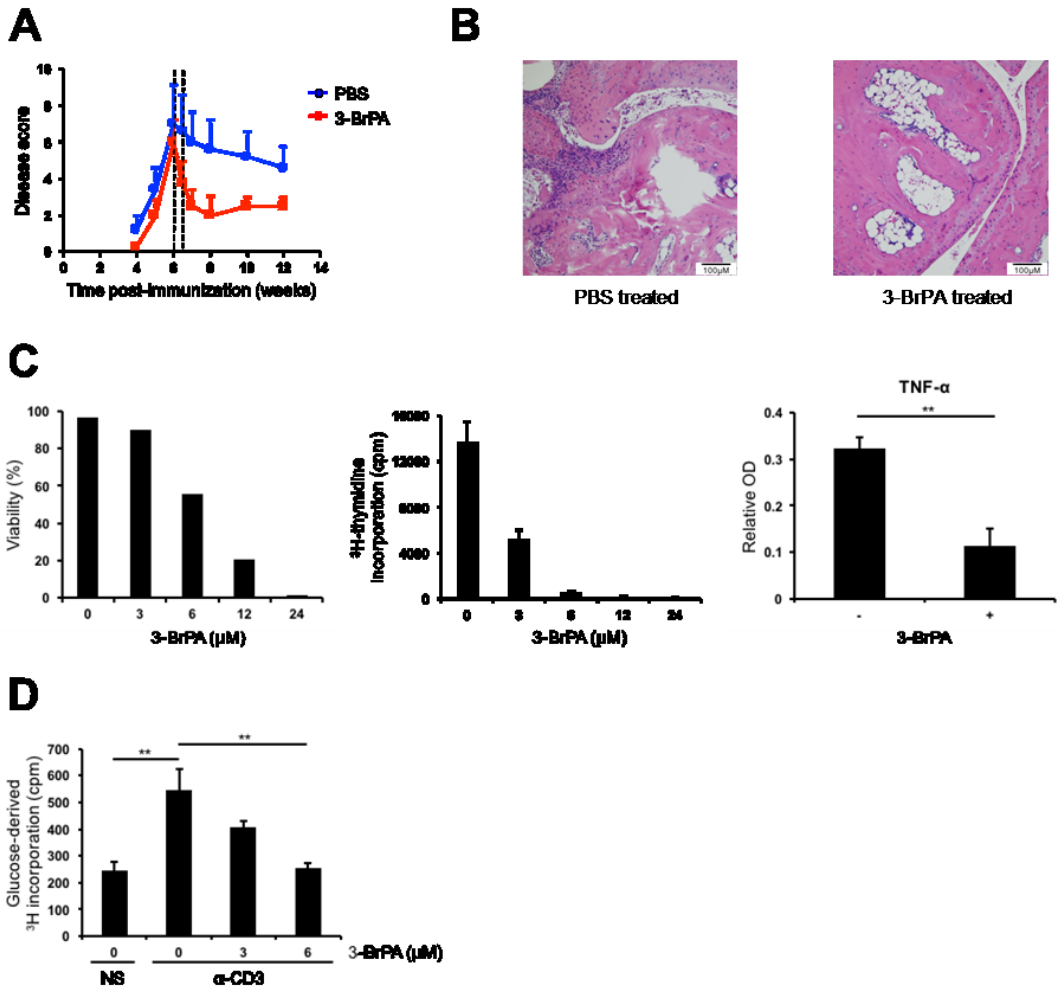
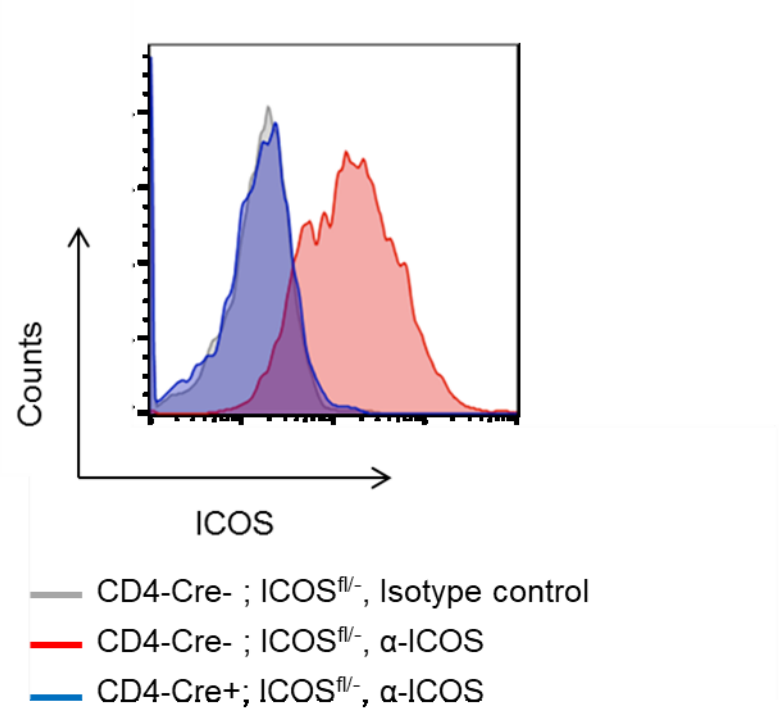
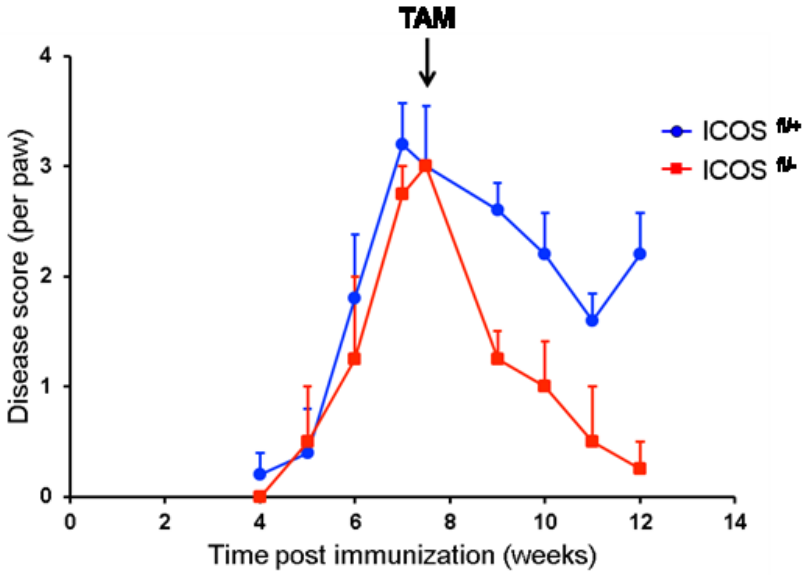


Figure 2.5 Glycolysis inhibitor ameliorates established collagen-induced arthritis. **(A)** Eight- to twelve-week-old WT DBA/1 were immunized with bCII + CFA, then treated with PBS or 5mg 3-BrPA/kg/day for 3 days at the peak of disease. Disease scores were given by visual inspection according to the criteria described in Materials and Methods. Data represents two independent experiments with similar results. n= 5 PBS and 4 3-BrPA. **(B)** Representative H&E stained histology images from mice in (A) at 12 weeks post-immunization. **(C)** Draining lymph node cells from WT DBA/1 mice were extracted two weeks post-immunization and restimulated *in vitro* for 48h with increasing concentrations of 3-BrPA. Viability was assessed by flow cytometry and a representative sample is shown. Proliferation was measured by ^{3}H-thymidine incorporation assay. TNF-α was detected by ELISA using culture supernatants taken from culture without (-) or with 3μM 3-BrPA (+). Data represents two independent experiments with similar results, n=3 for each group. **(D)** Lymph node cells from naïve WT DBA/1 mice were extracted and cultured for 48h with or without 1μg/mL α-CD3. During the last 4h, 3-BrPA was added at the indicated concentrations along with 1μCi/well D-[3-^{3}H]-glucose to measure glucose metabolism. Data pooled from two independent experiments. n=3 for α-CD3 + 6μM 3-BrPA and n=6 for the other groups. * $P < 0.05$, ** $P < 0.01$ by two-tailed Student's *t*-test (C) and one-way ANOVA with Tukey's multiple comparisons test (D).

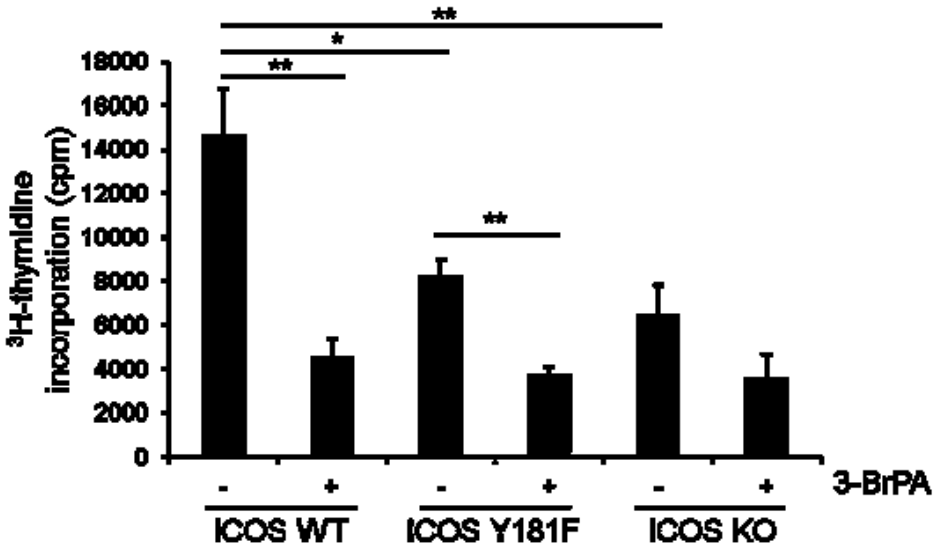
2.10 Supplementary figures and legends



Supplementary Figure 2.1 ICOS cKO allele is readily inactivated in T cells by Cre recombinase. Lymph node single cell suspensions isolated from from CD4-Cre^{-}; ICOS$^{fl/-}$ or CD4-Cre^{+}; ICOS$^{fl/-}$ mice were stimulated by soluble anti-CD3 antibodies (1 ug/ml) for 24 hours. ICOS expression levels on CD4 T cell blasts were assessed by flow cytometry.



Supplementary Figure 2.2 Regression of CIA in severely inflamed paws (score 3 or greater) after ICOS deletion. Data points represent mean scores ± SEM of five ICOS$^{fl/+}$ and four ICOS$^{fl/-}$ paws among the dataset described in Figure 3B.



Supplementary Figure 2.3 Dominant effect of 3-BrPA on T cell proliferation. Draining lymph node cells from ICOS WT and mutant mice were isolated two weeks post-immunization and restimulated *in vitro* with 100 μg/mL denatured bCII for 48h with or without 3μM 3-BrPA. ^{3}H-thymidine was added to the culture media for the last 8 h to assess cell proliferation. Data represent two independent experiments with similar results. n = 5 ICOS WT, 5 ICOS Y181F, and 4 ICOS KO. * $P < 0.05$, ** $P < 0.01$, by one-way ANOVA with Tukey's multiple comparisons test.

Chapter 3: ICOS costimulation is indispensable for the differentiation of T follicular regulatory cells

Chapter 3 presents original work in preparation for publication

ICOS costimulation is indispensable for the differentiation of T follicular regulatory cells

Vincent Panneton [1,2], Barbara C. Mindt [3,4], Yasser Bouklouch [1], Jinsam Chang [1,5], Mariko Witalis [1,5], Joanna Li [1,3], Albert Stancescu [1], Antoine Bouchard [1,5], John E. Bradley [6], Troy D. Randall [6], Jörg H. Fritz [3,4], Woong-Kyung Suh [1, 2, 3, 5, *]

[1] Institut de Recherches Cliniques de Montréal (IRCM), Quebec, Canada.

[2] Department of Microbiology, Infectiology and Immunology, University of Montreal, Quebec, Canada.

[3] Department of Microbiology and Immunology, McGill University, Quebec, Canada.

[4] McGill University Research Centre on Complex Traits (MRCCT), McGill University, Quebec, Canada.

[5] Molecular Biology Program, University of Montreal, Quebec, Canada.

[6] Department of Medicine, Division of Clinical Immunology and Rheumatology, University of Alabama at Birmingham, Alabama, United States.

* Correspondence: Woong-Kyung Suh, Institut de Recherches Cliniques de Montréal (IRCM), 110 av. des Pins Ouest, Montréal, Québec, Canada.

Email: woong-kyung.suh@ircm.qc.ca

Author contributions

V.P. and W-K.S. conceived and supervised the study. V.P., B.C.M., Y.B., J.C., M.W., J.L. and A.S. performed the experiments. V.P., Y.B., A.B. and W-K.S. analyzed the data. J.E.B., T.D.R. and J.H.F. contributed key reagents and resources. V.P. and W-K.S. wrote the manuscript. V.P., B.C.M., Y.B., M.W., J.L., A.B. and W-K.S. commented and revised the manuscript.

3.1 Abstract

The inducible costimulator (ICOS) is a T cell costimulatory receptor critical for humoral immunity. ICOS-deficient patients suffer from recurrent infections due to the lack of T follicular helper (Tfh) cells and protective antibodies. However, some patients also display signs of antibody-mediated autoimmunity. These findings may reflect a dual role of ICOS in facilitating the differentiation of Tfh and T follicular regulatory (Tfr) cells. While Tfh cells are known to provide help to B cells to produce high affinity antibodies, the main role of Tfr cells remains controversial. Using Foxp3-cre-mediated ICOS knockout (ICOS FC) mice, we show that T regulatory (Treg)-specific ICOS deletion drastically reduces the number of Tfr cells without altering Treg cell numbers. Importantly, we observed a lowered ratio of antigen-specific germinal center B (GC B) cells and increased anti-nuclear antibodies in ICOS FC mice, suggesting a rise of autoreactive GC B cells. We also noted variations in isotype composition of total and virus-specific antibodies in infected ICOS FC mice. Single-cell transcriptome analysis revealed shifts in transitory Tfr precursor populations in immunized ICOS FC mice. Mechanistically, our data suggest that ICOS promotes the Treg-to-Tfr transition by regulating KLF2 and NFAT2 with downstream impacts on Bcl6 and CXCR5 expression. Thus, our study demonstrates that ICOS is critical for Tfr cell generation and supports the role of Tfr cells in preventing the generation of autoantibodies during germinal center reactions.

3.2 Introduction

High affinity class-switched antibodies are essential for immune responses against pathogens. These antibodies arise from germinal centers (GCs) where T follicular helper (Tfh) cells facilitate the transition of GC B cells into antibody-secreting plasma cells (PCs) (69, 377). Tfh cells are defined by combined expression of their master transcription factor Bcl6 along with CXCR5, PD-1 and ICOS (378-381). Tfh cells mediate their helper functions through costimulation by CD40L and ICOS along with the production of the

cytokines IL-4 and IL-21 (382-384). Because dysregulation of Tfh cells and GC reactions can lead to humoral autoimmunity, they must be tightly controlled (385).

T follicular regulatory (Tfr) cells are a subset of $CD4^+$ $Foxp3^+$ regulatory T (Treg) cells found in and around germinal centers (183-186). Like Tfh cells, they express the chemokine receptor CXCR5 which is required for their migration towards B cell follicles (183-185). The transcription factor NFAT2 was recently shown to be required for CXCR5 upregulation by Tfrs, possibly to overcome BLIMP-1-mediated CXCR5 downregulation (193, 386, 387). There are no known lineage-defining factors specific to Tfr cells, although they require concomitant expression of Foxp3 and Bcl6 (187, 188). Despite other shared markers with Tfh cells such as PD-1 and ICOS, Tfr cells mostly originate from thymic Tregs and possess a T cell receptor (TCR) repertoire skewed towards self-antigens (183-185, 191). Under specific conditions, induced Tregs have also displayed the capacity to differentiate into Tfr cells that can be specific for the immunizing antigen (192). Interestingly, strong IL-2 signaling was shown to inhibit Tfr differentiation which is more akin to Tfh cells rather than Tregs (189). While the *in vivo* role of Tfr cells has been controversial, they display suppressive abilities on T cell proliferation, antibody secretion and cytokine production *in vitro* (190, 197). Several Tfr depletion models have been studied to understand their functions *in vivo*. Initially, adoptive transfer or mixed bone marrow (BM) chimera experiments showed that Tfr reduction had varying effects on GC responses, possibly due to unintended side effects such as impaired Treg function (183-185). More recently, Foxp3-specific deletion of Bcl6-expressing (Bcl6 FC) or CXCR5-expressing cells (Tfr-deleter) allowed for a more precise assessment of *in vivo* roles for Tfr cells (188, 198, 199, 202, 388). Results from these studies collectively suggest two roles for Tfr cells: suppression of autoantibody production and modulation of antibody responses more suggestive of “helper” functions (389).

The inducible costimulator (ICOS) is a member of the CD28 superfamily and is known to be expressed by activated T cells (2). ICOS was previously shown to be essential for the formation of Tfh cells and maintenance of Bcl6 expression (46, 95, 390). In both mice and humans, ICOS null mutations cause severe defects in GC reactions and antibody production driven by lack of Tfh cells (11-13, 332). Some ICOS-deficient patients

develop autoimmune symptoms such as rheumatoid arthritis and autoimmune neutropenia, suggesting a potential role for ICOS in Treg/Tfr compartments (391, 392). Indeed, ICOS deficiency in mice led to reduced Tfr cell numbers, although the underlying mechanisms have not been carefully analyzed (197, 203).

In this study, we used *Icos*$^{fl/fl}$ *Foxp3-cre* (ICOS FC) mice to evaluate the role of ICOS signaling in Treg and Tfr cells during GC reactions. Foxp3-specific loss of ICOS led to a significant decrease in Tfr populations after protein immunization or viral infection without affecting Treg cell numbers. Examination of antibody responses revealed significantly lowered IgG2b titers at steady state or after immune challenge along with increased anti-nuclear autoantibodies in ICOS FC mice. Single-cell transcriptomics and biochemical analyses suggest that ICOS may enhance the Treg to Tfr transition through KLF2 and NFAT2 regulation. Overall, our findings indicate that the major role of ICOS in regulatory T cell compartments during GC reactions is to control Tfr differentiation and highlight the importance of Tfr cells in preventing autoantibody generation.

3.3 Results

3.3.1 ICOS is required for Tfr cell generation during GC reactions against protein antigens

To assess the role of ICOS in Treg lineage cells, we used a Foxp3-cre system which allows for the specific abrogation of ICOS expression in all Treg and Tfr cells. Throughout this study, we used *Icos*$^{+/+}$ *Foxp3-cre*$^{+}$ controls (ICOS WT) for *Icos*$^{fl/fl}$ *Foxp3-cre*$^{+}$ mice (ICOS FC). First, we analyzed splenocytes 12 days after immunization with NP-OVA/alum by flow cytometry and subdivided the CD4^{+} Foxp3^{+} regulatory T cell compartment into Treg (CD4^{+} Foxp3^{+} CXCR5^{-} PD-1^{-}), PD-1^{-} Tfr (CD4^{+} Foxp3^{+} CXCR5^{+} PD-1^{-}) and PD-1^{+} Tfr (CD4^{+} Foxp3^{+} CXCR5^{-} PD-1^{+}) subsets (Fig. 3.1 A). PD-1^{+} Tfr cells form a GC-resident mature population with similarities to Tfh cells whereas PD-1^{-} cells may represent a precursor population (186, 195, 393). ICOS was expressed in both Tfh

($CD4^+$ $Foxp3^-$ $CXCR5^+$ $PD\text{-}1^+$) and Treg/Tfr cells, with $PD\text{-}1^+$ Tfr cells showing the highest surface levels (Supplementary Fig. 3.1 A).

We confirmed that ICOS deletion was limited to $Foxp3^+$ cells and did not occur in Tfh cells (Supplementary Fig. 3.1 B). We observed no change of total $Foxp3^+$ populations in ICOS FC mice (Fig. 3.1 B). However, we found ~2-fold and ~4-fold decreases in $PD\text{-}1^-$ and $PD\text{-}1^+$ Tfr cell proportions and numbers, respectively. Next, we examined Tfh, GC B cells and plasma cells since lack of Tfr cells has been shown to increase these populations in some experimental settings (183-185). We did not observe significant differences in Tfh cell proportion or absolute numbers (Fig. 3.1 A). We also detected no quantitative differences in GC B cell and plasma cell populations (Fig. 3.1 B-C). Consistently, NP-specific IgG1 antibodies in serum did not show changes in total (NP30) or high affinity (NP7) titers (Fig. 3.1 D). Taken together, these results indicate that loss of ICOS in $Foxp3^+$ cells leads to a reduced number of Tfr cells with proportionally increased Treg cells, suggesting an impaired Treg-to-Tfr transition.

3.3.2 ICOS is required for Tfr generation during anti-viral response

To evaluate the role of ICOS in regulatory T cell compartments during an anti-viral immune response, we infected mice with influenza A virus (IAV). We analyzed splenocytes by flow cytometry 30 days after infection since it has been shown that Tfr generation is delayed due to high levels of IL-2 present in the early stages of viral infection (189). We observed an increased proportion of Tregs and decreases in Tfr populations in spleens of ICOS FC mice reminiscent of results from protein immunization experiments (Fig. 3.2 A). As before, we did not detect significant differences in Tfh populations (Fig. 3.2 B). To exclude the possibility that ICOS only modifies CXCR5 expression levels measured by flow cytometry without affecting Tfr cells, we used the essential Tfr transcription factor Bcl6 as an alternative Tfr marker and confirmed significant Tfr decreases in ICOS FC mice (Supplementary Fig. 3.2). Next, we examined the expansion of GC B cell populations that recognize the IAV nucleoprotein using recombinant tetramers (Flu tetramer) (394). Interestingly, we observed a trend of increased total GC

B cells with a significantly decreased proportion of IAV-specific GC B cells in ICOS FC mice (Fig 3.2 C). This results in a significant increase of extraneous GC B cells, some of which could be autoreactive in nature. On the other hand, we did not observe significant differences in splenic plasma cells (Fig. 3.2 D). However, we found significant decreases of IAV-specific IgG2b serum titers (Fig. 3.2 E). Consistent with the role of anti-viral antibodies in the overall control of influenza virus (395), we observed that ICOS FC mice had more severe weight loss 9-10 days post-infection (Supplementary Fig. 3.3 A). This reduced anti-viral IgG2b titer was well correlated with a reduction of total IgG2b titers in infected ICOS FC mice (Supplementary Fig. 3.3 B). Further, we noticed that uninfected ICOS FC mice had decreased basal levels of IgG2b and IgG1 (Supplementary Fig. 3.3 C), suggesting that ICOS-expressing Treg or Tfr cells may work as “helper” T cells for certain antibody isotype switching. Thus, congruent with data from protein immunization experiments, this infection model confirmed the critical role of ICOS for efficient Tfr generation. Further, increases of non-viral specific GC B cells in ICOS FC mice confirms the regulatory role of Tfr cells in shaping GC responses.

3.3.3 Treg-specific ICOS deficiency leads to anti-nuclear antibody production

Since there is ongoing cell death and release of autoantigens within the GC, autoreactive GC B cell clones can expand and differentiate into PCs with help from Tfh cells if not restrained (385). Given that Foxp3-specific ICOS ablation results in reduced numbers of Tfr cells which are thought to suppress self-reactive antibody production, we investigated whether ICOS FC mice displayed signs of autoimmunity. We did not observe immune infiltration in the kidneys, lungs, spleen, pancreas, and salivary glands of 5-month-old ICOS FC mice (Fig. 3.3 A). Next, we used HEp-2 slides to look for the presence of anti-nuclear antibodies (ANAs) which are a hallmark of autoimmunity (Fig. 3.3 B) (396). We did not detect significant spontaneous increases of ANAs in serum samples of 6-month-old ICOS FC mice. However, we found that both single NP-OVA/alum immunization and secondary challenge with the same antigen resulted in significantly higher ANAs in ICOS FC mice. Additionally, ICOS FC mice infected with influenza A virus presented similar increases in ANA levels. These results suggest that immunization or

infection augments adventitious generation of autoantibodies which is normally suppressed by Tfr cells.

3.3.4 ICOS-deficient Treg cells show impaired transition to Tfr cells

To better understand the role of ICOS in Tfr cell differentiation, we performed single-cell transcriptome analysis of $CD4^+$ $Foxp3^+$ splenocytes sorted from ICOS WT and ICOS FC mice immunized with NP-OVA/alum. To collect cells in a dynamic Treg-to-Tfr transition stage, we prepared samples 6 days post-immunization, a timepoint where Tfr cells begin to appear (189, 197). After sorting, we added back sorted conventional $CD4^+$ $Foxp3^-$ T cells (~10% of total) into the sorted $CD4^+$ $Foxp3^+$ T cell pool to provide a reference point (Supplementary Fig. 3.4 A, cluster 4). We used Tfr-defining genes (*Cxcr5, Pdcd1, Foxp3, Bcl6*) to calculate a "Tfr identity score" and selected three clusters that are predicted to contain Tfr precursors and mature Tfr cells (Supplementary Fig. 3.4 B, clusters 3, 5, 8 black arrows). When compared amongst each other, these cells formed three distinct clusters with a spectrum of Tfr identity score (Fig. 3.4 A, clusters 1, 2, 3). Pseudotime trajectory analysis revealed a progressive differentiation from cluster 1 towards cluster 3 (Fig. 3.4 B). Interestingly, we observed that ICOS FC mice presented a 3-fold increase of cells in cluster 2 and a 3-fold decrease in cluster 3 (Fig. 3.4 A). This suggests that Tregs could be halted in their transition to Tfr cells due to loss of ICOS expression. To substantiate this idea, we compared gene expression profiles of the three clusters (Fig. 3.4 C, left). Cluster 3 had the highest levels of key Tfr signature genes (*Cxcr5, Pdcd1, Bcl6*), but reduced expression of typical Treg signature genes such as *Foxp3, Il2ra* (CD25), *Ctla4* and *Tnfrsf18* (GITR) when compared to clusters 1 and 2. However, when compared with $CD4^+$ $Foxp3^-$ conventional T cells, cluster 3 still mostly maintained higher levels of these Treg signature genes (Fig 4 C, right). Recent studies have identified CD25 downregulation as a key event in Tfr differentiation (189, 195). Similarly, we noticed that CD25 expression is inversely correlated with the levels of CXCR5 and PD-1 in Tfr cells, consistent with a previous report by Wing *et al.* (195) (Supplementary Fig. 5 A-B). Congruent with CD25 protein expression levels, *Il2ra* gene expression was significantly dampened in cluster 3 Tfr cells compared to those in clusters 1 and 2 (Fig. 4 D). Interestingly, cluster 2 cells from ICOS FC mice displayed significantly

higher *Il2ra* levels suggesting that ICOS could be involved in CD25 downregulation. ICOS deletion also resulted in significantly elevated CD25 protein expression in certain Tfr subsets (Supplementary Fig. 3.5 B). Apart from CD25, we noticed a significant decrease of *Tgfb1* expression in ICOS FC cluster 3 cells (Fig. 3.4 E). This could explain reduced IgG2b titers observed in ICOS FC mice since TGF-β1 is a known class switch factor for this isotype (397).

Another key event in the Treg-to-Tfr transition is CXCR5 upregulation (183-185, 193). ICOS was previously shown to regulate CXCR5 by suppressing KLF2 expression in Tfh cells (96). This mechanism may operate in Treg/Tfr cells since we observed that *Icos* and *Klf2* gene expression is inversely correlated and that all three clusters had higher *Klf2* expression in ICOS FC mice (Fig. 3.4 F). KLF2 was also shown to dampen Tfh differentiation by increasing S1PR1 and BLIMP-1 expression levels (97). We observed matching expression patterns of *Klf2, S1pr1 and Prdm1* with opposed *Bcl6* expression in all clusters (Fig. 3.4 G). Further, ICOS FC mice showed an accumulation of *Klf2*$^{+}$ *S1pr1*$^{+}$ *Prdm1*$^{+}$ cells in cluster 2 with reduced *Bcl6* expression in clusters 2 and 3. Thus, these results suggest that ICOS is required for a few key steps in the Treg to Tfr transition and that failure of these processes seem to lead to an accumulation of putative Tfr precursors.

3.3.5 ICOS ablation causes decreased NFAT2 activation and impaired CXCR5 expression in Tregs.

To test the potential role of ICOS in upregulating CXCR5 expression, we further analyzed our single-cell transcriptome data. We found that clusters 1 and 2 from ICOS FC mice had lowered proportions of *Cxcr5*$^{+}$ cells along with a diminished average *Cxcr5* expression (Fig. 3.5 A). Next, we investigated the potential impacts of ICOS signaling on NFAT2 (product of *Nfatc1* gene), a transcription factor known to directly bind to the promoter region of C*xcr5* (193). Importantly, we and others have previously shown that ICOS can potentiate TCR-mediated calcium flux, a key factor in NFAT activation (47, 398). Consistently, we found that the average expression levels of known NFAT target genes (Supplementary Table 1) was significantly decreased in ICOS FC cluster 2 cells

when compared to ICOS WT control (Fig. 3.5 B, top). Since Tfr precursor-like cells in cluster 2 also express the highest levels of *Nfatc1* (Fig. 3.4 C), we tested whether *Nfatc1* expression level was reduced in ICOS FC cluster 2 cells. However, we did not find significant differences in the expression level of the *Nfatc1* gene itself (Fig. 3.5 B, bottom). Nonetheless, we noticed that the protein levels of ICOS and NFAT2 trended higher in Tfr cells compared to Treg cells, suggesting a potential role of ICOS-NFAT2 in the Treg-to-Tfr transition (Fig. 3.5 C). Consistent with our single-cell transcriptomics data, we found that both PD-1$^-$ and PD-1$^+$ Tfr subsets from infected ICOS FC mice displayed significantly decreased CXCR5 expression (Fig. 3.5 D). Congruent with the unaltered *Nfatc1* mRNA levels in ICOS FC Tfr populations, we found that NFAT2 protein expression levels were not decreased in ICOS-deficient Tfr subsets (Fig. 3.5 E).

Because we observed that ICOS deletion led to decreased expression of NFAT target genes but not NFAT2 itself, we investigated other regulatory mechanisms. It has been established that NFAT2 activity depends on its dynamic nuclear-cytoplasmic shuttling controlled by its phosphorylation status (398). To evaluate if ICOS can regulate this process, we expanded purified splenic CD4$^+$ T cells *in vitro* and acutely restimulated them through the TCR (CD3) with or without ICOS costimulation. Cytoplasmic and nuclear NFAT2 proteins of varying migration properties were then harvested separately by fractionation and quantified by Western blot (Fig. 3.5 F). Without restimulation, 5-10 min incubation at 37ºC led to the increase of hyperphosphorylated (slower migrating) NFAT2 species in the cytoplasm with concomitant disappearance of hypophosphorylated (faster migrating) NFAT2 species in the nucleus (lanes 1 and 2 in cytoplasmic and nuclear fractions). This is presumably due to temperature-induced shifts in the activity of NFAT phosphatases and kinases (398). As predicted, CD3 ligation increased NFAT2 nuclear levels (lanes 3 and 4). Importantly, combined CD3/ICOS stimulation further augmented nuclear NFAT2 levels at 10 min when compared with CD3 stimulation alone (Fig. 3.5 G). This increase was dependent on ICOS since it was abolished when ICOS KO CD4$^+$ T cells were used (Fig. 3.5 H). Taken together, our biochemical data shows that ICOS costimulation can potentiate TCR-driven NFAT2 activation in CD4$^+$ T cells. Thus, we propose that ICOS could act upstream of the NFAT2-CXCR5 signaling axis known to be one of the key mechanisms for early Tfr differentiation (193).

3.4 Discussion

In this study, we identified ICOS as a critical costimulatory receptor for Tfr differentiation upon immune challenges. We show that Foxp3-specific ICOS deletion results in altered gene expression patterns at the single-cell level leading to an accumulation of Tfr precursor-like cells and a substantial reduction of the fully differentiated "GC Tfr" population. Mice with impaired Tfr differentiation showed an increase of extraneous GC B cells without increases of total GC B cell or Tfh cell numbers. ICOS FC mice also showed an elevated incidence of autoantibody production after GC reactions, indicating that autoreactive B cells can expand in the absence of Tfr cells. In contrast, total and virus-specific IgG2b antibody titers under steady-state and upon influenza infection were diminished in ICOS FC mice. Our single-cell data strongly suggests that ICOS-mediated downregulation of KLF2 plays a key role in shaping chemokine receptor expression and balancing BLIMP-1-Bcl6 levels in developing Tfr cells. Our biochemical analysis demonstrates that ICOS signaling can also augment NFAT2 nuclear localization, potentially counterbalancing the negative impacts of BLIMP-1 on CXCR5 expression (193, 386).

Our single-cell RNA transcriptome analysis indicates that dynamic changes in gene expression patterns drive the Treg-to-Tfr transition. Trajectory analysis reveals that the $CD25^+$ $BLIMP\text{-}1^+$ activated Treg subset progressively gains follicular features such as CXCR5 and Bcl6. Both single-cell transcriptomics and flow cytometry data demonstrate that Bcl6 and CXCR5 levels are highest in $CD25^-$ "GC-Tfr" cells, the main $Foxp3^+$ cells shown to be found within the GC (195). During this Treg-to-Tfr transition, ICOS seems to utilize a mechanism that has been shown to be critical for the differentiation and maintenance of Tfh cells – timely downregulation of KLF2 (97). As such, Tfr precursor cells reduced levels of *Klf2* and its main target genes *S1pr1* and *Prdm1* along the predicted Tfr trajectory. In contrast, *Cxcr5* and *Bcl6* expression levels were progressively elevated in cells that have dampened *Klf2* target genes, presumably due to the lack of BLIMP-1-mediated suppression of Bcl6. Importantly, this progression towards "GC-Tfr" was halted at the $Klf2^{hi}$ $Prdm1^{hi}$ stage in ICOS-deficient Treg cells. Therefore, we propose

that ICOS-mediated KLF2 downregulation is a key molecular event that initiates follicular T cell programing in Tfr cells.

In addition to KLF2, ICOS may utilize NFAT2-dependent pathways to support CXCR5 expression. Although NFAT2 is highly expressed in both Tfh and Tfr cells, abrogation of NFAT2 expression in T cells was shown to cause more pronounced defects in Tfr generation as opposed to Tfh differentiation due to compromised CXCR5 expression (193). This observation fits well with the idea that developing Tfr cells need higher concentrations of nuclear NFAT2 to overcome elevated levels of BLIMP-1 (known repressor of *Cxcr5* gene (386)) that are present in Tfr precursor cells. Our biochemical data indicate that ICOS ligation augments the amount of nuclear NFAT2 in TCR-activated $CD4^+$ T cells. Based on these, we speculate that ICOS costimulation reinforces CXCR5 expression in early Tfr populations leading to the establishment of "GC-Tfr" differentiation.

While we showed that ICOS signaling can maintain NFAT2 in the nucleus, the mechanism remains unclear. Nuclear transport of NFAT family members occurs through their dephosphorylation by calcineurin, a Ca^{2+} dependent phosphatase (398). We have previously shown that ICOS signaling can potentiate TCR-induced intracellular Ca^{2+} flux, although we did not determine if this resulted in increased NFAT activity (47). Conversely, nuclear export of NFAT2 is triggered by phosphorylation through several kinases including GSK3β (399). In turn, GSK3β activity can be inhibited by Akt-mediated phosphorylation of residue Ser9 (400). We and others have shown that ICOS stimulation can increase PI3K/Akt signaling, specifically through its Y^{181}MFM cytoplasmic tail motif (42, 43, 46). Thus, we suggest that ICOS could maintain NFAT2 nuclear localization by increasing its import through enhanced Ca^{2+} signaling and/or decrease its export by inhibiting GSK3β.

The biological roles of Tfr cells during GC reactions remain ill-defined. Tfr depletion studies using Bcl6 FC mice have shown small decreases of antibodies specific for the immunizing antigen (188, 200, 401). One study also showed spontaneous increases of autoantibodies and multi-organ lymphocytic infiltration in aged mice (199). Along the same line, high levels of anti-nuclear antibodies were generated in Bcl6 FC mice after influenza infection (189). ICOS FC mice do not display age-related

autoantibodies or lymphocytic infiltrations, suggesting that the impact of ICOS-deficiency on Treg cells is weaker than that of Bcl6-deficiency. In this context, Bcl6-deficient Treg cells (as opposed to bona-fide Tfr cells) could have contributed to some of these phenotypes considering recent reports that Bcl6-deficient Tregs have compromised suppressive functions in other immune settings (201, 402, 403). ICOS deficiency was shown to impair the suppressive ability of Tregs in asthma and type 1 diabetes murine models, but no defects in GC reactions were reported (56, 169). Combined with a ~4-fold reduction in the Tfr number, it seems likely that humoral immune defects in ICOS FC mice are mainly due to reduced Tfr numbers. Another potential role for Tfr cells is to promote the generation of antigen-specific antibodies. Bcl6 FC mice produce reduced amounts of IgE and IgG1 in certain immunization protocols (200, 401). We found that both basal IgG2b (in unimmunized mice) and anti-influenza IgG2b titers are lower in ICOS FC mice. This correlated well with reduced amounts of TGF-β1 mRNA in the GC-Tfr population. However, further work is required to clarify the mechanisms and biological implications of this finding.

In sum, we showed that ICOS is critically important for Tfr differentiation. ICOS-mediated downregulation of KLF2 and its target genes can shape the Bcl6-driven Tfr programming whereas an ICOS-NFAT2-CXCR5 signaling axis may reinforce CXCR5 expression during Tfr differentiation. Our data supports the view that the main role of Tfr cells is to suppress the expansion of self-reactive GC B cells during GC reactions, and we believe that our ICOS FC mouse provides a complementary model to dissect Tfr differentiation and function.

3.5 Methods

3.5.1 Mice and animal procedures

C57BL/6 and *Foxp3*$^{YFP\text{-}Cre}$ mice (Jax 016959) (404) were purchased from the Jackson Laboratory. ICOS conditional knockout mice were generated in C57BL/6 background as previously described (405). ICOS germline knockout mice have been

backcrossed onto C57BL/6 background for more than ten generations (13). All mice were housed in the Institut de Recherches Cliniques de Montréal animal care facility under specific pathogen-free conditions. Animal experiments were performed in accordance with animal use protocols approved by the Institut de Recherches Cliniques de Montréal Animal Care Committee. All mice were 8-12 weeks of age unless specified otherwise. For protein immunization, mice were injected intraperitoneally with 100µg of 4-Hydroxy-3-nitrophenylacetyl hapten-17 (NP17)-OVA (Biosearch Technologies, 1µg/mL) mixed with Imject Alum (Thermofisher) in a 1:1 ratio. For viral infections, mice were infected intranasally with a sublethal dose of influenza A virus H1N1 (strain A/Puerto Rico/8/34 (PR8), 10 PFU/20g body weight).

3.5.2 Flow cytometry

For analysis, single-cell suspensions were prepared by mechanical disruption of spleens unless specified otherwise. Viability was assessed by staining $1x10^8$ cells/mL with fixable viability dye eFluor 780 (ThermoFisher) for 20 mins at 4ºC. Fc receptors were blocked using anti-CD16/CD32 (BioXCell). For intracellular staining, cells were fixed and permeabilized using the Transcription Factor Staining Buffer Set (ThermoFisher). Surface or intracellular staining was performed at $1x10^8$ cells/mL for 20 mins at 4ºC. The following antibodies were used. BD Biosciences: anti-CD4 BUV395 (GK1.5), anti-PD-1 BV421 (RMP1-30), anti-CD95 PE-Cy7 (Jo2), anti-Bcl6 PE (K112-91). ThermoFisher: anti-B220 PerCP-eFluor 710 (RA3-6B2), anti-CXCR5 Biotin (SPRCL5), Streptavidin PE-Cy7, anti-ICOS FITC (7E.17G9), anti-Foxp3 APC (FJK-16s), anti-B220 eFluor 450 (RA3-6B2), anti-CD25 PE (PC61.5). BioLegend: anti-NFATc1 PE (7A6), anti-GL7 FITC (GL7). To identify influenza-specific B cells, we used tetramerized recombinant nucleoproteins conjugated with APC or PE (Flu tetramer) provided by Dr. Troy Randall (394). Data was acquired using a BD LSRFortessa and analyzed using FlowJo v10 (BD Biosciences).

3.5.3 ELISA

Serum samples were obtained from blood collected from the submandibular vein at the indicated timepoints. Plates were coated with either goat anti-mouse IgG (SouthernBiotech), NP30-BSA and NP7-BSA (Biosearch Technologies), or heat-inactivated influenza A viruses overnight at 4ºC. Serum samples underwent a 2-fold serial dilution starting from the indicated initial dilution. Bound antibodies were detected using alkaline phosphatase-conjugated anti-IgG1/2b/2c/3, IgM or IgA and *p*-nitrophenyl phosphate substrate (SouthernBiotech). The reaction was stopped by adding 1.5 N NaOH solution and optical density was measured at 405 nm.

3.5.4 Histology

Organs were dissected and fixed in 10% neutral buffered formalin for 12 h at 4ºC. Organs were then washed in 1x PBS, embedded in paraffin, and cut into 5 µM sections. Slides were stained with H&E to examine immune cell infiltration of organs.

3.5.5 Anti-nuclear antibody assay

Serum samples were obtained from blood collected from the submandibular vein at the indicated timepoints and incubated on Kallestad HEp-2 slides (Bio-Rad) according to the manufacturer's instructions. Bound antibodies were detected using goat anti-mouse IgG Alexa Fluor 555 (ThermoFisher). Fluorophore signals were visualized using a DMRB fluorescence microscope (Leica). Nuclear MFI was quantified using ImageJ.

3.5.6 Single-cell RNA sequencing

Splenocytes from *Foxp3*$^{YFP\text{-}cre}$*Icos*$^{+/+}$ (ICOS WT) and *Foxp3*$^{YFP\text{-}cre}$*Icos*$^{fl/fl}$ (ICOS FC) male mice were isolated 6 dpi with NP-OVA/alum and stained with anti-CD4 Alexa Fluor

647 (GK1.5, BioLegend), anti-TCRβ PE-Cy7 (H57-597, BioLegend) and propidium iodide (ThermoFisher). Live (PI^-) conventional (YFP^-) and regulatory (YFP^+) $CD4^+TCR\beta^+$ T cells were sorted with a BD FACSAria (BD Biosciences) to >95% purity. Sorted conventional and regulatory T cells were mixed in a 1:10 ratio to provide an internal control. A total of 13500 cells from ICOS WT and ICOS FC mice were sent for library preparation. Libraries were generated using the following components from 10x Genomics: Chromium Next GEM Chip G Single Cell kit, Chromium Next GEM Single Cell 3' GEM, Library & Gel Bead kit v3.1, Chromium i7 Multiplex kit. Sequencing was performed by Genome Québec using a NovaSeq 6000 (Illumina) with a flow cell S1 PE28*91.

Reads alignment

Using *Cellranger* 4.0.0 (from *10xGenomics*®), we generated a custom reference genome using the GRCm38.p6 (mm10) assembly procured from *Ensembl* to which we added the *Ires-Yfp-iCre* sequence as described in its design map (404). The alignment of the reads was performed using the same software and the resulting expression matrix was loaded into *R* version 3.6.1 (from the R Foundation for Statistical Computing) to conduct analysis.

Single cell expression matrix analysis

The expression matrices were stored into an R Seurat object available in the package *Seurat* version 3.0 (406) to ease the analysis. ICOS WT and ICOS FC samples were merged during the filtering phase which consisted of the elimination of any cell that presented more than 10% mitochondrial RNA contamination as well as any cell with less than 200 unique genes expressed. The expression matrix was then log normalized and scaled. We identified the most differentially expressed genes within the samples and proceeded with a dimensional reduction using a principal component analysis (PCA) approach based on the 2000 most variable features. We selected the first 30 most important eigenvectors produced by the PCA to construct a *Shared Nearest Neighbor* (SNN) graph and used *Modularity Optimizer version 1.3.0* (407) to identify 13 clusters. The cells were projected on a 2D space using a *Uniform Manifold Approximation and Projection UMAP* method (408). We isolated 3 clusters of interest based on their markers and moved the normalized expression matrix into an R *cell_data_set* object

available in the package *Monocle3* version 0.2.3 (409). Using the dimensionally reduced matrices of expression, a differentiation trajectory was constructed. The cells were then ordered along the trajectory and pseudotime was computed. We further confirmed the consistency of our trajectory analysis using a diffusion map-based approach (available in package *destiny* version 2.0.4) which has proven to be more robust to noise (410). We computed NFAT signaling gene expression score using the *AddModuleScore* function available in the *R* library *Seurat v3.0*. The list of NFAT target genes was established using the PANTHER classification system combined with data from literature and can be found in Supplementary table 3.1 (193, 411-413).

3.5.7 $CD4^+$ T cell activation and Western blot analysis

$CD4^+$ T cells were isolated from spleens and lymph nodes using the EasySep mouse $CD4^+$ T cell isolation kit (StemCell Technologies) according to the manufacturer's instructions. Purified T cells were stimulated from 2 days in complete RPMI 1640 (10% FBS, 1 unit/mL penicillin, 1 µg/mL streptomycin, 55 µM β-mercaptoethanol and 10 mM HEPES) with plate-bound anti-CD3 (BioXCell, 3 µg/mL) and soluble anti-CD28 (ThermoFisher, 2 µg/mL). For restimulation, $CD4^+$ T cell blasts were incubated for 3 mins at room temperature with the indicated combination of the following antibodies: 1 µg/mL Armenian hamster IgG isotype control (BioXCell), 1 µg/mL anti-CD3e (145-2C11, ThermoFisher) and 2 µg/mL anti-ICOS (C398.4a, BioLegend). Goat anti-hamster IgG (20 µg/mL, SouthernBiotech) was added for crosslinking and cells were immediately incubated for the indicated timepoints in a 37ºC waterbath. Restimulation was stopped using ice-cold STOP buffer (PBS, 10% FBS, 1 mM Na_3VO_4, 1 mM EDTA). Cytoplasmic and nuclear fractions of restimulated T cells were obtained using the NE-PER kit (ThermoFisher) according to the manufacturer's instructions. Lysates were boiled in Laemmli buffer and samples were run on SDS-PAGE gels. Proteins were transferred to Amersham nitrocellulose membranes (GE Healthcare). Membranes were blocked using 3% BSA in TBS-T. The following antibodies were used for detection according to manufacturer's instructions: anti-NFAT2 (D15F1, Cell Signaling Technology), anti-α-tubulin (2144, New England Biolabs), anti-Histone H3 (4499, New England Biolabs) and

anti-mouse IgG-HRP (Santa Cruz Biotechnology). Detection was performed using Amersham ECL prime kits (GE healthcare) and images were captured using a ChemiDoc imaging system (Bio-Rad). Band quantification was performed using ImageJ. NFAT2 band intensity was normalized based on intensity of loading controls (α-tubulin for cytoplasmic and histone H3 for nuclear). Band intensity values are presented as a percentage of the highest intensity NFAT2 band for each fraction.

3.5.8 Statistical analysis

Data is presented as mean ± SEM unless specified otherwise. For single comparisons, statistical significance was judged using two-tailed Student's *t*-tests. For multiple comparisons, one-way ANOVA with Tukey's multiple comparison tests or two-way ANOVA with Holm-Sidak multiple comparison tests were used. For single-cell gene expression comparisons between clusters, the Wilcoxon signed-rank test was used. R^2 values were obtained by linear regression. Statistical significance was judged based on *p* values and is indicated as follows: **p*<0.05, ***p*<0.01, ****p*<0.001. Analysis was performed using Prism 7 (GraphPad software).

3.5.9 Data accessibility

Single-cell transcriptome data have been deposited in the GEO database under the accession number GSE164995.

3.6 Figures and figure legends

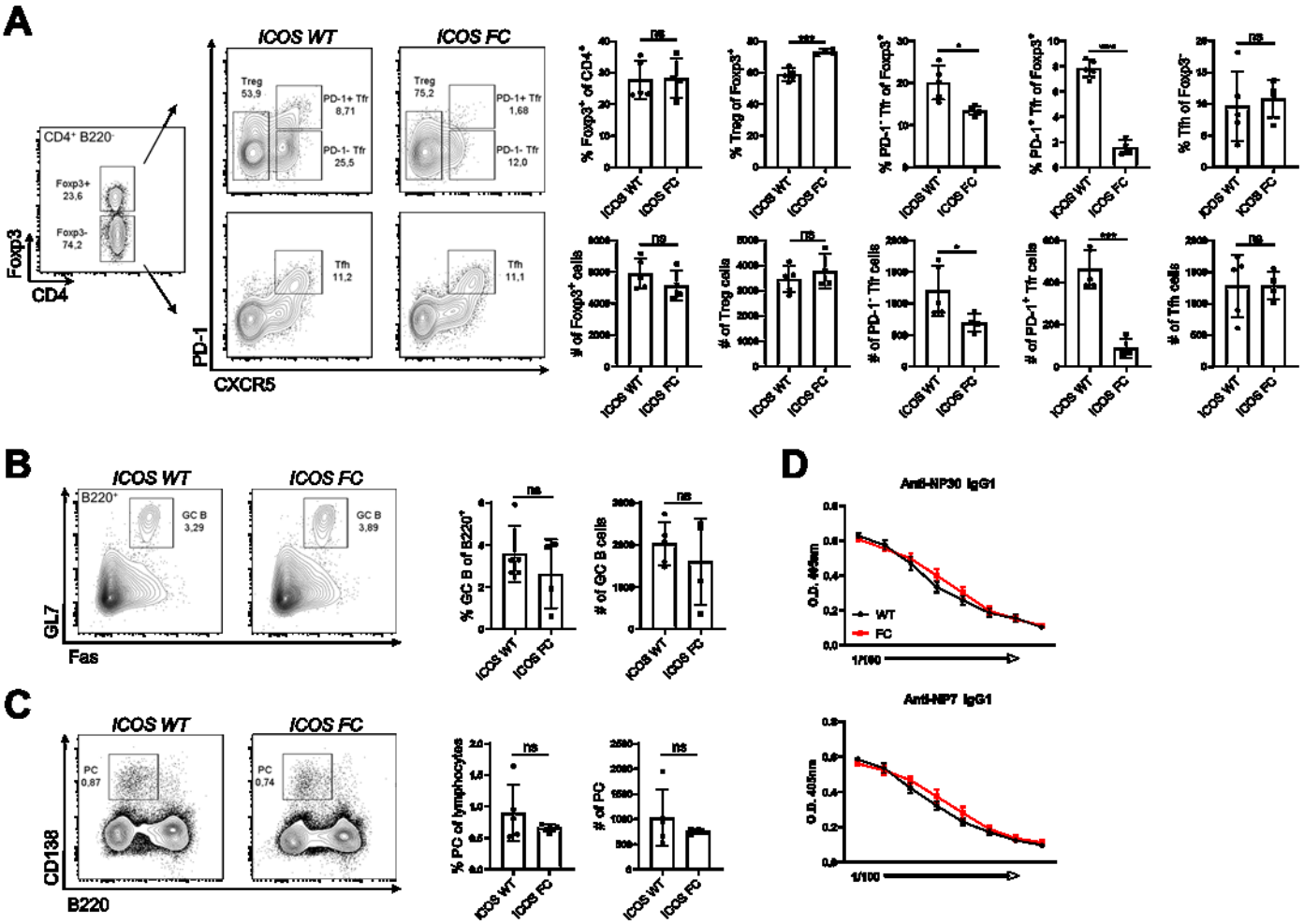


Figure 3.1 Foxp3-specific ICOS ablation decreases Tfr populations in a protein immunization model.

Splenocytes from ICOS WT (n=5) and ICOS FC (n=4) mice were harvested 12 days post-immunization (dpi) with NP-OVA/alum and analyzed by flow cytometry. (A) Treg, Tfr and Tfh cell percentages and numbers were evaluated using a combination of Foxp3, PD-1 and CXCR5 staining. (B) $B220^+$ Fas^+ $GL7^+$ GC B cell populations and (C) $B220^-$ $CD138^+$ plasma cells populations were analyzed from the same splenocyte pool 12 dpi. (D) Total (NP30) and high affinity (NP7) anti-NP IgG1 titers were measured by ELISA using serum from ICOS WT (black, n=5) or ICOS FC mice (red, n=5) obtained 28 dpi. Data shown as mean ± SEM, *p<0.05, **p<0.01, ***p<0.001 by two-tailed Student's t-test. All data are representative of at least three independent experiments.

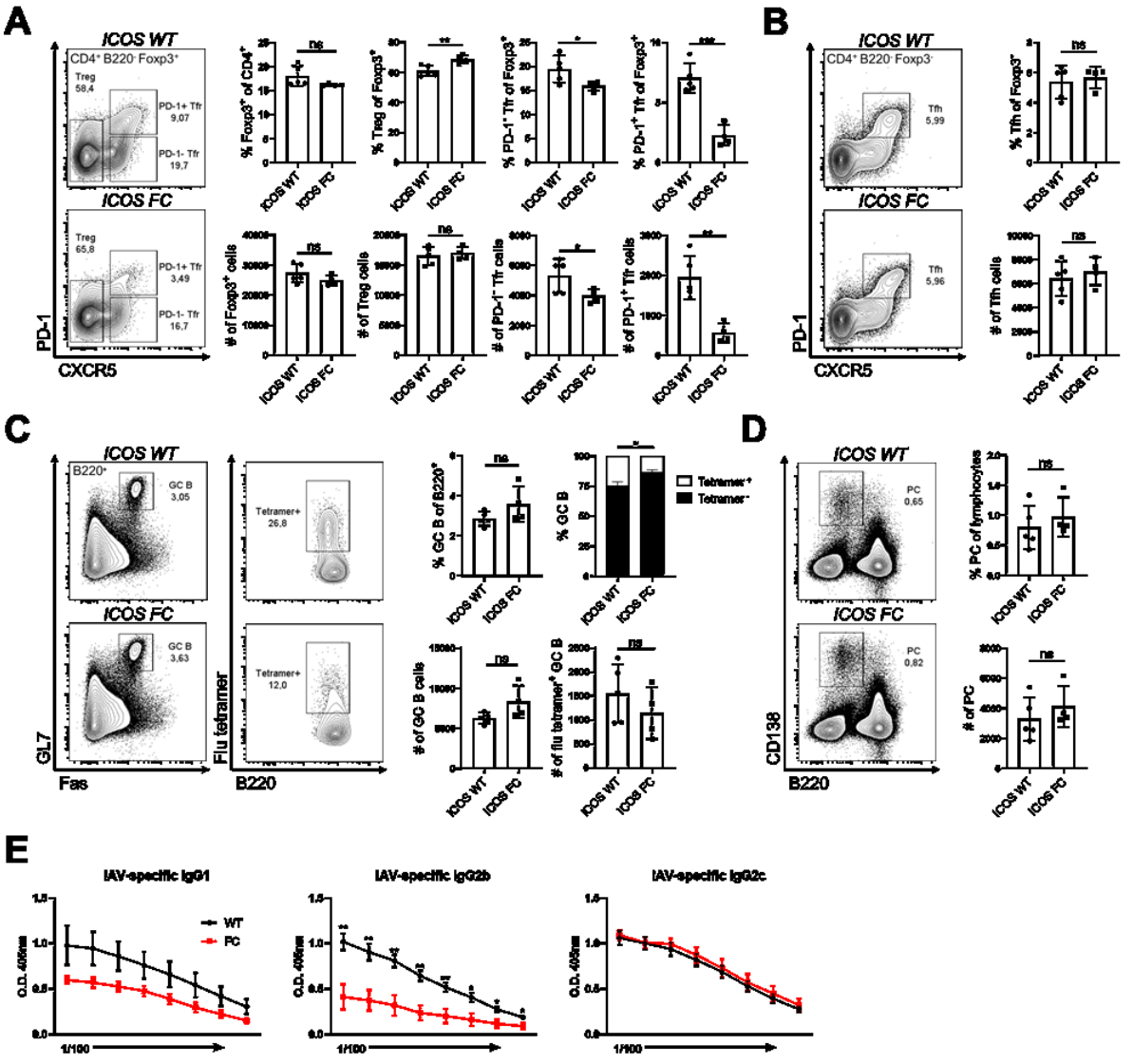


Figure 3.2 ICOS FC mice display reduced Tfr populations along with increased extraneous GC B cells during anti-viral responses.

Splenocytes from ICOS WT (n=5) or ICOS FC (n=4) were harvested 30 days post-infection (dpi) with influenza A virus (IAV) and analyzed by flow cytometry. (A) Foxp3$^+$ Treg and Tfr cells were classified using CXCR5 and PD-1 staining. (B) Foxp3$^-$ CXCR5$^+$ PD-1$^+$ Tfh cells were analyzed from the same splenocyte pool. (C) B220$^+$ Fas$^+$ GL7$^+$ GC B cells were harvested from spleens 30 dpi and stained with influenza nucleoprotein-specific tetramers (flu tetramers). (D) B220$^-$ CD138$^+$ plasma cells were analyzed using the same splenocyte pool. (E) IAV-specific IgG1, IgG2b and IgG2c titers were measured by ELISA using serum samples from ICOS WT (black) and ICOS FC mice (red) obtained 30 dpi. Data shown as mean ± SEM, *p<0.05, **p<0.01, ***p<0.001 by two-tailed Student's t-test (A, B, C, D) and two-way ANOVA with Holm-Sidak multiple comparisons test (E). All data are representative of two independent experiments.

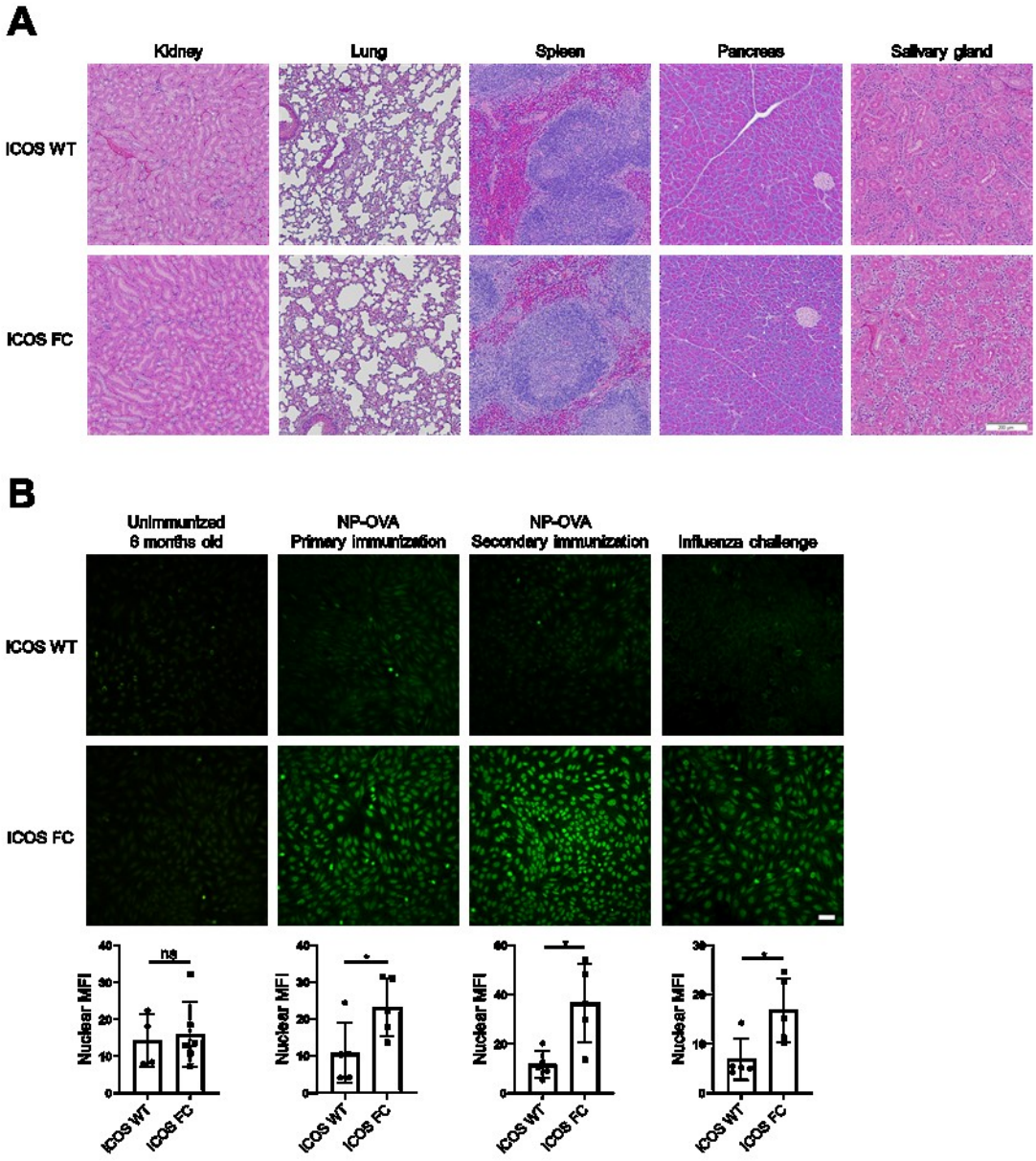


Figure 3.3 ICOS FC mice produce increased anti-nuclear antibodies following immune challenge.

(A) Representative images of H&E-stained tissue sections from 5-month-old ICOS WT (n=3) and ICOS FC mice (n=3). Scale bar 200 μM. (B) Anti-nuclear antibodies were detected by staining HEp-2 slides with serum samples collected from mice after the following treatments. Unimmunized ICOS WT (n=4) and ICOS FC (n=6) mice at 6 months of age. Primary immunization of ICOS WT (n=5) and ICOS FC (n=5) mice with NP-OVA/alum (serum harvested 12 dpi). Secondary NP-OVA/alum injection 30 days after the primary injection (serum harvested 44 days post secondary challenge). IAV infection of ICOS WT (n=5) and ICOS FC (n=5) mice (serum harvested 30 dpi). Scale bar 50μM. Nuclear fluorescence intensity was quantified using ImageJ. Data shown as mean ± SEM, *p<0.05 by two-tailed Student's t-test. All data are representative of two independent experiments.

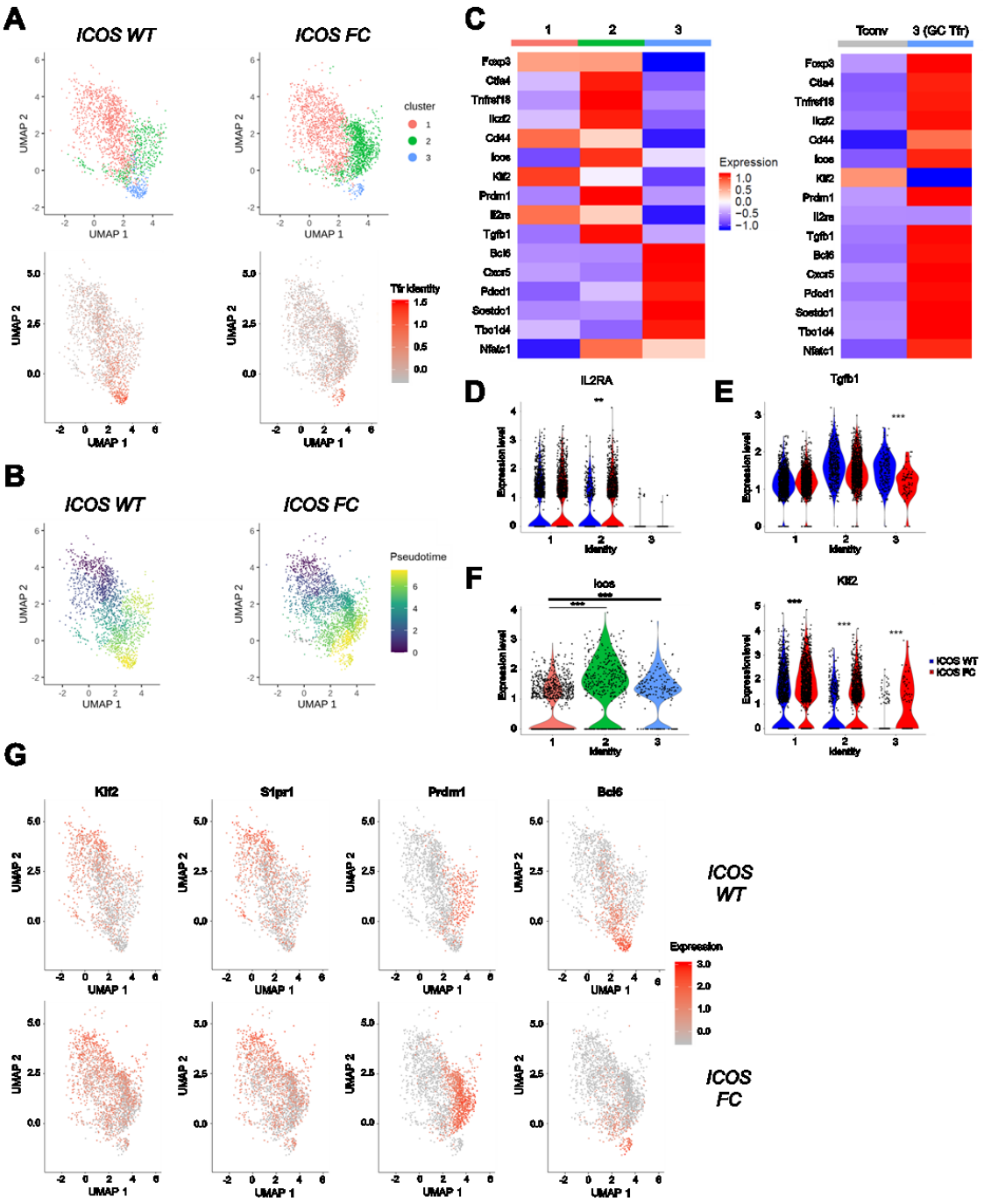
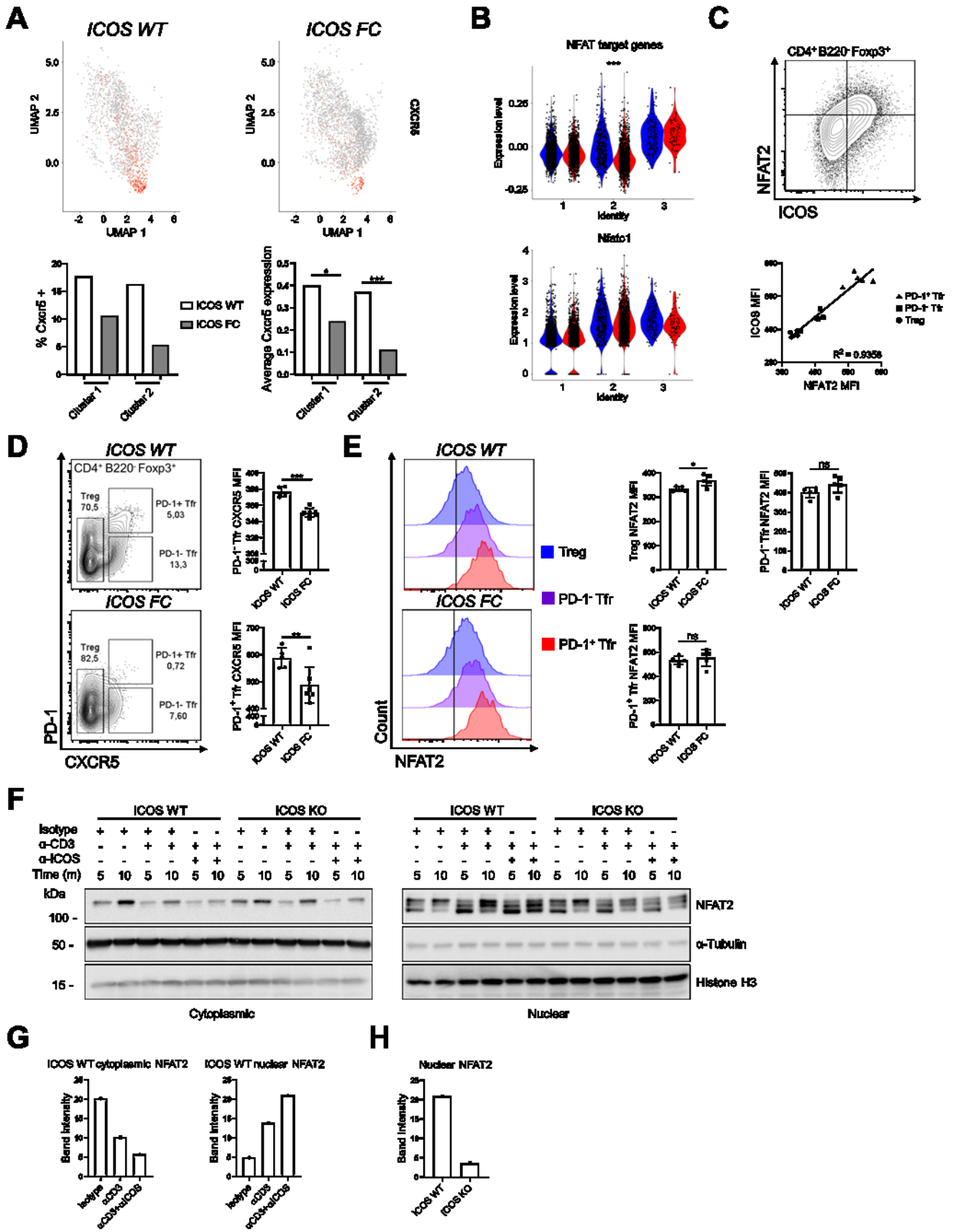


Figure 3.4 ICOS-deficient Treg cells show impaired Treg to Tfr transition.

Single-cell transcriptomes of FACS-sorted CD4$^+$ Foxp3$^+$ splenocytes from an ICOS WT or ICOS FC mouse harvested 6 days after protein immunization. (A) Selection and sub-clustering of Foxp3$^+$ cells based on positive Tfr identity scores. (B) Pseudotime analysis showing the differentiation trajectory of selected Foxp3$^+$ splenocytes. (C) Mean expression of regulatory and follicular genes by the indicated subpopulation. (D) *Il2ra* (E) *Tgfb1*, (F) *Icos* and *Klf2* violin plots showing the gene expression levels subdivided by cluster identities defined in (A). (G) Feature plots of *Klf2, S1pr1, Prdm1* and *Bcl6* expression. Each dot represents one cell. *p<0.05, **p<0.01, ***p<0.001 by Wilcoxon signed-rank test.


A
ICOS WT
ICOS FC
UMAP 1
UMAP 2
CXCR5
% Cxcr5 +
ICOS WT
ICOS FC
Cluster 1
Cluster 2
Average Cxcr5 expression
B
NFAT target genes
Expression level
Identity
Nfatc1
C
CD4+ B220- Foxp3+
NFAT2
ICOS
ICOS MFI
NFAT2 MFI
PD-1+ Tfr
PD-1- Tfr
Treg
R² = 0.9358
D
ICOS WT
ICOS FC
CD4+ B220- Foxp3+
Treg 70,5
PD-1+ Tfr 5,03
PD-1- Tfr 13,3
Treg 82,5
PD-1+ Tfr 0,72
PD-1- Tfr 7,60
PD-1
CXCR5
PD-1- Tfr CXCR5 MFI
PD-1+ Tfr CXCR5 MFI
E
ICOS WT
ICOS FC
Treg
PD-1- Tfr
PD-1+ Tfr
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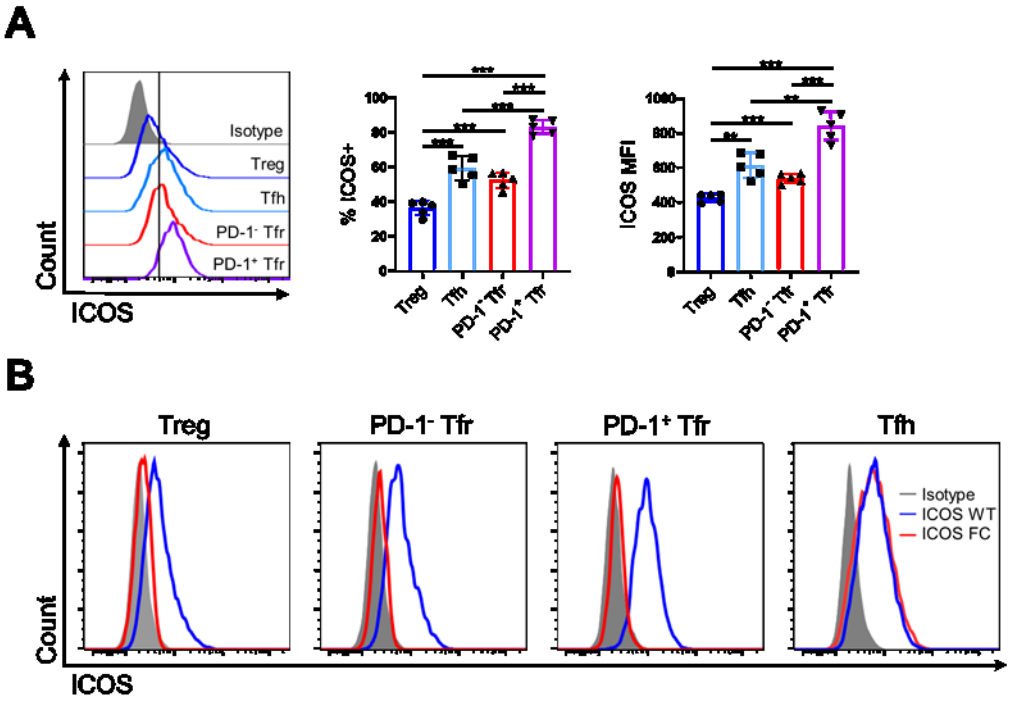
NFAT2
Treg NFAT2 MFI
PD-1- Tfr NFAT2 MFI
PD-1+ Tfr NFAT2 MFI
F
ICOS WT
ICOS KO
Isotype
α-CD3
α-ICOS
Time (m)
kDa
100 -
50 -
15 -
NFAT2
α-Tubulin
Histone H3
Cytoplasmic
Nuclear
G
ICOS WT cytoplasmic NFAT2
ICOS WT nuclear NFAT2
Band Intensity
Isotype
αCD3
αCD3+αICOS
H
Nuclear NFAT2
ICOS WT
ICOS KO

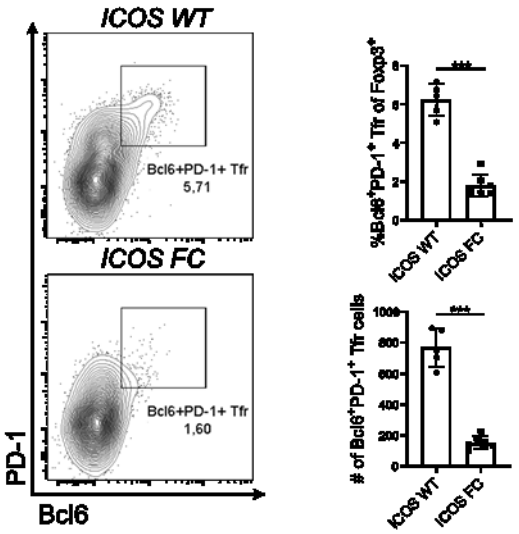
Figure 3.5 ICOS-NFAT2 signaling may regulate CXCR5 expression in regulatory T cells.

(A) Feature plot of *Cxcr5* gene expression (top) and quantification of *Cxcr5*$^{+}$ cells along with average *Cxcr5* expression in clusters 1 & 2 cells (bottom). (B) Average expression of NFAT target genes (top) and expression of *Nfatc1* (bottom) subdivided by cluster identity. (C) Splenocytes from ICOS WT (n=5) and ICOS FC mice (n=7) were analyzed 30 dpi with IAV. ICOS and NFAT2 expression was compared in Treg, PD-1^{-} Tfr and PD-1^{+} Tfr cells coming from ICOS WT mice. (D) MFI values for CXCR5 in PD-1^{-} Tfr and PD-1^{+} Tfr cells from the same splenocyte pool. (E) NFAT2 MFI values in ICOS WT vs. ICOS FC Treg, PD-1^{-} Tfr and PD-1^{+} Tfr cells were measured using the same splenocyte pool. (F) ICOS WT and ICOS germline KO CD4^{+} T cells were isolated from spleens by magnetic sorting and cultured for 2 days with α-CD3/CD28 stimulation. Cells were then restimulated for the indicated times with combinations of isotype control, α-CD3 and α-ICOS antibodies after which cytoplasmic and nuclear fractions were extracted and analyzed for NFAT2 protein content by Western blot. (G) Bar graph representing normalized cytoplasmic vs. nuclear NFAT2 band quantification of the indicated samples at the 10min timepoint. Only the bottom NFAT2 band was quantified to represent hypophosphorylated nuclear NFAT2. (H) Normalized nuclear NFAT2 band intensity of α-CD3/α-ICOS stimulated samples from ICOS WT vs. KO cells at 10 min. Data shown as mean ± SEM, *p<0.05, **p<0.01, ***p<0.001 by Wilcoxon signed-rank test.(A, B) or two-tailed Student's t-test (D,E). All data are representative of two independent experiments.

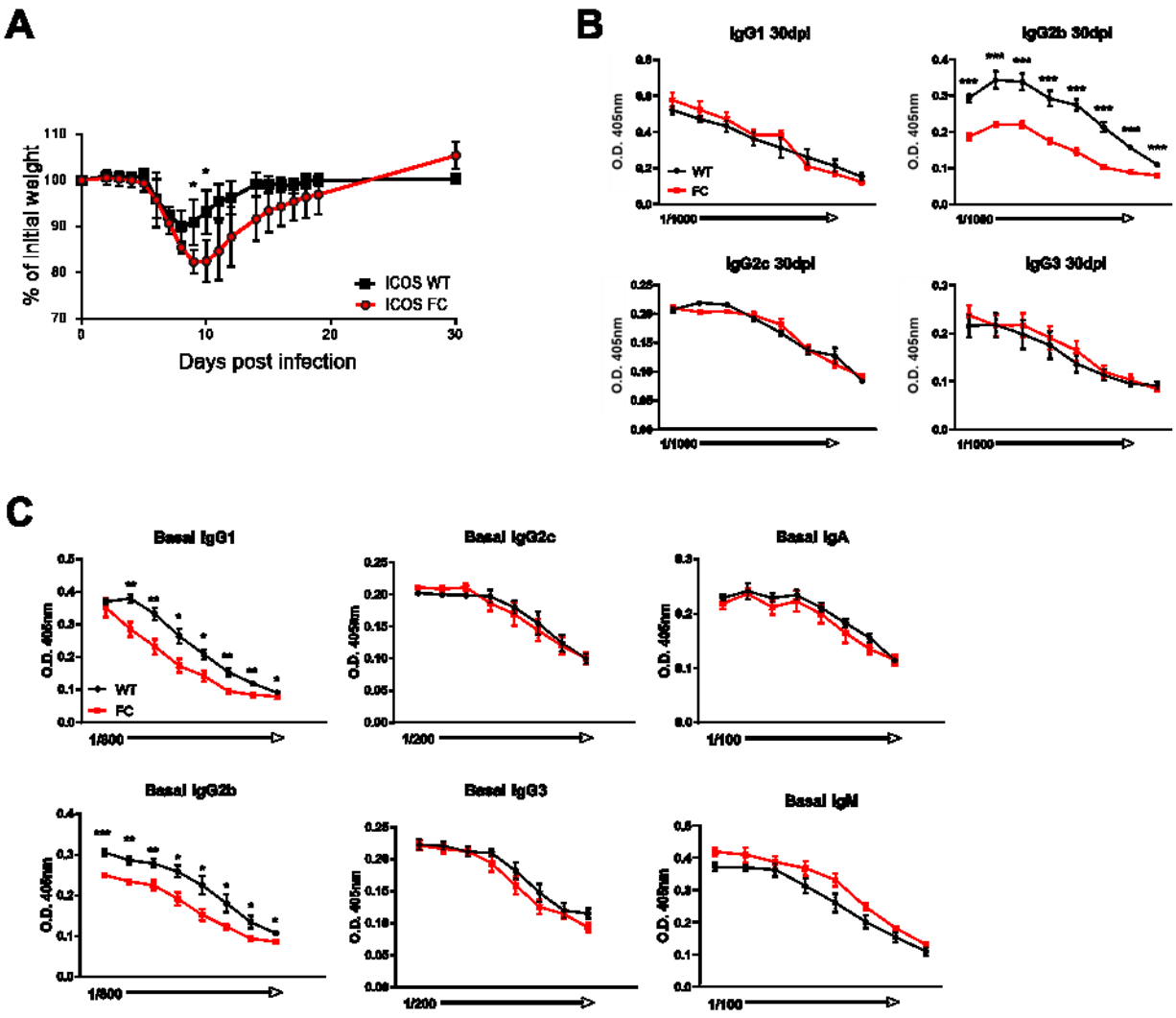
3.7 Supplementary figures and legends



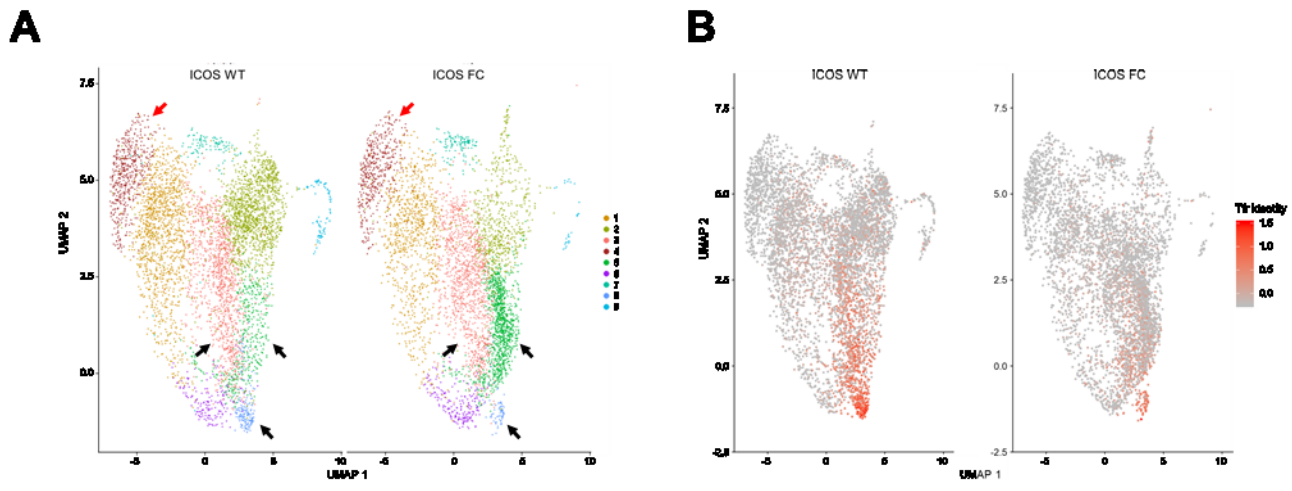
Supplementary Figure 3.1 Foxp3-cre mediated ICOS gene deletion is specific to regulatory T cells.
(A) Splenocytes from ICOS WT mice (n=5) were analyzed 12 dpi with NP-OVA/alum to measure ICOS expression in the indicated T cell subsets by flow cytometry. (B) Representative histograms of ICOS expression in the indicated T cell subsets in ICOS WT (blue) vs. ICOS FC (red) splenocytes. Data shown as mean ± SEM, **p<0.01, ***p<0.001 by one-way ANOVA with Tukey's multiple comparisons test. All data are representative of three independent experiments.



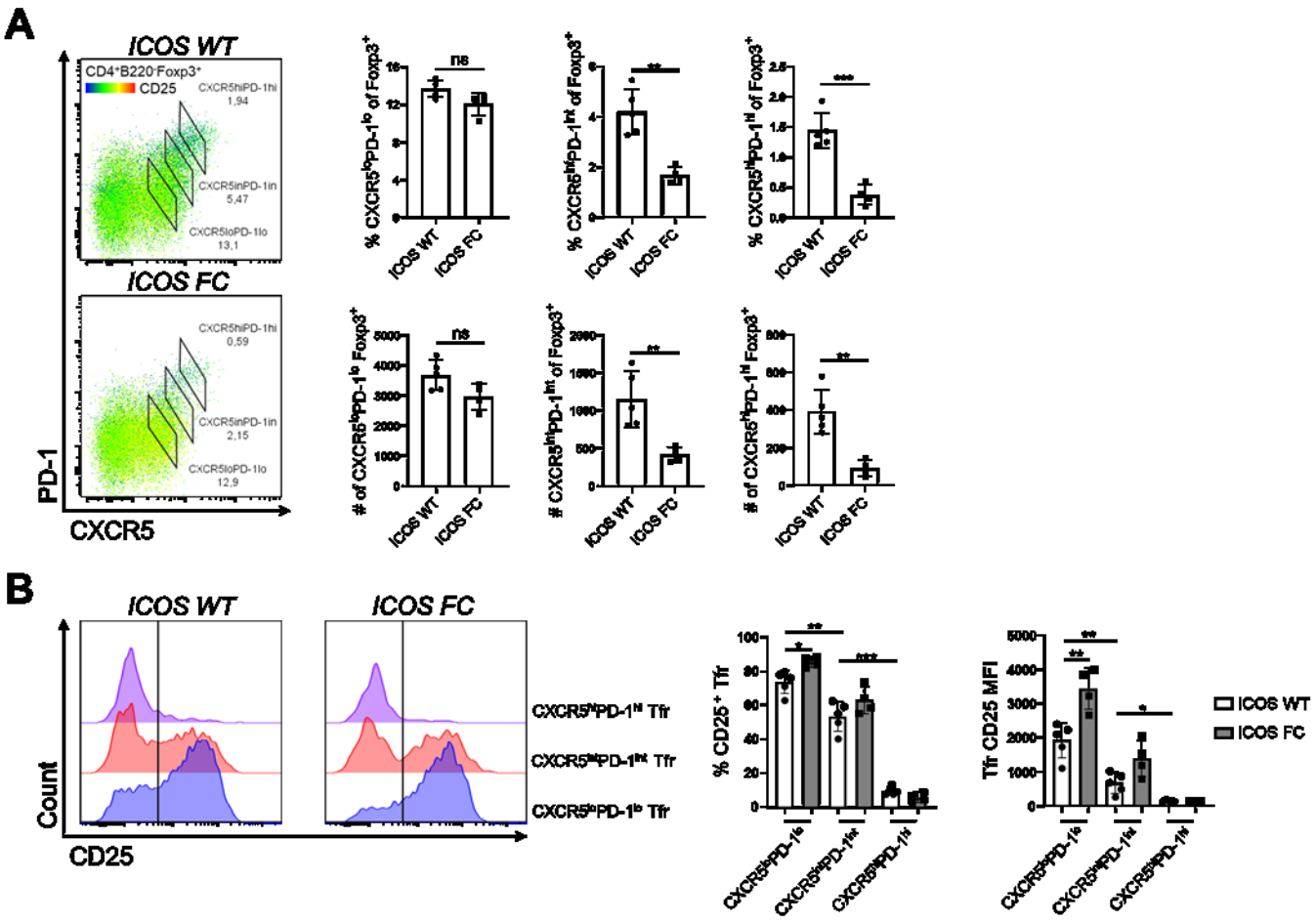
Supplementary Figure 3.2 $CD4^+$ $Foxp3^+$ $Bcl6^+$ $PD\text{-}1^+$ Tfr cells are reduced in ICOS FC mice Splenocytes from ICOS WT (n=5) and ICOS FC (n=7) mice were analyzed 30 days after IAV infection using Bcl6 instead of CXCR5 to define Tfr cells. Live $CD4^+Foxp3^+$ lymphocytes were pregated and analyzed for Bcl6 and PD-1 expression. Data shown as mean ± SEM, ***p<0.001 by two-tailed Student's t-test. All data are representative of two independent experiments.



Supplementary Figure 3.3 Impaired anti-viral responses in ICOS FC mice.
(A) Relative body weight loss over time following influenza A virus (IAV) infection in ICOS WT (n=5) and ICOS FC mice (n=7). (B) Serum samples from ICOS WT (black) and ICOS FC mice (red) was harvested 30 dpi with IAV and total titers of the indicated antibody isotypes were measured by ELISA with 2-fold serial dilutions. (C) Basal antibody titers were measured by ELISA with 2-fold serial dilutions using serum samples from unimmunized ICOS WT (n=6) or ICOS FC mice (n=6). Data shown as mean ± SEM, *p<0.05, p<0.01, ***p<0.001 by two-way ANOVA with Holm-Sidak multiple comparison tests. All data are representative of two independent experiments.



Supplementary Figure 3.4 Identification of putative Tfr precursor populations from Treg clusters. (A) UMAP projections of $CD4^+$ $Foxp3^+$ cells isolated from an ICOS WT or ICOS FC mouse 6 dpi with NP-OVA/alum. $CD4^+$ $Foxp3^-$ conventional T cells (cluster 4, red arrows) were added back after sorting to provide an internal control. Black arrows represent Tfr-like clusters (3, 5, 8) selected for further analysis. (B) Feature plots of Tfr identity scores based on expression levels of *Foxp3, Cxcr5, Pdcd1 and Bcl6*.



Supplementary Figure 3.5 CD25 is downregulated in CXCR5^{hi} PD-1^{hi} GC Tfr cells.
(A) Splenocytes from ICOS WT (n=5) and ICOS FC (n=4) mice were analyzed 30 dpi with IAV and Tfr subsets were defined by their relative expression levels of CXCR5 and PD-1. (B) CD25 expression levels in the Tfr subsets defined in (A) were analyzed by flow cytometry. The black bar in the histograms defines CD25^{-} and CD25^{+} populations. Data shown as mean ± SEM, **p*<0.05, ***p*<0.01, ****p*<0.001 by Student's *t*-test (A) or one-way ANOVA with Tukey's multiple comparisons test (B). All data are representative of two independent experiments.

Reference	Genes
PANTHER pathway analysis	Actc1, Acta1, Acta2, Actg2, Adssl1, Adss, Edn1, Myh1, Myh2, Myh3, Myh6, Myh7, Myh7b, Myh8, Myh13
Mognol, J., et al.	Bcl2, Fasl, Myc, Ccna2, Ccnd1, Ccnd3, Cdkn1a, Tnf, Tnfsf10, Ddias
Herman-Kleiter, N., et al.	Il2, Ifng, Il17a, Il17f, Il22, Il4, Il5, Il13, Il21
Vaeth, M., et al.	Cxcr5

Supplementary Table 3.1 List of NFAT target genes

Chapter 4: Conclusion

4.1 Summary

The overarching goal of research presented in this thesis was to better understand the role of ICOS in autoimmunity. In Chapter 2, we demonstrated that ICOS is required for the initiation and maintenance of CIA, a murine model of RA. During the priming phase of this disease, ICOS was indispensable for the production of anti-collagen antibodies and the proliferation of inflammatory T cells. This is in line with the established roles of ICOS in supporting humoral immunity and effector T cell functions. We were also able to specify that ICOS-PI3K signaling was responsible for these impacts. Next, we established an inducible ICOS knockout system and found that ICOS-PI3K signaling was important for the maintenance of established CIA in an anti-collagen antibody-independent manner. In parallel, we showed that glycolysis inhibition using 3-bromopyruvate led to CIA amelioration. This suggests potential overlaps between ICOS-PI3K and glucose metabolism.

In Chapter 3, we reported that ICOS is necessary for the differentiation of Tfr cells. We found that Foxp3-specific ICOS knockout (ICOS FC mice) led to a substantial reduction of Tfr cell numbers in protein immunization or viral infection models. Interestingly, Treg populations were not lowered in these settings which suggested a Treg-to-Tfr transition defect. We did not detect significant differences of total Tfh, GC B, and plasma cell numbers in immunized or infected ICOS FC mice. However, by using influenza-specific tetramers, we found that infected ICOS FC mice displayed an increased number of extraneous (non-viral specific) GC B cells. Further, immunized or infected ICOS FC mice presented increased amounts of ANAs, a hallmark of autoimmunity. Additionally, ICOS FC mice exhibited compromised anti-viral responses and IgG2b production defects. Taken together, these results support the view that Tfr cells may have B cell helper functions while also preventing autoantibody production. To further investigate the function of ICOS in Tfr cells, we performed single-cell transcriptome analysis of regulatory T cells isolated from immunized ICOS WT or FC mice. This data revealed an accumulation of Tfr precursors accompanied by a paucity of Tfr cells in ICOS FC mice, reinforcing the view that ICOS can promote the Treg-to-Tfr transition.

Mechanistically, we found that ICOS can promote Tfr differentiation by regulating KLF2 and NFAT2. ICOS-mediated KLF2 downregulation was already known to be important for Tfh lineage commitment and appears to be required by Tfr cells as well (96). While single-cell transcriptomics indicated that ICOS signaling can increase NFAT target gene expression in regulatory T cells, we did not observe alterations of NFAT2 expression levels in ICOS FC mice. Instead, we found that ICOS can increase the nuclear residence time of NFAT2, thus promoting the upregulation of NFAT2-dependent genes. Therefore, our data suggests that ICOS signaling acts upstream of a previously reported NFAT2-dependent CXCR5 upregulation mechanism shown to be required for Tfr differentiation (193).

Overall, our work provides insights into the double-edged nature of ICOS signaling. Since ICOS can play dual roles in supporting effector and regulatory T cells, non-specific blockade therapies might cause unintended side effects. By dissecting the involvement of specific ICOS-dependent pathways in autoimmunity, we provide avenues for future research on targeted therapeutic options.

4.2 Outstanding questions

4.2.1 Mechanisms of ICOS-dependent maintenance of autoimmune arthritis

While we showed that ICOS is required for CIA maintenance, we did not resolve which mechanisms are involved. We found that anti-collagen antibodies were not sufficient to maintain established CIA, but we did not investigate whether other autoantibodies generated through processes such as epitope spreading could have played a role. Indeed, epitope spreading was shown to occur in RA patients and is a predictor of disease progression (414, 415). Autoantigen arrays have been used to screen a wide variety of self-reactive antibodies and could reveal that CIA maintenance relies on novel autoantibodies (202). Additionally, ACPAs and RF are features of CIA and could participate in CIA progression (268). Thus, additional antibody screening may show that,

like CIA initiation, disease maintenance also requires autoantibody production. Whether ICOS could be involved in the diversification of autoantibodies during disease progression remains to be tested. Other murine models of autoimmune disorders involving autoantibody production such as T1D, SLE, and MS may be informative in this regard.

Studies on the role of ICOS have mostly focused on resolving its impact on germinal center reactions. Some early reports have mentioned that ICOS seems to be important for Th2 differentiation and function (42, 106-108). However, impacts of ICOS signaling on Th1 subsets remain partially understood due to their context dependent nature. Interestingly, our study using the CIA murine model of arthritis provides a window into ICOS-dependent Th1 regulation. Indeed, the complete Freund's adjuvant used in these experiments contains particles of *Mycobacterium tuberculosis* which trigger a Th1-dominated response. Our data suggests that CIA initiation mostly relies on the production of anti-collagen antibodies in an ICOS-dependent manner. However, CIA maintenance also requires ICOS for antibody-independent processes. Amongst these, ICOS may promote the survival of Th1 cells inside inflamed joints. Of note, ICOS costimulation could be of greater importance than CD28 in synovial tissues due to ligand availability. Unlike CD80/CD86, ICOSL was found to be expressed by a host of non-immune cells that could be present in articular tissues (32, 33). Along this line, elevated levels of ICOSL mRNA were found in synovial tissues of RA patients (302). Expression of ICOSL can be driven by TNF-α, the latter of which is produced upon macrophage – T cell contacts in synovial tissues (6, 416). Thus, inflammatory conditions present inside arthritic joints can increase ICOSL expression levels which may in turn promote inflammatory T cell survival in an ICOS-dependent manner. Interestingly, a study reported that ICOS-PI3K signaling can protect inflammatory T cells from apoptosis in a murine lupus model (313). This effect was dependent on local ICOSL expression by $CD11c^+$ macrophages and dendritic cells. To test whether this mechanism occurs in CIA mice, we could use our inducible ICOS knockout model to specifically isolate effects stemming from the ICOS-PI3K pathway. Then, we could harvest immune cells from inflamed joints and look for signs of apoptosis and indicators of PI3K signaling strength such as Akt phosphorylation

Our data suggests that glucose metabolism is a key component of CIA disease progression. This is supported by *in vivo* imaging showing increased glycolytic activity in the arthritic joints of K/BxN STA mice (359). Subsequent research showed that $CD4^+$ T cells from K/BxN mice display enhanced metabolic activity when compared to controls (417). Further, glycolysis inhibition using 2-deoxyglucose reduced the severity of K/BxN STA and dampened autoantibody production (417). Enhanced glycolytic activity in synovial tissues and inflammatory T cells is also a feature of human RA (357, 358, 418). Whether ICOS can modulate glucose metabolism to promote CIA maintenance remains to be tested. Studies have shown that ICOS can activate glycolytic pathways through mTOR signaling, which is likely downstream of ICOS-PI3K signaling (94, 419, 420). Using our inducible ICOS knockout system, we could inject CIA mice with fluorescent glucose analogs to track the impact of ICOS-PI3K signaling on glycolytic activity in arthritic joints. Alternatively, ^{13}C-glucose can be infused in arthritic mice which allows for the analysis of radio-labeled glucose metabolites through mass spectrometry, thus providing additional insights into glucose-derived carbon usage for pathways such as nucleotide biosynthesis (421). Because T cells are known to display metabolic plasticity dependent on glucose availability, this technique may provide novel research avenues to study mechanisms responsible for arthritis progression (422).

Lastly, we did not evaluate the impact of ICOS signaling on regulatory T cells in the context of autoimmune arthritis. Studies on Treg proportions in RA patients have produced contradicting results due to discrepancies in methods used to identify human Tregs (423, 424). The use of more recent definitions of Treg identity have suggested an overall decrease of Treg populations in the blood of RA patients, but increased Treg numbers in synovial fluid (423). However, these Tregs often lack CTLA-4 expression and display impaired suppressive function (425). Interestingly, a study has shown that Tregs can convert to proinflammatory Th17 cells in CIA mice (426). These converted Tregs possess pathogenic potential since they are often self-reactive. Adoptive transfer of $CD25^{lo}$ Tregs from arthritic to naïve mice caused accelerated arthritis onset and increased disease severity upon secondary collagen immunization (426). Subsequent analysis showed that most of the transferred Tregs lost Foxp3 expression. However, adoptive transfer of $CD25^{hi}$ Tregs resulted in osteoclast inhibition, thus suggesting functional

heterogeneity of the regulatory T cell compartment in the context of autoimmune arthritis. Of note, IL-17$^+$ Tregs were observed in synovial tissues of RA patients which suggests that Treg-to-Th17 conversions may occur in humans (426). Hypoxia-induced HIF-1α expression in arthritic joints was suggested as a potential mechanism causing Foxp3 downregulation and favouring Th17 differentiation (427, 428).

It is difficult to predict the impacts of Foxp3-specific ICOS abrogation at different stages of autoimmune arthritis. The reduction in Tfr cell numbers due to ICOS ablation could lead to increased autoantibody production as a result of dysregulated GC reactions. This mechanism would be relevant during disease initiation but might not influence disease maintenance if autoantibody titers become irrelevant in later stages of CIA. Next, ICOS-deficient Tregs were found to have reduced suppressive abilities in certain settings (56, 169). Thus, Foxp3-specific ICOS ablation could aggravate established CIA due to compromised Treg function. Further, loss of ICOS expression causes Foxp3 instability and may promote the aforementioned pathogenic Treg-to-Th17 transition (172). Finally, a subset of ICOS$^+$ Tregs was found to preferentially express CXCR3 in a T-bet-dependent manner which allows them to migrate towards sites of type I inflammation in the context of murine autoimmune diabetes (331, 429). Therefore, ICOS may be required for Tregs to migrate towards arthritic joints in CIA mice in order to suppress inflammatory T cells. Further, we have found that ICOS is required for Tregs to acquire a Th1-like phenotype which may be a necessary adaptation for Treg survival in inflammatory settings (unpublished data).

In sum, additional work is required to understand how ICOS signaling affects the regulatory T cell compartment in autoimmune arthritis. Improved knowledge in this regard may benefit Treg adoptive transfer therapies under investigation for the treatment of autoimmune diseases (430, 431).

4.2.2 Unresolved aspects of ICOS-dependent Tfr differentiation and function

Our work presented in Chapter 3 suggests that Foxp3-specific ICOS abrogation severely depletes Tfr populations. However, it is possible that the apparent loss of Tfr cells is artificially caused by a downregulation of surface markers used in flow cytometry such as CXCR5. To investigate this possibility, we used alternative gating strategies which seemed to confirm our original findings. Nevertheless, it would be interesting to perform imaging studies to evaluate changes of $Foxp3^+$ populations inside GCs of ICOS FC mice. A recent report has shown that mice with Foxp3-specific CXCR5 deficiency have Tfr-like cells within their GCs (432). This suggests that ICOS FC mice may still possess GC-resident $Foxp3^+$ Tfr-like cells that escape detection by flow cytometry. However, ICOS FC mice also display increased ANA production which implies defects in the Tfr compartment. Thus, we can hypothesize that any subsisting Tfr-like cell in ICOS FC mice would exhibit impaired suppressive abilities.

We did not investigate whether ICOS is required for the function of Tfr cells due to the significant depletion of Tfr populations measured by flow cytometry in ICOS FC mice. However, some Tfr cells (~2% of $Foxp3^+$ cells) can still be found after Foxp3-specific ICOS abrogation and could be isolated by cell sorting using the YFP Foxp3 reporter present in ICOS FC mice. Some *in vitro* studies have shown that functional Tfr cells can suppress Tfh responses and B cell activation (190, 197). Our team has previously established a T-B co-culture system which uses an AID-GFP reporter as a readout of GC B cell activation (103). By introducing ICOS-sufficient or deficient Tfr cells along with Tfh cells in this system, we could evaluate whether ICOS signaling affects the suppressive abilities of Tfr cells.

Early reports indicated that Tfr cells do not express key Tfh cytokines such as IL-4 and IL-21 (183, 184). However, these studies used LN-derived Tfr cells which were later shown to express high levels of CD25 when compared to splenic or PP Tfr populations (195). More recent studies suggest that $CD25^{lo}$ Tfr cells are the true “GC-Tfr” population and that $CD25^+$ Tfr cells are either $CD25^{lo}$ Tfr precursors or a functionally separate population (189, 195, 196). Interestingly, $CD25^{lo}$ Tfr cells were found to express high

levels of key T follicular signature genes such as *Bcl6* and *Cxcr5*, but also genes such as *Sostdc1* and *Tbc1d4* which were thought to be restricted to Tfh cells (195). Our single cell transcriptome analysis indicates that Foxp3-specific ICOS abrogation caused a severe depletion of $CD25^{lo}$ Tfr cells. Further, we found that this Tfr population expressed IL-4 and IL-21 which suggests a potential Tfh origin. So far, the scientific consensus is that Tfr cells differentiate from $Foxp3^{+}$ precursors. This is substantiated by adoptive transfer experiments which show that the Tfr compartment can be reconstituted from transferred Tregs but not from naïve T cells (183-185, 195). However, Aloulou *et al.* found that Tfr cells can differentiate from $Foxp3^{-}$ precursors under specific conditions, and at least one report found *in vitro* evidence of Tfh-to-Tfr conversion using Tfh cells isolated from SLE patients (192, 433). Thus, we hypothesize that ICOS signaling may mediate the differentiation of a Tfh-derived Tfr population. It is unclear whether this population would function differently from Treg-derived Tfr cells. It is possible that Tfh-like Tfr cells could provide antigen-independent B cell help through IL-4 and IL-21, thus promoting increased diversity of the antibody response.

Lastly, we did not investigate the relative contribution of different ICOS signaling arms in Tfr cells. The engagement of PI3K signaling was found to be correlated with Treg-to-Tfr conversion (205). Also, ICOS was shown to promote the formation of complexes between the PI3K subunit p85α and OPN-i which in turn protects Bcl6 from degradation (95). Our data shows that ICOS may promote CXCR5 upregulation in Tfr cells by enhancing NFAT2 activity. This mechanism may rely on ICOS-calcium signaling since NFAT2 activation is Ca^{2+}-dependent (398). Moreover, ICOS-PI3K signaling could prevent nuclear export of NFAT2 by inhibiting GSK3β (399, 400). Finally, ICOS-calcium signaling may promote Tfr differentiation through other mechanisms since direct interference with the calcium pathway was shown to cause Tfr depletion and autoantibody production (206). The specific impacts of ICOS-PI3K signaling in Tfr cells could be tested by adapting our inducible ICOS knockout model presented in Chapter 2 with an inducible Foxp3-cre system. However, the Tfr-specific contribution of ICOS-calcium signaling or other mechanisms involving ICOS-TBK1 or the ICOS TM segment will require additional tools for *in vivo* studies.

4.2.3 Clinical implications of ICOS signaling

Studies on ICOS polymorphisms in human disease have been complicated due to genetic proximity with CD28 and CTLA-4 (434). Several autoimmune diseases have been associated with the 20 kb region of chromosome 2q33 which includes genes encoding these receptors (20, 435, 436). However, most early studies have focused on associations between autoimmune disorders and mutations around the CTLA-4 gene. No deleterious ICOS mutation was specifically identified by these analyses. However, it is possible that CTLA-4-proximal mutations may have impacted ICOS due to their adjacent chromosomal location. More recent studies have identified ICOS polymorphisms associated with risks of developing coeliac disease, breast cancer, and colorectal cancer (437-439). However, no specific mechanism has been identified and more work is needed to elucidate if and how ICOS polymorphisms influence disease progression.

ICOS blockade remains understudied in the context of human autoimmune disorders. Some studies have found positive impacts of ICOS or ICOSL blockade in murine RA and SLE models, but data from human patients remains limited (297, 317, 440, 441). However, several clinical trials utilizing ICOS agonists or antagonists for cancer immunotherapy are currently underway (442). Since ICOS is known to promote the function of effector T cells and regulatory T cells, results from these trials provide invaluable insights into the net impact of promoting or inhibiting ICOS signaling. Preclinical data indicate that ICOS agonists can potentiate the impact of CTLA-4 blockade (443, 444). Conversely, ICOS-deficient mice are unable to respond adequately to anti-CTLA-4 treatment (443). Current phase I clinical trials indicate that ICOS agonists are generally well tolerated and have shown promising clinical activity (445, 446). ICOS antagonists have shown limited ability to improve antitumor activity by suppressing Treg function (442). However, these must be carefully dosed to find a therapeutic window in which the antitumor function of effector T cells is preserved. Thus, more studies are required to understand the net effect of manipulating ICOS signaling in the field of cancer immunotherapy. Lessons from these studies may also inspire future therapies geared towards treating autoimmune disorders.

Several studies including our own have identified multiple signaling pathways controlled by specific motifs found in the transmembrane and intracellular domains of ICOS (17, 45-47). These findings may inform the design of future generations of chimeric antigen receptors (CARs). Currently, CARs utilizing the ICOS intracellular domain have shown the ability to maintain an IL-17-producing effector T cell population able to mediate potent antitumor responses (447). Interestingly, these cells also acquired Th1-like features such as expression of IFN-γ and T-bet. Recently, it was found that combining intracellular domains of ICOS and 4-1BB in a third-generation CAR resulted in increased *in vivo* persistence, a critical aspect of CAR-mediated tumor suppression (448). Specifically, optimal persistence was achieved when the ICOS cytoplasmic domain was proximal to the membrane. This finding raises the possibility that interactions between transmembrane and cytoplasmic ICOS domains can have functional consequences. This underlines the importance of increasing our understanding of ICOS signaling mechanisms to improve CAR-based therapies.

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