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The Role of Inducible T-cell Co-stimulator in Regulatory T Cell Homeostasis and Function

par

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Abstract

Inducible T-cell co-stimulator (ICOS) is a member of the CD28 family of T cell costimulatory receptors that is induced upon activation in $CD4^+$ and $CD8^+$ T cells. It has been established that ICOS plays a critical role in humoral immunity by supporting the generation and function of T follicular helper cells. Thus, ICOS deficiency in humans and mice leads to immunodeficiency due to impaired germinal center reaction and antibody production. ICOS can also promote expansion, survival, and cytokine expression of inflammatory T cells. However, given that ICOS is also constitutively expressed in $Foxp3^+$ regulatory T (Treg) cells, interruption of ICOS signaling may lead to different outcomes depending on the context of immune reactions. Due to the potential opposing roles of ICOS in overall T cell immune response, the T cell subset-specific role of ICOS needs to be clarified.

In order to address the intrinsic roles of ICOS in Treg homeostasis and function, we generated mice (termed ICOS FC) in which *Icos* gene is specifically deficient in $Foxp3^+$ T regulatory cells. Using flow cytometry and single-cell transcriptome analysis, we show that ICOS FC mice do not have any severe alterations in the Tcon cell activation status and subset compositions of Treg cells under a steady state condition. Consistently, no spontaneous autoimmune symptoms developed in aged ICOS FC mice. In contrast, when the mice were challenged with chemically induced skin inflammation, ICOS FC mice mounted more severe inflammatory responses. In parallel, the number of Treg cells was reduced allowing increase of inflammatory $CD4^+$ and $CD8^+$ T cells in the draining lymph nodes and the skin. Although very small, our single-cell transcriptome analysis identified a cluster of Treg cells coexpressing T-bet and CXCR3 (termed Th1-Treg) in the draining lymph nodes in an ICOS-dependent manner. Therefore, Treg-intrinsic ICOS deficiency had minimal impact on the overall Treg homeostasis, but weakened Treg cells' capacity to control Th1-driven skin inflammation likely due to the impaired differentiation of Th1-Treg cells.

A dual role of ICOS in T cell-driven autoimmune disease has been modeled in NOD mice. In pure NOD mice in which polyclonal T cells control the disease, ICOS germline deficiency reduced disease progression by dampening activation of pathogenic autoreactive T cells. In contrast, when the TCR repertoire was highly restricted to autoantigens in BDC2.5 TCR transgenic

NOD line, ICOS-expressing Treg cells appear to play a dominant role by halting progression of insulitis to overt diabetes. However, previous studies could not exclude the possibility of altered TCR repertoire by ICOS deficiency in germline. In this study, we tested the impact of *Icos* gene deletion in adult NOD mice using an inducible ubiquitous Cre system once peripheral T cell repertoire had been established. We observed reduced incidence of diabetes in pure NOD mice but accelerated disease in BDC2.5-NOD mice, very similar to germline ICOS-deficiency. These results support the prevailing view that the main function of ICOS is to regulate effector and regulatory T cells in the periphery.

In sum, we demonstrated that ICOS-deficient Treg cells retain the capacity to prevent spontaneous autoimmune disease but have a compromised ability to dampen Th1-driven skin inflammation. We further confirm the notion that ICOS mainly regulates mature T cells as opposed to thymic selection process.

Keywords

Inducible T-cell Co-stimulator, ICOS, Regulatory T cells, Foxp3, Autoimmune disease, Inflammation, Homeostasis, Type I diabetes, NOD, BDC2.5.

Résumé

Le co-stimulateur inductible des lymphocytes T (ICOS) est un membre de la famille CD28 des récepteurs co-stimulateurs qui est induit suite à l'activation des lymphocytes T $CD4^+$ et $CD8^+$. Il a été établi que ICOS joue un rôle essentiel dans l'immunité humorale en soutenant la génération et la fonction des cellules T auxiliaires folliculaires. Le déficit en ICOS chez l'homme et la souris conduit à une immunodéficience due à une altération de la réaction du centre germinatif et de la production d'anticorps. ICOS peut également favoriser l'expansion, la survie et l'expression des cytokines des cellules T inflammatoires. Cependant, étant donné que ICOS est également exprimé de manière constitutive dans les cellules T régulatrices $Foxp3^+$ (Treg), l'interruption de la signalisation ICOS peut conduire à des résultats différents selon le contexte des réactions immunitaires. En raison des rôles potentiellement opposés de ICOS dans la réponse immunitaire globale des lymphocytes T, le rôle spécifique de ICOS chez les sous-ensembles de lymphocytes T doit être clarifié.

Afin d'étudier les rôles intrinsèques de ICOS dans l'homéostasie et la fonction des Tregs, nous avons généré des souris (appelées ICOS FC) dans lesquelles le gène *Icos* est spécifiquement aboli chez les cellules T régulatrices $Foxp3^+$. À l'aide de la cytométrie en flux et de l'analyse du transcriptome unicellulaire, nous démontrons que les souris ICOS FC ne présentent aucune altération grave de l'état d'activation des cellules Tcon et des compositions de sous-ensembles de cellules Treg dans des conditions d'équilibre. De plus, aucun symptôme auto-immun spontané ne s'est développé chez les souris ICOS FC âgées. En revanche, lorsque les souris ont été confrontées à une inflammation cutanée induite chimiquement, les souris ICOS FC ont présenté des réponses inflammatoires plus sévères. En parallèle, le nombre de cellules Treg a été réduits permettant une augmentation des cellules T $CD4^+$ et $CD8^+$ inflammatoires dans les ganglions lymphatiques drainants et la peau. Notre analyse du transcriptome unicellulaire a identifié un petit groupe de cellules Treg coexprimant T-bet et CXCR3 (appelé Th1-Treg) dans les ganglions lymphatiques drainants d'une manière ICOS-dépendante. Par conséquent, le déficit en ICOS intrinsèque aux Tregs a eu un impact minimal sur l'homéostasie globale des Tregs, mais a affaibli la capacité des cellules Treg à contrôler l'inflammation cutanée induite par les cellules Th1, probablement en raison de la différenciation altérée des cellules Th1-Treg.

Un double rôle de ICOS dans les maladies auto-immunes induites par les cellules T a été modélisé chez les souris NOD. Dans les souris NOD pures chez lesquelles des cellules T polyclonales contrôlent l'apparition du diabète auto-immun, un déficit de ICOS réduit la progression de la maladie en atténuant l'activation des cellules T autoréactives pathogènes. En revanche, dans un contexte où le répertoire du TCR est fortement restreint aux auto-antigènes (lignée NOD transgénique BDC2.5 TCR), les cellules Treg exprimant ICOS semblent jouer un rôle dominant en arrêtant la progression vers le diabète manifeste. Cependant, des études antérieures n'ont pas pu exclure la possibilité d'un répertoire de TCR altéré par un déficit en ICOS dans la lignée germinale. Dans cette étude, nous avons testé l'impact de la délétion inductible du gène *Icos* chez des souris NOD adultes à l'aide d'un système Cre ubiquitaire une fois que le répertoire des cellules T périphériques a été établi. Nous avons observé une incidence réduite du diabète chez les souris NOD pures, mais une accélération de la maladie chez les souris NOD BDC2.5 très similaire au phénotype causé par une ablation de *Icos* de la lignée germinale. Ces résultats soutiennent l'opinion dominante selon laquelle la fonction principale de ICOS est de réguler les cellules T effectrices et régulatrices dans la périphérie.

En résumé, nous avons démontré que les cellules Treg déficientes en ICOS conservent la capacité de prévenir les maladies auto-immunes spontanées, mais démontrent une capacité réduite à atténuer l'inflammation cutanée provoquée par les cellules Th1. Nous confirmons de plus l'hypothèse que ICOS régule principalement les cellules T matures au lieu du processus de sélection thymique.

Les mots-clés

Co-stimulateur inductible des lymphocytes T, ICOS, lymphocytes T régulateurs, Foxp3, maladie auto-immune, inflammation, homéostasie, diabète de type I, NOD, BDC2.5.

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List of Acronyms and Abbreviations

Abbreviation	Full Name
AD	Atopic dermatitis
AHR	Airway hypersensitivity
AIRE	Autoimmune regulator
APC	Antigen Presenting Cell
aTreg	Activated Treg
B7-H2	B7 homolog 2
B7RP1	B7-related protein 1
BAFF	B cell activating factor
Bcl-2	B cell lymphoma 2
BCR	B cell receptor
BLIMP-1	B lymphocyte-induced maturation protein 1
CHS	Contact hypersensitivity
CNS2	Conserved non-coding sequence 2
cTEC	Cortical thymic epithelial cells
CTLA-4	Cytotoxic T lymphocyte-associated protein 4
cTreg	Central Treg
CXCR5	C-X-C motif chemokine receptor 5
DC	Dendritic cell
DNFB	2,4-dinitro-fluorobenzene
DP	Double Positive
EAE	Experimental autoimmune encephalomyelitis
ER	Endoplasmic reticulum
eTreg	Effector Treg
FC	Foxp3-Cre
FOXP3	Forkhead box P3

Abbreviation	Full Name
GC	Germinal center
GVHD	Graft-versus-host disease
HSCT	Hematopoietic stem cell transplantation
IBD	Inflammatory bowel disease
ICOS	Inducible T-cell co-stimulator
ICOSL	Inducible T-cell co-stimulatory ligand
Ig	Immunoglobulin
IL	Interleukin
IL2Rα	Interleukin-2 receptor alpha chain
ILC	Innate lymphoid cell
Ip	Intraperitoneal
IPEX	Immunodysregulation, polyendocrinopathy, enteropathy X-linked
IRF4	Interferon regulatory factor 4
iTreg	Induced Treg
kDa	Kilodalton
LEF1	Lymphoid enhancer binding factor 1
LN	Lymph node
mAb	Monoclonal antibody
MAPK	Mitogen activated protein kinase
MHC	Major histocompatibility complex
mRNA	Messenger RNA
mTEC	Medullary thymic epithelial cell
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NOD	Non-obese diabetic

Abbreviation	Full Name
Nrp1	Neuropilin-1
nTreg	Natural Treg
OVA	Ovalbumin
PD-1	Programmed cell death protein-1
PI3K	Phosphoinositide 3-kinase
PIP3	phosphatidylinositol 3,4,5-triphosphate
PLC-γ	Phospholipase C-gamma
pTreg	Peripheral Treg
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
RORγ	RAR related orphan receptor gamma
rTreg	Resting Treg
SAP	SLAM-associated protein
SLE	Systemic lupus erythematosus
SP	Single positive
T1D	Type 1 diabetes
T-bet	T-box expressed in T cells
TBK1	TANK-binding kinase 1
TCF-1	Transcription factor T cell factor-1
Tcon	Conventional T cell
TCR	T cell receptor
Tfh	T follicular helper (cell)
Tfr	T follicular regulatory (cell)
TGF-β	Transforming growth factor beta
Th	T helper (cell)

Abbreviation	Full Name
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
Treg	T regulatory (cell)
Tregp	Treg progenitor cells
TSA	Tissue specific self antigen
TSDR	Treg-specific demethylated region
tTreg	Thymic Treg
UTR	Untranslated region
ZAP70	Zeta-chain-associated protein kinase 70

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First of all, I would like to thank **God** for being able to write this thesis. I hope that the thesis and the research I have done becomes a necessary tool in this world and glorifies the name of our Lord.

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Contribution of Authors

All chapters of this thesis have been proofread under the guidance of **Dr. Woong-Kyung Suh**, who always gave me insightful guidance and feedback.

Chapter 2

The contents of Chapter 2 was submitted to *Journal of Immunology* (in revision): **Chang J.**, et al. ICOS-deficient Treg cells can prevent spontaneous autoimmunity but are impaired in controlling acute inflammation.

Jinsam Chang performed and completed the majority of the experiments such as mouse experiments, flow cytometry, contact hypersensitivity experiments, Treg suppression assay, microscopy image capturing and genotyping. **Jinsam Chang** and **Dr. Woong-Kyung Suh** designed the experiments and analyzed the data. **Jinsam Chang** prepared all the figures with **Dr. Woong-Kyung Suh**'s guidance. **Dr. Woong-Kyung Suh** conceptualized and supervised this project, and the manuscript was written and edited by **Jinsam Chang** and **Dr.Woong-Kyung Suh. Antoine Bouchard** performed single cell RNA sequencing data analysis for the fugures. **Vincent Panneton** and **Joanna Li** performed genotyping and gave valuable comments for the experiments and proofread the manuscript. **Nikoletta Diamantopoulos** and **Saba Mohammaei** performed genotyping. **Dr. Cirriaco Piccirillo** provided experimental advice for this study.

Chapter 3

The data presented in Chapter 3 will be used for a future publication. **Jinsam Chang** performed all the flow cytometry experiments using the autoimmune diabetes mouse model, captured microscopy images for immunohistochemistry, and *in vitro* T cell activation. Type 1 diabetes mice were monitored by **Jinsam Chang** and animal technicians in the IRCM animal facilities. This study was conceptualized and supervised by **Dr. Woong-Kyung Suh**. **Jinsam Chang** and **Dr. Woong-Kyung Suh** designed the experiments and analyzed data. **Jinsam Chang** prepared all the analyzed figures with comments from **Dr. Woong-Kyung Suh**. The manuscript

was written by **Jinsam Chang** and proofread and edited by **Dr. Woong-Kyung Suh. Jinsam Chang**, **Vincent Panneton**, **Antoine Bouchard**, **Joanna Li**, **Nikoletta Diamantopoulos** and **Saba Mohammaei** performed genotyping. **Dr. Cirriaco Piccirillo** provided insightful comments on BDC2.5-NOD mice experiments.

Jinsam Chang was also involved in collaborations with other lab members and laboratories, which resulted in the following publications.

1. Dong M*, **Chang J***, Lebel M, Gervais N, Suh WK, Melichar H. "The ICOS-ICOSL pathway tunes thymic selection" submitted to *Immunology & Cell Biology* ICB-21-SC-0189. * Equal contribution
2. Bouchard A, Witalis M , **Chang J** , Panneton V, Li J, Bouklouch Y , Suh WK. "Hippo Signal Transduction Mechanisms in T Cell Immunity" *Immune Network.* 2020. 20 (5)
3. Witalis M, **Chang J,** Zhong M-C, Panneton V, Li J, Buch T, Kim SJ, Kim WS, Ko YH, Veillette A, Suh WK Progression of AITL-like tumors in mice is driven by Tfh signature proteins and T-B crosstalk. *Blood Advances*. 2020. 4(5), 868-879
4. Panneton V, **Chang J**. Witalis M, Li J, Suh WK. "Inducible T cell co-stimulator: Signaling mechanisms in T follicular helper cells and beyond" *Immunological reviews* 2019. 291 (1), 91-103
5. Yazdchi SB, Witalis M, Meli AP, Leung J, Li X, Panneton V, **Chang J,** Li J, Nutt SL, Johnson RL, Lim DS, Gu H, King IL, Suh WK. "Hippo Pathway Kinase Mst1 Is Required for Long-Lived Humoral Immunity" *Journal of Immunol*. 2019. 202(1), 69-78
6. Zhang M, Wu Y, Bastian D, Iamsawat S, **Chang J**, Daenthanasanmak A, Nguyen HD, Schutt S, Dai M, Chen F, Suh WK, Yu XZ. "Inducible T-cell Co-stimulator Impacts Chronic Graft-Versus-Host Disease by Regulating Both Pathogenic and Regulatory T Cells". *Frontiers in Immunology*. 2018. 9,1461

7. Panneton V, Bagherzadeh Yazdchi S, Witalis M, **Chang J**, Suh WK. "ICOS Signaling Controls Induction and Maintenance of Collagen-induced Arthritis" *Journal of Immunology*. 2018. 200(9):3067-3076.

Contibutions to the co-authorship papers.

1. Jinsam Chang performed the initial profiling of ICOS and ICOSL expression in the thymus : the optimiztion of staining conditions for mTEC (medullary thymic epithelial cells), cTEC (cortical thymic epithelial cells) and DC (dendritic cells) in the thymus. Jinsam Chang carried out some experiments and prepared thymic samples from ICOS and ICOSL-deficient mice. Jinsam Chang verified the analytical methods and conceptual framework of the final version of the manuscript.

2 & 4. Jinsam Chang wrote one chapter and provided critical feedback on the manuscript.

3 & 5. Jinsam Chang prepared mice and reagents and contributed to the interpretation interpretation of the results. Jinsam Chang provided critical feedback and helped shape the research, analysis and the manuscript.

6 & 7 Jinsam Chang prepared mice and reagents and contibuted to the interpretation of the results. Jinsam Chang discussed the results and commented on the manuscript.

Chapter 1. General Introduction

1.1 Inducible T-cell Co-stimulator (ICOS)

Three signals are required for activation of naïve T cells while they interact with antigen presenting cells: T cell receptor (TCR) signal, cosignaling and cytokines [1-3]. TCR ligation controls T cell clonal expansion, survival and differentiation into several different T cell subsets [4]. Cosignaling can determine T cell function and fate. When APCs recognize non-self pathogens or damaged tissue, the expression of costimulatory ligands is increased to induce T cell immunity. On the other hand, lack of costimulation results in T cell tolerance [5]. Cosignaling receptors play an important role to amplify or inhibit TCR mediated signaling not only in the activation of mature T cells, but they also facilitate T cell thymic selection [6, 7]. The representative costimulatory protein for T cells is CD28. The CD28 ligation provides the second signal to induce cellular proliferation and survival following T cell activation. The discovery of CD28 led to the identification of a variety of cosignaling molecules, including costimulatory and coinhibitory receptors and ligands. Unlike CD28, cytotoxic T lymphocyte antigen 4 (CTLA-4) served to mitigate the T cell activation as a coinhibitory molecule.

Inducible T-cell co-stimulator (ICOS) is a member of the CD28 family of cosignaling receptors expressed in activated human T cells [8] and it turned out to be the human homolog of the murine protein termed H4 [9, 10]. ICOS was considered as being inducible only when $CD4^+$ and $CD8^+$ T cells are activated and matured. However, most recently, ICOS is found to be expressed in a constitutive manner, not only in an inducible manner [11]. The expression of ICOS is associated with T cell proliferation [12, 13], and leads to the augmentation of cytokine secretion [8, 14-17]. It is also important for the generation of an effective humoral immune response by supporting the generation and function of Tfh (T follicular helper) cells [18, 19].

ICOS deficiency in humans causes common variable immunodeficiency (CVID), a disease characterized by a severe reduction in serum gamma globulin [20], leading to high susceptibility to bacterial infection of the respiratory tracts [21]. Subsequent studies have also been published supporting that ICOS can be a potential therapeutic target for autoimmunity and cancer immunotherapy [11]. ICOS deficiency in human disease is an important component of my thesis, so I will dedicate a whole section to ICOS and disease relevance (*see Section 1.1.4*).

My work mainly focuses on the characterization of the role of ICOS in the regulatory T cell's (Treg) homeostasis and function. In this section, I will review what is known regarding ICOS based on published papers and outline the project that I have done for my Ph.D.

1.1.1 Structure and expression of ICOS

Similar to CD28, ICOS is a type I transmembrane glycoprotein with one extracellular immunoglobulin domain, one transmembrane region (~23 amino acid) and a cytoplasmic tail (35 amino acid) [8] (Figure 1.1). ICOS was discovered on the cell surface as a homodimeric disulfide-linked protein, that was 55-60 kDa in humans and 47-57 kDa in mice. Human ICOS mRNA encodes a 199 amino-acid chain, whereas murine ICOS encodes 200 amino-acid chain [22]. The amino-acid sequence of ICOS in humans and mice share approximately 70% identity [12, 22]. The amino-acid sequence of ICOS shows 17% and 19% of similarity with CD28 in humans and mice, respectively [8, 12]. The gene encoding ICOS is closely located to the genes for CD28 and CTLA-4, as they are all located on the same chromosome in both mice and humans [8]. Due to the genetic and biochemical relationship between ICOS and other CD28 family members, initial research was done to investigate whether ICOS plays similar roles to those of CD28 for T cell activation. The cytoplasmic tail of ICOS consists of 35 amino acids, which is of similar length compared to CD28 and CTLA-4. All of these have a common binding motif for phosphoinositide 3 kinase (PI3K), Tyrosine– X– X– Methionine: YMFM for ICOS, YMNM motif at the corresponding site of CD28 and YVKM for CTLA-4. ICOS binds PI3K upon phosphorylation of the tyrosine reside in the YMFM motif [23-26], but the YXXM motifs in CD28 and CTLA-4 are not potent activators of PI3K.

Although ICOS and CD28 have similar structure, ICOS has critical non-redundant functions in multiple immune responses due to its unique features. First of all, the ligand for ICOS (ICOSL) is expressed on antigen presenting cells (APC) [27, 28] and non-hematopoietic tissues such as endothelial [29] and epithelial tissues [30]. Second, unlike CD28, ICOS is constitutively expressed in innate lymphoid cells (ILCs) [31]. Lastly, ICOS can activate PI3K, PLCγ1-mediated intracellular calcium signaling and TBK1 (Tank Binding Kinase 1). Although CD28 also activates PI3K, ICOS is much more potent PI3K activator than CD28 [24, 32], and ICOS and PI3K signaling axis plays a critical function in the differentiation of Tfh cells.

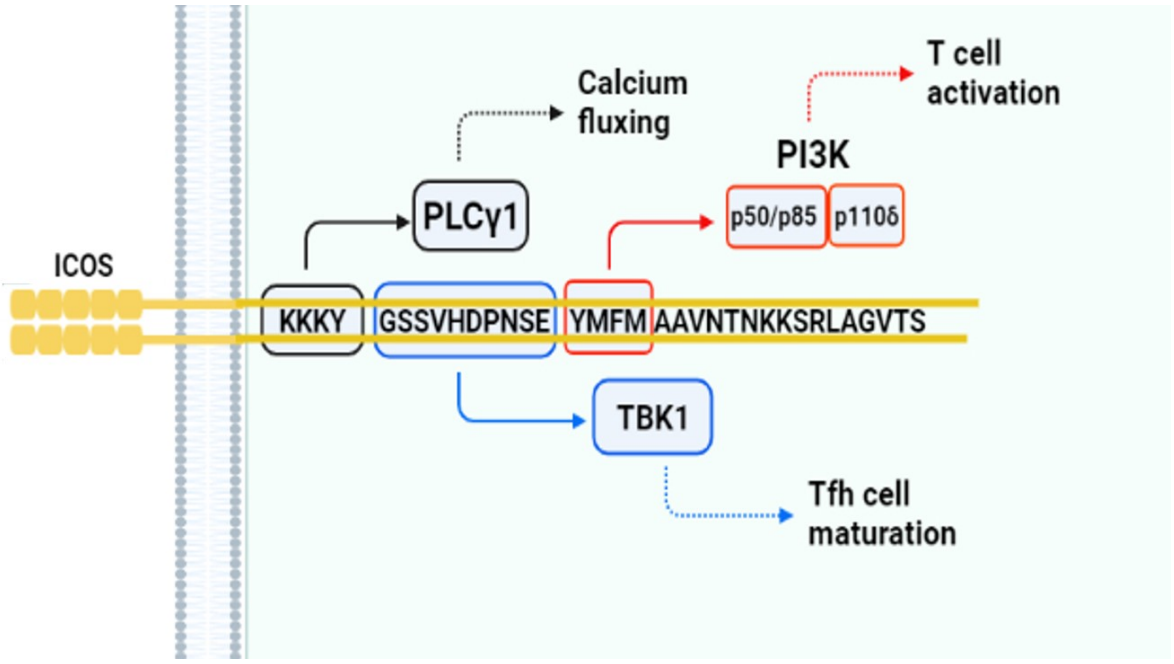


Figure 1.1 Signaling motifs in the cytoplasmic tail of murine ICOS. Amino acid sequences serving as binding sites for phosphoinositide 3-kinase (PI3K), phospholipase Cγ1 (PLCγ1), and TANK-binding kinase 1 (TBK1) are marked along with functional consequences in T cell activation.

Naïve T cells express low levels of ICOS, but ICOS expression is rapidly upregulated in activated T cells after TCR and CD28 stimulation. However, the molecular mechanisms that control ICOS expression is not clearly elucidated. The Src family kinase Fyn and MAP kinase Erk can regulate ICOS expression at the transcriptional level [33]. Calcineurin can be activated by Fyn, which leads to NFATc2 (Nuclear factor of activated T cells, cytoplasmic 2) dephosphorylation and its nuclear localization [34]. NFATc2 and Erk bind to the *Icos* promoter region independently, and induce the transcription of ICOS [33]. An early study reported that Th2 cells expressed higher levels of ICOS than Th1 cells [35], suggesting ICOS expression may be associated with IL-4 production. A later study showed that Th1 promoting cytokine IL-12 can upregulate ICOS expression, indicating that ICOS expression is not limited to Th2 conditions [36]. It is now clear that ICOS is expressed in Th1, Th2, Th17, Treg and Tfh cells in a context dependent manner and many studies consider ICOS as a marker of activated T cells. As the T cell subset-specific role of ICOS is too broad topic to be covered in this subsection, I will dedicate *"Section 1.1.5"* to this topic

ICOS can be negatively controlled by ubiquitin ligase Roquin [37]. ICOS mRNA is degraded by Roquin-dependent mechanisms, which is important to dampen T cell activation and prevent lupus-like autoimmunity [38, 39]. Roquin proteins post-transcriptionally degrade the mRNA of *Icos*, *Ox40*, and *Tnf* by recognizing stem-loop structures and linear sequences found in the 3'-untranslated regions of targets genes [40-42]. Additionally, it is also reported that Roquin proteins interfere with expression of *Icos* through the interactions with certain microRNAs [43]. Roquin increases Dicer-mediated activation of the pre-miR-146a, which is a microRNA that targets *Icos* mRNA [43]. Mice with the *Sanroque* mutation in the gene encoding Roquin-1 (*Rc3h1*) show increased ICOS expression with increased Tfh cell numbers and systemic lupus erythematosus (SLE)-like autoimmune symptoms [37, 44, 45]. In addition, ICOS internalization after interacting with ICOSL is another negative regulatory mechanism of ICOS expression [46].

Thus, ICOS is a T cell costimulatory receptor with multiple signaling motifs. Despite its wide expression in many T cell subsets, its deficiency or dysregulated overexpression can lead to humoral immune disorders indicating that it has an important role in the Tfh compartment.

1.1.2 ICOS ligand (ICOSL)

The ICOS ligand (ICOSL, B7RP-1, CD275, B7-H2) is a member of the B7 family [12, 47, 48]. ICOSL has two extracellular immunoglobulin domains, one transmembrane domain, and one cytoplasmic tail [49]. ICOS and ICOSL are a unique receptor-ligand pair and there is no binding competition for ligands between ICOS and other CD28 family. This is different from CD80 and CD86, which interact with CD28 and CTLA-4 [50]. This finding suggests that ICOSL expression could be regulated in different manner from ligands for CD28 and CTLA-4 (CD80 and CD86). Whereas the expression of CD80 and CD86 are restricted to APCs, ICOSL is expressed not only on APCs, including B cells, macrophages and dendritic cells (DC), but also on cells of non-hematopoietic origin and ILCs (innate lymphoid cells) [28-31, 51]. Recently, ICOSL was shown to be upregulated in mesenchymal stem cells (MSCs) under inflammatory conditions [52]. In addition, normal muscle fibres also expressed low levels of ICOSL constitutively. However, in inflammatory myopathy, ICOSL expression is significantly increased in muscle fibres [51]. Human endothelial cells express ICOSL *in vitro* and *in vivo* [29]. In particular, naïve B cells abundantly express ICOSL, which is regulated by B cell activating factor (BAFF) receptor dependent non-canonical NFκB signaling [53, 54]. Importantly, ICOSL is readily downregulated after binding to ICOS, but this process is not observed in CD28-B7-1/B7-2 interaction [27, 55]. ICOS-ICOSL interaction is mostly in the context of T cell interaction with APCs. However, current studies reveal that ICOSL may have ICOS independent roles in mediating both immunological and non-immunological responses through its broad expression pattern [56]. For instance, ICOSL in mice can bind activated αvβ3 integrin on renal podocytes to dampen its response to kidney injury [57].

The control of ICOSL expression is different from CD80 and CD86. CD80 and CD86 are expressed at low levels under steady state, and are upregulated upon toll-like receptor (TLR) stimulation [2]. In contrast, ICOSL is constitutively expressed, and its expression level is increased when B cell receptor (BCR) stimulation and CD40 signals are provided [48, 58]. Upon binding of ICOS, the majority of ICOSL is shed from B cell surface by ADAM family proteases [55, 59]. This shedding can be prevented by TLR7/8 and TLR9 signaling [55]. However, the level of CD86 was not changed under similar experimental setting, supporting that ICOSL shedding may play a unique physiological role [55]. Transgenic mice which overexpress ICOS in all T cells exhibited a phenotype resembling ICOSL deficient mice in terms of antibody responses [27]. This was due to a

constant downregulation of ICOSL from the B cell surface through ICOS-induced shedding [55]. Likewise, overexpressed ICOSL on B cells demonstrated dysregulated T cell responses [59, 60]. When ADAM10 proteases were selectively removed in B cells, the ICOSL levels were elevated. This, in turn, caused excessive ICOS internalization in T cells leading to severe defects in T follicular helper development and Th2 polarization [59]. Loss of ICOSL in humans also showed hypogammaglobulinemia and respiratory infections [54, 61]. Compared to ICOSL-sufficient counterparts, ICOSL-deficient human B cells displayed reduced antibody production and memory B cell generation [61]. These results suggest that ICOS and ICOSL expression is tightly regulated on the surface of T cells and B cells to coordinate Tfh-GC B cell collaboration and antibody production.

Different from T cells that only express ICOS but not ICOSL, human and murine ILCs express both ICOS and ICOSL in a constitutive manner [31]. ICOS-ICOSL interaction plays a crucial role in the homeostasis of ILC2, as blocking the ICOS-ICOSL interaction impairs the activation of STAT5 and cytokine production in ILC2s [31]. Similar to T cell-APC interaction, ICOS and ICOSL expression levels were downregulated upon engagement in ILCs. Blocking the ICOS-ICOSL interaction increased ICOSL expression levels in wildtype ILCs, and *Icos*$^{-/-}$ ILC2s showed elevated levels of ICOSL compared to their wildtype counterparts [31].

1.1.3 Downstream of ICOS costimulation

ICOS stimulation induces the recruitment of PI3K, which leads to the production of membrane-bound phosphatidylinositol 3,4,5-triphosphate (PIP_3), to promote cellular proliferation and survival upon the activation of the kinase Akt [62]. Similar to CD28, ICOS ligation recruits p50α and p85α subunits of PI3K [63]. Interestingly, ICOS preferentially binds to the p50α, which induces more potent kinase activity than p85α, which may explain why ICOS has a stronger PI3K signaling capability than CD28 [63]. PI3K activation is implicated in many aspect of T cell biology, including T cell development, differentiation, homeostasis and migration [64]. Its signaling is indispensable for the proper function of most T cell subsets.

For T cell activation, a rise in intracellular calcium concentration is essential. TCR engagement initiates intracellular calcium flux, and this can be potentiated by ICOS signaling [65-67]. Upon co-ligation of ICOS and TCR, signaling proteins such as ZAP70 (zeta chain associated protein kinase 70) are recruited to CD3ζ chains to initiate signaling cascades leading to the activation

of PLCγ. This cascade induces the release of calcium stored in the endoplasmic reticulum (ER) and subsequent influx of extracellular calcium. Increase of intracellular calcium activates the phosphatase calcineurin, leading to the dephosphorylation of NFAT (nuclear factor of activated T cells) in the cytoplasm. This results in the translocation of NFAT to the nucleus and induction of NFAT target genes such as IL-4, IL-10, IL-21 and IFNγ [68, 69]. Thus, ICOS can induce NFAT-related genes through the calcium signaling pathway.

Different from PI3K and calcium signaling that are sharing with ICOS and CD28, TBK1 is not recruited to the signaling complexes although TCR and CD28 are co-ligated [70]. This result suggests that ICOS and TBK1 axis may have unique ICOS specific signaling pathways [11]. Of note, the downstream of TBK1 signaling pathways are not clarified yet.

Akin to CD28, ICOS can enhance T cell proliferation [12, 13] and augments T cell survival by regulating Bcl2 family proteins. ICOS cosignaling results in the increased secretion of cytokines including IL-4, IL-21, IFN-γ, IL-10, IL-17 and IL-21 [8, 14-17]. Contrasting to CD28, IL-2 is not highly produced in response to ICOS stimulation and, ICOS-mediated T cell proliferation seems to be minimal in the presence of CD28 costimulation [13, 71]. Many studies were performed to elucidate the effects of ICOS costimulation at the cellular level. However, most of the studies on ICOS have investigated its role by exploring the phenotype of mice lacking ICOS ubiquitously. Some of T cell's subset-specific ICOS function await more rigorous approaches such as tissue-specific knockout.

One of the distinct roles of ICOS is the maintenance of germinal centers (GC) in lymphatic tissues. ICOS plays a crucial role by providing a survival signal for $CXCR5^+$ Tfh cells [72] and enhances migration of Tfh cells from T-B cell border into the follicle [73]. ICOS costimulation is responsible for the induction of IL-10 and IL-21 in Tfh cells for the maintenance of GCs and differentiation of B cells. These results explain why ICOS deficiency leads to severely impaired memory B cell formation.

1.1.4 ICOS and human disease

ICOS deficiency is an autosomal recessive disorder and a very rare disease. It was the first monogenetic defect described in common variable immunodeficiency (CVID)-like disease in 2003 [74]. The symptoms of ICOS-deficient patients are much broader than initially expected and

categorized as a combined immunodeficiency, hypogammaglobulinemia, vulnerability to infection, autoimmunity, lymphoproliferation [20, 72, 75, 76].

1.1.4.1 Common variable immune deficiency (CVID)

Homozygous autosomal mutations of the ICOS gene in humans cause a subset of CVID [74]. Some patients experience frequent infections or inflammation of the gastrointestinal tract, which causes diarrhea and weight loss. Accumulation of immune cells leads to enlarged lymph nodes (lymphadenopathy) or spleen (splenomegaly) in some patients. CVID patients suffer from recurrent bacterial infections mainly in the airways due to combined reductions of serum IgG, IgA and IgM, or IgG/A/M [77]. Pneumonia is common in CVID patients and recurrent infections lead to chronic lung disease. [76, 78]. Impaired GC reactions with reduced Tfh cells characterize CVID [76]. Along with these reports, targeted disruption of ICOS or ICOSL in mice caused impaired Tfh generation and defective GC reactions leading to reduction in class-switched antibodies to T cell-dependent antigens [79, 80]. It is well documented that ICOS-mediated PI3K and calcium signaling pathways play an important role in Tfh cell generation and function [66, 74, 81-84].

In addition to antibody deficiency, ICOS-deficient CVID patients develop autoimmunity and enteropathy. While antibody defects by impaired Tfh generation are well known, the reasons for autoimmunity and enteropathy are not clear yet.

1.1.4.2 Systemic lupus erythematosus (SLE)

Roquin-1 (encoded by *Rc3h1*) post-transcriptionally regulates *Icos* mRNA in $CD4^+$ T cells [38]. In activated $CD4^+$ T cells, ICOS expression is upregulated partially due to the reduction of Roguin-1 expression through microRNA-induced degradation of Rc3h1 mRNA. Dysregulation of the signaling pathway by a point mutation in *Rc3h1* gene (*Sanroque*) leads to the overexpression of ICOS, thereby promoting spontaneous Tfh cell induction, GC formation and autoantibody production. This s*anroque* roquin-mutant mice ($Rc3h1^{san/san}$) develop a lupus-like systemic autoimmune disease in mice [38, 85].

SLE is a multisystem autoimmune disease with currently unclear causes. SLE affects mainly women of childbearing potential. The hallmark of this disease is the presence of class-switched IgG autoantibodies [86-88]. *In vitro* ICOS stimulation of peripheral T cells from SLE showed increased IFNγ expression compared to normal controls, with an elevated level of anti-dsDNA titre [89, 90]. In the murine SLE model (BXSB-*Yaa*), the production of IL-21 from splenic $ICOS^+$ $CD4^+$ T cells was related to renal disease and early mortality [91].

These results suggest that overt activation of ICOS costimulation promotes SLE.

1.1.4.3 Rheumatoid arthritis

Rheumatoid arthritis (RA) is one of the most common autoimmune diseases, and it is characterized by synovial joint inflammation and progressive bone destruction caused by immune cell infiltration [92]. The disease gradually deteriorates cartilage and bone, affecting patient's ability to perform basic tasks and lowers the quality of life. An estimated 0.5%- 1% of the world's population is afflicted with RA [93]. One possible explanation for the pathogenesis of RA is based on the observation of T and B cell infiltration in synovial tissues in RA. Also, DCs are recruited to the joints of RA and serve as APCs, with inflammatory cytokines such as tumor necrosis factor alpha (TNFα) and IL-17A secreted in synovial tissues [92, 94]. The pro-osteoclastogenic cytokines erode bones, and chondrocyte apoptosis leads to cartilage damage [95]. However, the pathogenesis of RA is not completely elucidated. Understanding of the mechanisms of RA pathogenesis is difficult because the patients are diagnosed in the late stage of RA. Thus, animal models are commonly used for RA research.

The collagen-induced arthritis (CIA) mouse model is the most widely used autoimmune model of RA [96]. Immunizing type II collagen to CIA-susceptible DBA/1 mice induces an autoimmune reaction resembling RA, and autoantibodies against type II collagen can be detected in this mouse model [97]. Also, the immune cell infiltration of synovial tissues and bone destruction is similar to the features of human RA [98].

ICOS is involved in class-switched antibody generation. The level of $ICOS^+$ T cells is increased in synovial tissues from RA patients [99]. Thus, a study has shown that the blockade of ICOS reduced the severity of CIA by reducing titers of anti-type II collagen antibodies [100]. Our

group also confirmed that ICOS-mediated PI3K signaling is necessary to induce and maintain CIA [101]. Taken together, these findings suggest that ICOS signaling is involved in RA.

1.1.4.4 Asthma

Asthma is a chronic airway inflammation characterized by airway hyperreactivity and bronchoconstriction, associated with dysregulated type 2 immune responses. Th2 and type 2 cytokines including IL-4, IL-5 and IL-13 have been considered as the central factor for this disease [102]. However, other immune cells can produce the type 2 cytokines and play important roles in regulation of asthma.

First, characterization of ILC2 cells changed our understanding of the pathogenesis of asthma by demonstrating the role of ILC2 in type 2 response [103]. ILC2 reacts to stimuli such as IL-25, IL-33 and TSLP (thymic stromal lymphopoietin) and secretes Th2 cytokines (IL-5, IL-13 and IL-9) causing airway inflammation. As mentioned above (*section 1.1.2*), ILCs express not only ICOS but also ICOSL. ICOS and ICOSL interaction is necessary for murine and human ILC2-mediated airway inflammation and hyperreactivity in a mouse model [31].

Second, Tfh cells are crucial in T cell dependent B cell response, a response implicated in allergic airway disease. Tfh cells are required for allergen-specific IgE production [104, 105]. Isolated Tfh cells from lung draining lymph nodes can migrate to the lungs and become Th2 cells which enhances allergic airway disease [106]. After ICOSL-blockade, reduced Tfh cells lead to reduced GC B cell responses, lung plasmablasts and allergen-specific IgE [107]. This result presented the potential of targeting Tfh cells to ameliorate the IgE-mediated disease. Taken together, these findings suggest that ICOS signaling is involved in asthma and that ICOS could be a therapeutic target.

1.1.5 The role of ICOS in T cell compartments

In addition to Tfh and antibody responses, many studies suggest that ICOS promotes $CD4^+$ T cell responses in a mouse model [108]. $CD4^+$ T cells are differentiated into different subsets: Th1,

Th2, Th17, Treg and Tfh cells (Figure 1.2). $ICOS^{-/-}$ mice showed impaired Th1 and Th2 response [109]. Th17 cell development is also affected by defects in ICOS signaling. [16, 17].

To address the role of ICOS in the development of the polarized Th cells, $ICOS^{-/-}$ mice were infected with *Trichuris muris* or *Toxoplasma gondii* [110]. In these parasite infection models, ICOS knockout mice failed to clear worms and produced reduced levels of Th2 response [110]. ICOSL deficient mice also showed decreased number of Th2 cells [69]. Furthermore, ICOS ablation reduces the Th2-mediated airway inflammation in an autoimmune mouse model [111]. Deficiency of ICOS signaling led to Th2 response defects, characterized by the impaired pathogen clearance and reduced severity of Th2-mediated airway inflammation [110-112]. Thus, ICOS signaling is a key factor for Th2 differentiation and function.

Studies characterizing the role of ICOS in Th1 cells describe a conflicting role of ICOS in this T cell subset. In some Th1 inducing infection models, ICOS signaling is necessary for a protective Th1 response. For instance, *Salmonella* or *Listeria* infected ICOS knockout mice showed reduced Th1 responses and increased bacterial burden [113, 114]. It should be noted, however, that there are contradictory studies reporting that ICOS or ICOSL knockout mice show increased Th1 responses in pulmonary infection of *C. muridarum*.[17, 115]. Despite the increased Th1 response, lung pathology was exacerbated with the increased bacterial burden. Our group showed that this absence of ICOS is correlated with insufficient Th17 response [17]. Another group also reported that ICOS knockout mice infected with *Mycobacterium tuberculosis* showed elevated Th1 response with reduced bacterial burden [116]. It remains to be clarified why ICOS knockout mice show elevated Th1 responses in lung infection models. One intriguing possibility is that reduced lung ILC2 response in ICOS knockout mice skewed the T cell responses towards Th1 responses.

The impact of ICOS on Th17 cells remains controversial. Our group has shown that Th17 response against chlamydial lung infection is controlled by ICOS-PI3K signaling axis. *In vitro* data also suggested that ICOS signaling is correlated with initial differentiation and IL-23 mediated expansion of Th17 cells [17]. However, another study showed that ICOS is not required for initial Th17 cell differentiation [16]. Furthermore, blocking of ICOS signaling in EAE (experimental autoimmune encephalomyelitis), a disease whose pathology is dependent on Th17 cells, abrogates the disease severity. [117].

Tfh cells perform important roles for the germinal center (GC) reactions. Inside GC, Tfh cells provide key mediators for the selection and survival of B cells expressing high-affinity B cell

receptors [118]. Tfh cells express high levels of CXCR5 and PD-1 which guide Tfh cells into the GC. Tfh cells can produce IL-4 and IL-21 as key cytokines and express high levels of CD40L that promotes GC B cell expansion and differentiation [119, 120]. Many studies highlighted the importance of ICOS in Tfh cell generation. Our group also confirmed that ICOS-PI3K signaling is required for Tfh cells using $ICOS^{Y181F}$ mutant mice that have a selective defect in the ICOS-PI3K pathway. $ICOS^{Y181F}$ mutant mice showed reduced numbers of Tfh cells and impaired GC reactions similar to ICOS knockout mice [81]. ICOS and ICOSL interaction increased the T cell and B cell contact, leading to affinity maturation, B cell survival and plasma cell differentiation [121]. As mentioned in *section 1.1.4.*, ICOS deficiency in humans can cause severe reduction in antibodies and impaired specific antibody response to vaccination or infection [20, 77]. Similarly, ICOS or ICOSL deficient mice also showed severe defects in Tfh cell generation and GC reaction. [19, 79, 122]. On the other hand, increased expression of ICOS increased Tfh cell numbers which can lead to autoimmunity [39, 123].

Therefore, ICOS costimulation can influence Th1, Th2, Th17 and Tfh responses with the most dominant effect on the Tfh generation and function. Of note, some of the conflicting results generated by ICOS germline knockout mice could have stemmed from complex interplay between ICOS-expressing T cell subsets and ILCs. This complexity could be resolved by using cell-specific ICOS knockout mouse models.

The majority of data indicates that ICOS is critical for the activation and differentiation of effector T cells such as Tfh cells and inflammatory T cells. However, there are several studies showing that ICOS deficient mice have reduced $CD4^{+}Foxp3^{+}$ Treg cells in various autoimmune mouse models [124-127]. However, because of the dual role of ICOS in effector T cells and Treg cells, conflicting results have been reported depending on the disease model. Another Foxp3 expressing cell type, Tfr (T follicular regulatory) cells also express a high level of ICOS at the cell surface [128, 129]. (*see section 1.3.3*). As the role of ICOS in Treg is a key component of my thesis, I will devote a whole section to the role of ICOS in Treg cells in this introduction (*see Section 1.3*)

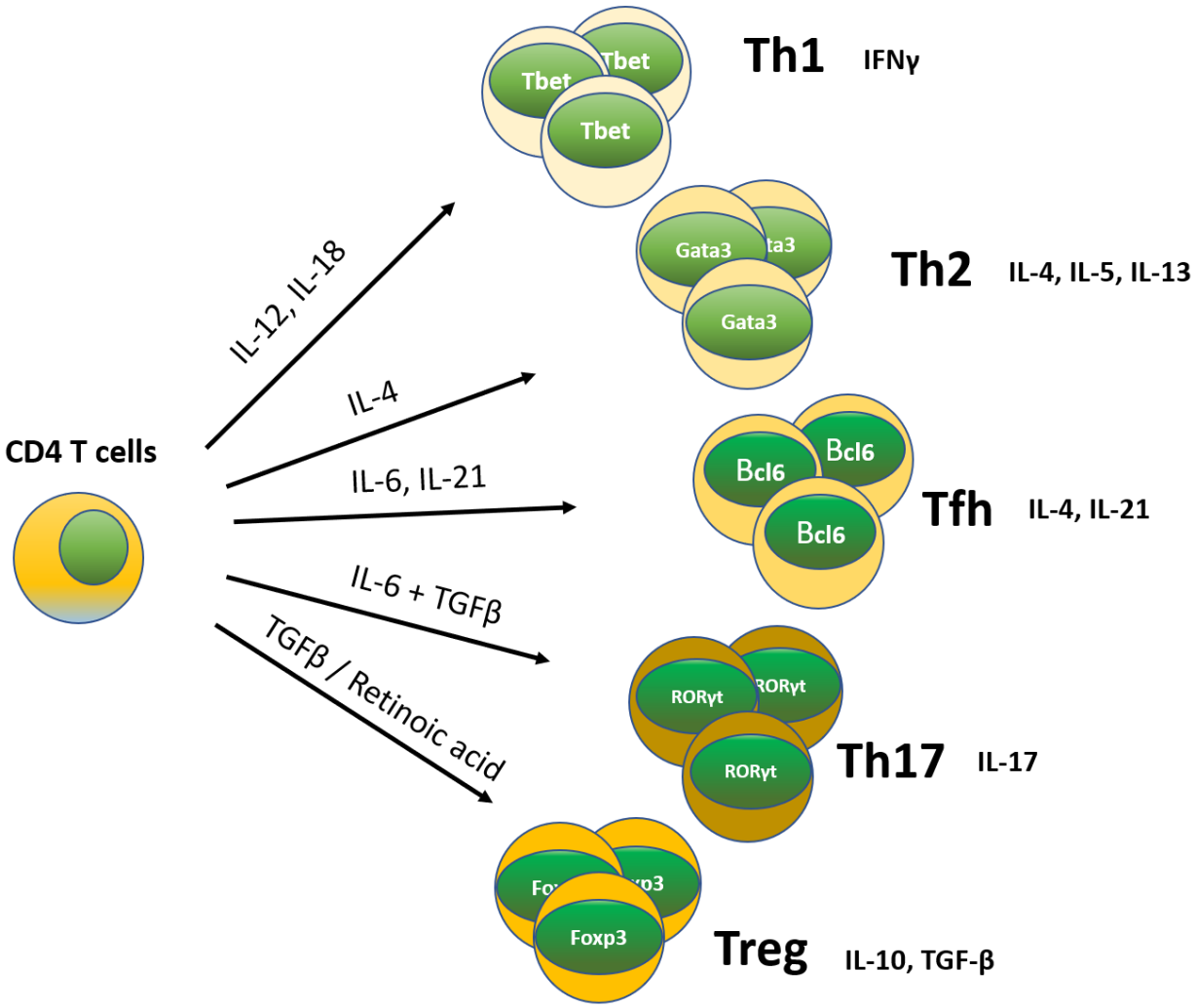


Figure 1.2. CD4$^+$ T cell subsets. Each subset is differentiated under the influence of different cytokines as indicated. For each subset, a distinct lineage-specific transcription factor has been identified. Fully differentiated CD4$^+$ T cell subsets produce unique sets of signature cytokines to mediate various immune responses.

1.2 Regulatory T cells

Peripheral tolerance is necessary to protect our body from autoreactive T cells raised against self-antigens or allergic reactions towards innocuous environmental antigens such as food. Regulatory T cells are the key immune cell type for maintaining peripheral tolerance [130]. Treg cells were first discovered by the Sakaguchi group. They identified a $CD4^+$ T cell subset constitutively expressing CD25 (α-chain of IL-2 receptor) as an immunosuppressive T cell population in 1995 and named regulatory T cells (Tregs) to distinguish from conventional $CD4^+$ T cells (Tcons). These $CD4^+CD25^+$ Treg cells maintained self-tolerance by down-regulating immune response to self and non-self antigens [131]. However, some Tcons also express CD25 when they are activated, thus the identification of a definitive Treg marker was necessary. Less than a decade later, the forkhead box transcription factor, Foxp3 was identified as a lineage-defining transcription factor for Treg cells [132, 133]. Since then, Foxp3 has been the most widely accepted marker for Treg cells.

The *Foxp3* gene is located on the X chromosome. In humans, the lack of the *FOXP3* gene in males causes immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), a severe autoimmune disease. In females, there are two X chromosomes. X-chromosome inactivation occurs randomly for one of the X chromosomes. If *FOXP3* gene on one of X chromosomes is mutated, this leads to impaired Treg cells. But the other X chromosome can generate normal Treg cells to protect this female from IPEX [134]. The Scurfy mouse model has an X-linked frameshift mutation in the *Foxp3* gene, and they develop the fatal lymphoproliferative disease with multi-organ inflammation.

Most $Foxp3^+$ Treg cells are generated during thymic development (thymus-derived Treg, tTreg or natural Treg, nTreg), but some Treg cells can be converted from Tcons in the periphery (peripherally derived Treg, pTreg). I will cover the tTreg development in *section 1.2.1*. *In vitro* studies have shown that Tcons can be differentiated into Foxp3-expressing Treg cells in the presence of IL-2 and TGF-β. This type of Tregs is known as induced Treg (iTreg) [135]. These iTreg cells have the similar suppressive function as tTreg cells *in vitro* and *in vivo*. [136, 137]. pTreg cells as well as tTreg express Foxp3, CD25, CTLA-4, GITR, CD39 and CD73. Some studies reported that tTreg and pTreg have different roles in different places. tTreg cells are mainly to maintain immune tolerance, while pTreg cells play a role in mucosal tolerance. To distinguish thymic versus

peripherally derived Treg, some markers such as Helios and Nrp-1 (neuropilin-1) have been proposed as tTreg markers [138, 139]. But 3 years later, tTreg cells have been found to contain $Foxp3^{+}Helios^{+}$ and $Foxp3^{+}Helios^{-}$ subpopulations, suggesting that Helios is not a specific marker to distinguish tTreg and pTreg. Nrp-1 is highly and specifically expressed on tTreg cells not on pTreg cells [139]. But another group demonstrated Nrp-1^{low} pTreg cells could be changed into Nrp-1^{high} pTreg cells in highly inflammatory environments [140]. Thus, more specific surface markers are yet to be discovered to distinguish tTreg and pTreg cells.

It is well documented that the level of Foxp3 expression correlates with Treg stability. When the 3'-UTR (untranslated region) of Foxp3 locus in C57BL/6 mice was replaced by DNA encoding luciferase and eGFP, Foxp3 expression level was reduced by ten-fold. The suppressive activity of Treg cells in these mutant mice was impaired *in vitro* and *in vivo*, leading to the development of an aggressive autoimmune syndrome [141]. This suggested that Foxp3 expression and Treg's suppressive functions are closely associated. Another component is epigenetic changes of *Foxp3* locus. The methylation status of *Foxp3* promoter region regulates the expression of Foxp3 in Treg cells. Within the *Foxp3* locus, there are three conserved non-coding sequences (CNS). Among them, CNS2, also called Treg specific demethylated regions (TSDR), is highly demethylated in tTreg cells, and methylated in naïve $CD4^{+}CD25^{-}$ T cells. The demethylated region is positively correlated with stable Foxp3 expression [142].

1.2.1 Development of Treg cells

Like other T cells, tTreg cells are differentiated from progenitor cells that migrate from the bone marrow into the thymus. Although the proportion of tTreg cells among single positive CD4 thymocytes is less than 1%, they make up to 15% of $CD4^{+}$ T cells in secondary lymphoid organs, and the depletion or functional alterations of tTreg cells lead to the development of a fatal autoimmune disease in mouse models [131, 143, 144]. These thymic derived Tregs cells migrate to the periphery very early in life– within three days after birth in mice [145]. Thus, the removal of the thymus in three-day-old mice induces organ-specific autoimmunity, but the same procedure in seven-day-old mice does not [146]. Over the past 20 years, immense amounts of research have reported the identification and understanding of Treg cells. In this section, I will cover how these tTreg cells are developed in the thymus.

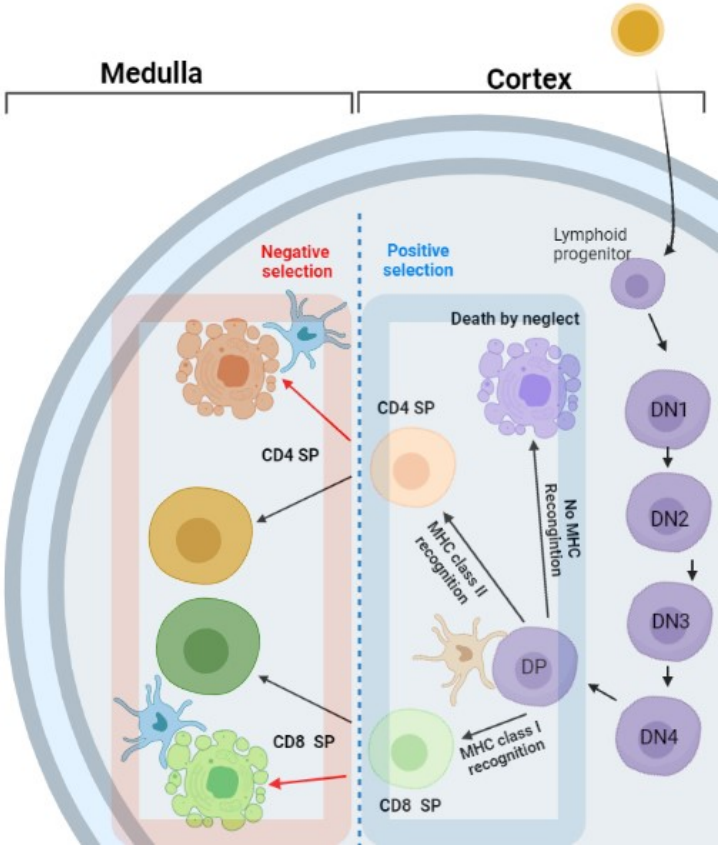
The current tTreg development paradigm is a two-step process [147, 148]. The first step is strong TCR stimulation. This induces the high-affinity IL-2R, CD25, GITR, OX40 and TNFR2 (Tumor necrosis factor receptor 2), leading to $CD25^{+}Foxp3^{-}$ Treg progenitor cells (TregP). The second step is the conversion of the TregP to mature Treg cells depending on IL-2 and IL-15 [147]. Thus, higher TCR self-sensitivity allows TregP cells to compete more effectively for IL-2, leading to a selective advantage for Treg repertoire [149]. More recently, another group reported alternative TregP cells, defined by low Foxp3 and lack of detectable CD25 expression ($CD25^{-}Foxp3^{lo}$ Treg). They demonstrated that this TregP cell develops into mature Treg cells efficiently *in vitro* or *in vivo.* Intriguingly, both $CD25^{+}Foxp3^{-}$ TregP and $CD25^{-}Foxp3^{lo}$ TregP were able to be differentiated into mature Treg cells at similar efficiencies. These TregP cells have a common TCR repertoire with mature Treg cells, but much less so with each other [150]. This result suggests that distinct interaction with self-antigens selects unique TregP cells, contributing unique TCRs to the mature Treg cell repertoire. They also observed that their protective roles are different from each other. Tregs from $CD25^{+}Foxp3^{-}$ TregP could protect mice from EAE (experimental autoimmune encephalomyelitis), but Tregs derived from $CD25^{-}Foxp3^{lo}$ TregP was capable of suppressing colitis. In sum, these data showed that both $CD25^{+}Foxp3^{-}$TregP and $CD25^{-}Foxp3^{lo}$ TregP contribute to the mature Treg cell repertoire.

It was controversial whether tTreg cell TCR repertoire is enriched in self-reactive TCRs. Nevertheless, early work supports the idea that the Treg cell TCR repertoire is more self-reactive than Tcons [149]. This view was confirmed in later studies that TCR transgenics are associated with thymic Treg cell development when the thymus has the cognate Ag [151]. Later work showed the relationship between TCR affinity and Treg cell development. They observed that TCR affinity and Treg cell niche size are closely related with higher-affinity TCRs characterized by increased Treg numbers [152]. TCR signaling strength is also related to the competency of developing TregP cells to respond to the low level of IL-2 in the thymus [153]. In summary, Treg cell interaction with thymic Ag is necessary for initiating thymic Treg cell development.

The thymus is composed of the outer cortex and inner medulla. While the cortex represents the early T cell development and positive selection of $CD4^{+}CD8^{+}$ thymocyte, the thymic medulla plays a key role in T cell maturation (Figure 1.3A) [154]. Tcons experience a selection process in the thymus depending on the strength of the received signal from thymic APCs such as DCs, mTEC (medullary thymic epithelial cell), or cTEC (cortical thymic epithelial cell) presenting self-peptides.

Positive selection occurs when a double positive thymocyte can bind cTEC expressing MHC: self peptide complexes with enough affinity as a survival signal. No or very low signals result in thymocyte death by neglect. Strong signals between the TCR and MHC: peptide in mTEC lead to negative selection (Figure 1.3B). Like Tcons, tTreg cells also have been selected on self-peptides. However, in addition to TCR signal strength and affinity for MHC/peptide, control of the tissue-specific self-antigens (TSA) repertoire expressed in the thymus affects Treg cell development. mTEC promiscuously expresses TSA and *Aire* (autoimmune regulator gene) is associated with the expression of TSA in mTECs. *Aire*-deficient mice have compromised negative selection, developing multi-organ autoimmunity. Some studies reported that Aire-expressing cells not only promote the deletion of self-reactive Tcons, but they may also enhance Foxp3 expression in $CD4^+$ thymocyte [155, 156]. However, other groups have shown that the number, frequency and function of tTreg is not changed in the absence of the *Aire* gene [157-159]. Like *Aire*, *Fezf2*, a transcriptional regulator of TSA in mTECs also plays an indispensable role in the expression of TSA independently of Aire [160]. It seems to be possible that *Aire* or *Fezf2*- dependent TSA is linked to the tTreg cell development. Thus, these findings are important to understand the immune tolerance and mechanisms underlying autoimmune disease.

A


Medulla
Cortex
Negative selection
Positive selection
Lymphoid progenitor
Death by neglect
CD4 SP
CD4 SP
DN1
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DN4
No MHC Recognition
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DP
MHC class I recognition
CD8 SP
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B.

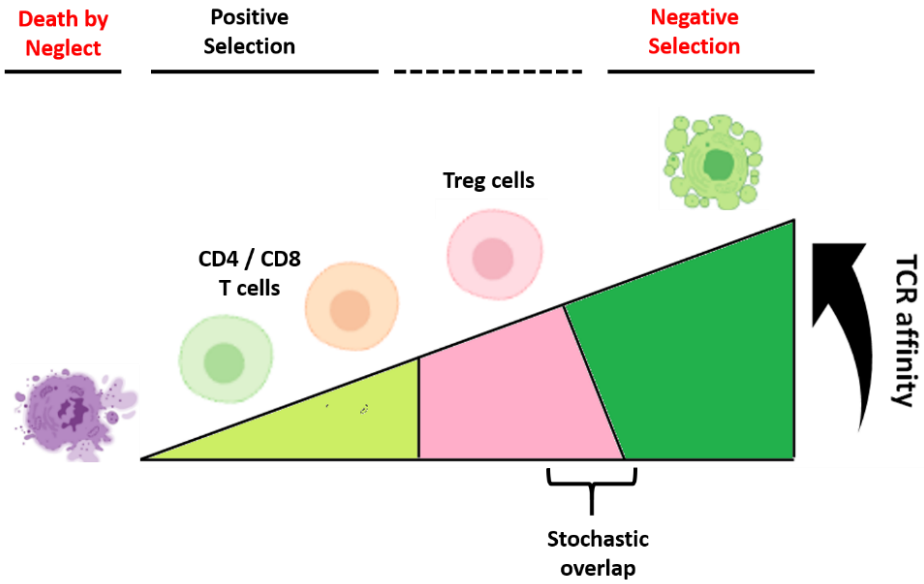

Death by Neglect
Positive Selection
Negative Selection
Treg cells
CD4 / CD8 T cells
TCR affinity
Stochastic overlap

Fig 1.3 Scheme of T cell development in the thymus and TCR affinity-based on selection model. **(A)** Committed lymphoid progenitor cells migrate to the thymus. Early T cells which lack expression of TCR, CD4 and CD8, are named as double-negative (DN) thymocytes. DN thymocytes can be divided into DN1 to DN4 stages. As these DN cells progress, they express the pre-TCR, then they become double positive (DP). These DP thymocyte can interact with cortical epithelial cells expressing MHC I or MHC II molecules associated with self-peptides. The fate of these DP cells will be determined based on signaling that is mediated by interaction of TCR with these self-peptide MHC ligands. Too weak signaling leads to death by neglect, whereas too much signaling promotes apoptosis (negative selection). This negative selection is the most common event in the medullar upon encounter with strongly activated self-peptides on DC or mTEC. Of note, TCRs expressed on Treg cells can recognize self-peptides with high affinity, and this high affinity is required for Treg development in the thymus. The intermediate level of TCR signaling leads to effective maturation and positive selection. Thymocytes that express TCR which can bind self-peptide on MHC I become $CD8^+$ T cells, whereas those that express TCR that interact with self-peptide on MHC II become $CD4^+$ T cells. Then these cells are ready to leave thymus to go to the periphery. **(B)** Double positive (DP) thymocytes expressing TCRs with no affinity for self-peptide-MHC complexes die by neglect. Thymocytes with intermediate affinity for self-peptide-MHC complexes survive through the positive selection process, then commit to the CD4 or CD8 T cells. High-affinity binding of the TCR to self-peptide-MHC complexes induces cell death by apoptosis known as negative selection. Some high functional affinity T cells become Treg cells to achieve significant suppressive effect on the periphery.

1.2.2 Homeostasis of Treg

Treg homeostasis represents the activity maintaining an appropriate balance between effector and regulatory T cells with preserving their functional specialization. This way, our body can prevent unwanted immunopathology and autoimmune disease. This homeostatic balance is achieved by a combination of cytokine signals and interactions between various cell types. The mechanism of Treg homeostasis is well established.

The mechanisms of Treg homeostasis are based on the continuous signaling from TCR that recognizes self-peptides [161, 162], co-stimulation signals mediated by CD28 [163, 164] or ICOS and the effects of cytokines, mainly IL-2 [165, 166]. The combined effects of these factors could be observed in T cell zones of secondary lymphoid organs, and the effects are DC-mediated.

First, TCR signaling is a key element in the homeostatic maintenance. TCR cloned from Treg cells propel a high degree of homeostatic proliferation [167]. The high degree of self-reactive Treg cells demonstrate the possibility to encounter antigens in the periphery, and control the size and composition of the Treg cell pool in different tissues [168]. Interestingly, downstream of the TCR complex, the PI3K-Akt pathway is essential for proper activation of Tcons, but is inhibitory to Treg cell differentiation and expansion. Decreased Akt activation induced Treg development [169], whereas overexpression of constitutively active Akt led to reduced Treg cell frequencies [170]. In summary, Tcons and Treg cells share similar molecular machinery to respond to TCR signals, but the result of these signals could have different consequences depending on the cell types.

Second, costimulation plays a role in Treg homeostasis. For T cell activation, naïve T cells need at least two signals, the first one is delivered through the TCR, and the second one is provided by costimulatory receptors. CD28 is the first identified costimulatory receptor, the interaction between CD28-CD80/CD86 is well known costimulatory pathway. Deficiency of CD28 exacerbated diabetes incidence in NOD (Non-obese diabetic) mice, and was rescued by transfer of wildtype Treg cells [171]. The abrogation of CD28 leads to decreased numbers of tTreg and pTreg cells [172]. Treg-specific CD28 knockout mice also developed severe autoimmunity with increased numbers of activated effector cells [173]. ICOS is another member of costimulatory receptors to control the development and functions of $CD4^+$ T cells subsets. ICOS deficient mice showed reduced Treg cell numbers. However, the molecular mechanisms of CD28 and ICOS controlling Treg cell development and homeostasis remain to be elucidated.

Lastly, IL-2 is a critical regulator of immune homeostasis of Treg and effector T cells. Originally IL-2 was known as a potent T cell growth factor, characterized by promoting the expansion of antigen-activated T cells. This cytokine is secreted by $CD4^+$ and $CD8^+$ T cells in secondary lymphoid tissues. IL-2 binds to their receptor, which has three components. IL-2 receptor α chain (CD25), IL-2 receptor β (CD122) and IL-2 receptor γ (CD132) [174]. All three chains form a complex to bind IL-2 with high affinity on activated T cells including Treg cells [174, 175]. IL-2 signaling is associated with cell cycle progression and the expression of anti-apoptotic protein, Bcl2 [176]. Additionally, IL-2 signaling can promote CD25 expression through Stat5 [177]. Since Treg cells do not produce IL-2, Treg cells rely on paracrine IL-2 secreted by other T cells [178]. The importance of IL-2 in Treg homeostasis has been supported by autoimmune diseases that occur in IL-2 deficient mice. Mice lacking IL-2 demonstrate increased active T cell responses, lymphoproliferation, lymphadenopathy and splenomegaly before 8 weeks of age [179, 180]. Intriguingly, IL-2 controls a specific Treg subset, named as central Treg (or cTreg) characterized by $CD4^+CD44^{lo}CD62L^{hi}Foxp3^+$ which are localized in T cells zone of secondary lymphoid tissues [181]. cTreg cells receive IL-2 from activated T cells and inhibit APC and autoreactive T cells [182]. However, effector Treg cells (eTreg, $CD4^+CD44^{hi}CD62L^{lo}Foxp3^+$) can be maintained in an IL-2 independent manner [181]. These eTreg cells are localized in non-lymphoid organs, where they function as suppressive Treg cells to dampen the tissue inflammatory response [183]. Based on these data, IL-2 is not a survival factor for all Treg cells, but rather it has a specific role in certain Treg subtypes (Figure 1.4).

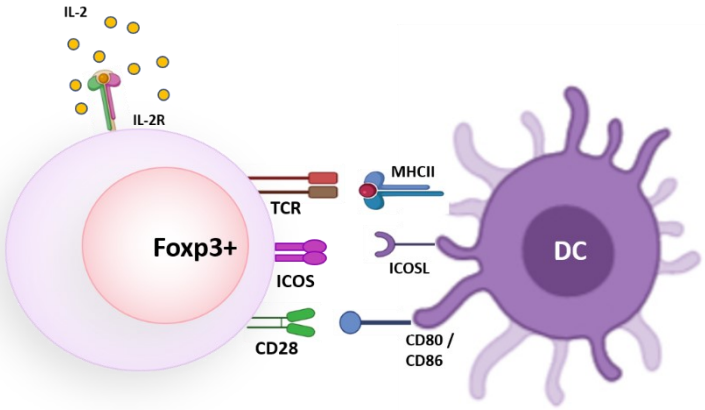


Figure 1.4 Signals for Treg homeostasis. Treg cells require multiple signals for homeostasis. These include TCR stimulation, costimulatory signals such as CD28 (and possibly ICOS) as well as IL-2 produced by neighboring autoreactive $CD4^+$ T cells.

1.2.3 Suppressive mechanisms

A tremendous amount of research on the mechanisms by which Treg cells suppress the immune response has revealed a broad spectrum of suppressive mechanisms [131].

Interactions between Treg and DCs initiate the antigen-specific suppression by specific recognition of antigens. This interaction induces an antigen-specific tolerogenic DC or inhibits DCs from presenting a specific antigen. CTLA-4 on Treg cells binds to costimulatory ligands CD80/CD86 on the DCs, and through a process of trans-endocytosis removes CD80/CD86 from DCs [184]. In addition, CTLA-4 mediated increase in the expression of IDO (Indoleamine 2,3-dioxygenase), an enzyme that degrades tryptophan, lowers tryptophan concentration which is necessary for the proliferation of effector T cells [185]. These mechanisms impair antigen presentation, causing inactivation of effector T cells (Figure 1.5A).

Antigen-non-specific mechanisms were also reported. Hydrolysis of extracellular ATP to AMP and adenosine is a well-known suppressing mechanism mediated by Treg cells. Treg cells highly express ectoenzyme, CD39 and CD73. CD39 induces the hydrolysis of ATP (Adenosine triphosphate) to AMP (Adenosine monophosphate), CD73 catalyze AMP to adenosine following CD39 activity [186]. Adenosine and adenosine A_{2A} receptors (A2AR) can blunt the TCR activation via generation of cyclic AMP and activation of protein kinase A (PKA). PKA suppresses the TCR signaling by phosphorylation of C-terminal Src kinase (Csk), nuclear factor of activated T cells (NF-AT) and cyclic AMP response element binding proteins (CREB) [187]. Increased concentration of adenosine suppresses antigen presentation by DC, and inhibits the proliferation of effector T cells [188] (Figure 1.5B).

Treg cells produce immune inhibitory cytokines such as IL-10, IL-35 and TGF-β. IL-10 has been described as an immunomodulatory cytokine, which can inhibit the secretion of inflammatory cytokines such as IL-2 with suppressing Th1 response [189]. IL-10 can increase phagocytic activity of phagocytes, removing the cellular debris in the inflammation site. Furthermore, IL-10 suppresses tyrosine phosphorylation in response to CD28 costimulation [190]. This inhibition leads to impaired PI3K/AKT signaling activation, which in turn dampens NF-κB nuclear translocation [190, 191]. Treg cells deficient in one of the IL-35 chains showed reduced suppressive capacity *in vivo* [192]. IL-35 deficient Treg could not control the homeostatic expansion of effector T cells and have an impaired capacity to cure the established colitis when compared to wildtype Treg cells [193]. TGF-β can

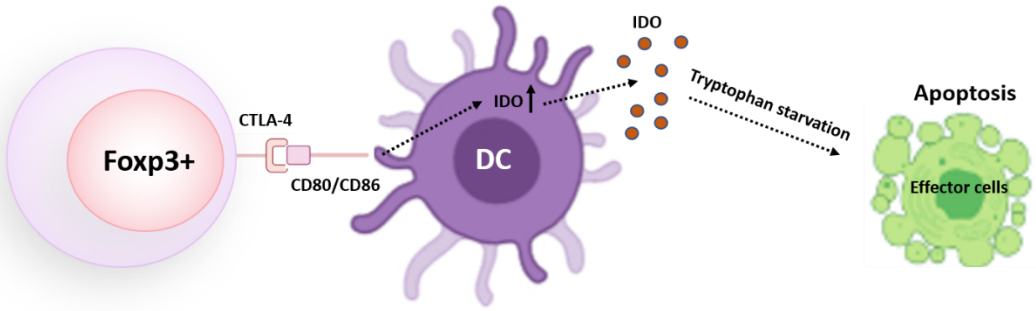
regulate effector T cell differentiation. TGF-β receptors are expressed on monocytes and DCs, thus regulating DC maturation and differentiation. TGF-β is necessary to generate tolerogenic DC, which inhibits T cell proliferation [194]. Like IL-10, TGF-β also inhibits IL-2 production [194] (Figure 1.5C).

Treg cells secrete a serine protease, granzyme B, inducing apoptosis in effector T cells [195]. In Treg-effector T cell interactions, Treg granules containing granzymes and perforin are exocytosed. Perforins insert into the lipid membrane of the target cells and forms a pore through which granzyme can enter the cells. Perforin-granzyme cytolysis is another contact-dependent suppression mechanism (Figure 1.5D).

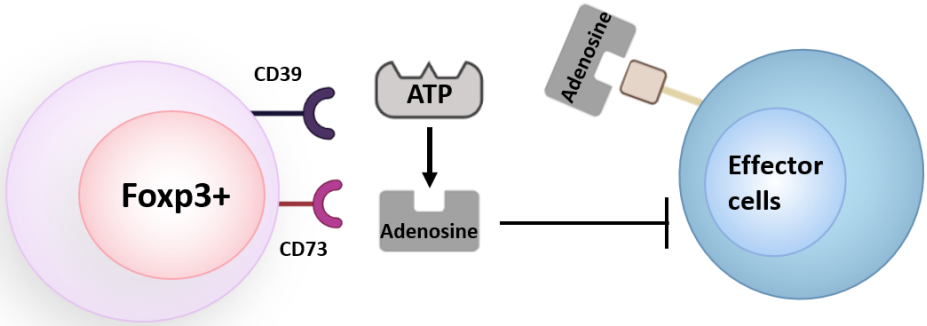
Treg cells constitutively express high levels of IL-2 receptor alpha chain, CD25, which has a high affinity to IL-2. They compete with proliferating effector T cells for this growth factor. Treg cells deprive proliferating $CD8^+$ T cells from IL-2, and leave them with metabolic interruption and cell death [196]. However, a different IL-2 receptor transcriptional signatures between thymic Tregs and peripheral Treg cells are observed, indicating that IL-2R signaling in the thymus and periphery leads to different effects on Treg function [197]. In addition, deficiency in IL-2 receptors results in reduced expression of Foxp3 expression in Treg cells, which makes it complicated to understand the role of IL-2 receptor in the suppressive function of Treg cells. Based on the genetic gain- or loss-of -function approaches, capture of IL-2 is dispensable for the control of $CD4^+$ T cells but is necessary for limiting the activation of $CD8^+$ T cells. IL-2 receptor dependent activation of STAT5 has an important role in the suppression [198].

The suppressive mechanisms of Treg cells described above can perform together or independently based on the requirements of the immune system.

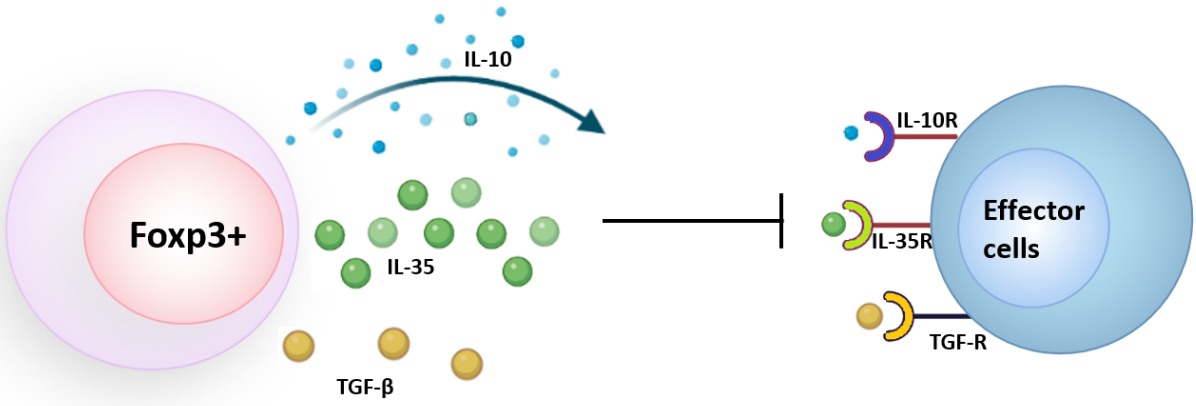
A.


Foxp3+
CTLA-4
CD80/CD86
DC
IDO
IDO
Tryptophan starvation
Apoptosis
Effector cells

B.


CD39
ATP
Adenosine
Foxp3+
CD73
Adenosine
Effector cells

C.


IL-10
Foxp3+
IL-35
TGF-β
IL-10R
IL-35R
TGF-R
Effector cells

D.

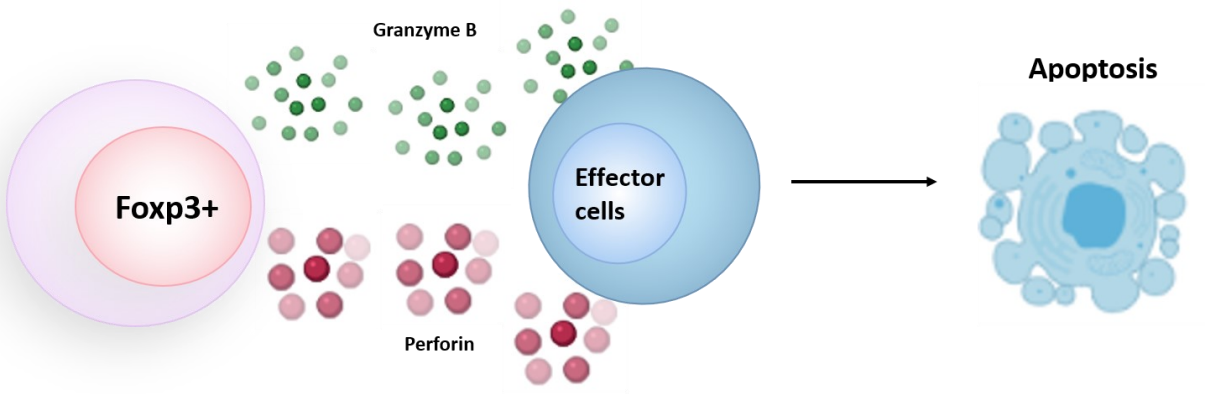


Figure 1.5 Mechanisms of Treg suppression. (A) Controlling APC maturation and function. The interaction of CTLA-4 and CD80/86 on APCs has a negative impact on T cell activation. CTLA-4 captures its ligands CD80/86 on APC and subsequently trans-endocytoses its ligands. The upregulation of IDO and degradation of tryptophan lead to starvation of effector cells. **(B)** Disruption of metabolic pathways. The ectoenzyme CD39 and CD73 on Treg cells lead to the metabolism of ATP to AMP and producing immunoregulatory adenosine. **(C)** Anti-inflammatory cytokine production. The secretion of anti-inflammatory cytokines such as IL-10, TGF-β and IL-35 is associated with inhibition of T cell activation. **(D)** Induction of apoptosis. Treg cells are able to induce apoptosis via granzyme B and perforin, which damage target cells membrane leading to apoptosis.

1.2.4 Heterogeneity of Treg cells

As described earlier, Treg cells could be divided into two subgroups, tTreg and pTreg. tTreg cells develop in the thymus during the thymic selection process, whereas pTreg develops in the periphery from naive T cells following antigenic stimulation. tTreg cells have the constitutive expression of Foxp3 and TCR of high auto-affinity, and they are located predominantly in the blood stream and lymph nodes, providing tolerance to autoantigens [130]. pTreg cells are in the peripheral tissue, and are involved in suppressing local inflammation. TCR repertoire for tTreg and pTreg cells are distinct. The TCR repertoire for tTreg cells is mainly self-recognition, and pTreg TCR repertoire is biased toward foreign antigen recognition [199]. Thus, tTreg cells are implicated in the development of autoimmunity. On the other hand, pTreg cells function to control local inflammation in the mucosal barriers and placenta-fetal interface [200, 201].

Treg cells are further characterized by surface markers and localization [181, 202]. Based on the expression level of CD44 and CD62L, the Campbell group divided Treg cells into $CD44^{lo}CD62L^{hi}$ central Treg (cTreg) and $CD44^{hi}CD62L^{lo}$ effector Treg (eTreg) cells [181]. cTreg cells are quiescent, reside in T cell zones of secondary lymphoid tissues, and are IL-2 dependent. In contrast, eTreg cells are a dominant population in nonlymphoid tissues with IL-2 independence. eTreg cells are highly proliferative, but prone to apoptosis. Interestingly, there is evidence that the maintenance of eTreg cells depends on TCR and the co-stimulatory signal, ICOS but not IL-2 [181].

Another group identified two Treg populations with different degrees of self-reactivity and distinct regulatory function, $GITR^{hi}$ $PD\text{-}1^{hi}$ $CD25^{hi}$ ($Triple^{hi}$) Treg and $GITR^{lo}$ $PD\text{-}1^{lo}$ $CD25^{lo}$ ($Triple^{lo}$) Treg. $Triple^{hi}$ Treg cells are self-reactive in peripheral lymph nodes, whereas $Triple^{lo}$ Treg cells were less self-reactive and promote differentiation of $CD4^+$ T cells into peripherally induced Treg cells (pTreg cells) and prevent the development of colitis [199]. Based on the expression of these proteins, Triple Tregs are different from cTreg and eTreg populations.

Currently, a revised model of peripheral Treg cells has been proposed. They reported that gradient expression of TCF1(T cell factor 1) and LEF1 (Lymphoid enhancer binding factor 1) separates Tregs cells into three distinct populations. TCF1 and LEF1 are transcription factors to cooperate with Foxp3 [203]. They found that all $CD44^{lo}CD62L^+$ Treg cells were $TCF1^+$, which they named as resting Treg (rTreg) cells. In contrast, $CD44^{hi}CD62L^{lo/-}$ Treg cells can be divided two

subsets, $TCF1^+$ (activated Treg, aTreg) and TCF^- (effector Treg, eTreg). Although TCF1 and LEF1 are dispensable for Treg's suppressive function, they are essential for Treg's survival [204].

Treg cells can acquire the expression of transcription factors typical of effector cells such as Th1, Th2 or Th17 cells based on their adaptation to the immune response [205]. The binding of IFN-γ on IFN-γ receptor on Treg cells activates STAT1 and triggers T-bet and CXCR3 expression, allowing Treg cells migration to the sites of Th1-mediated inflammation [206]. TCR repertoire analysis revealed that $CXCR3^+$T-bet^+ Treg cells and $CXCR3^-$T-bet^- Treg cells have different antigenic specificity, reflecting the functional differences between them [207]. Interferon regulatory factor 4 (IRF4) was reported as the transcription factor responsible for the generation of Treg cells controlling Th2 responses [208]. IRF4-deficient Treg cells in murine models showed autoimmune syndrome mediated by Th2 type of immune response [208]. Similar to regulation of Th1 and Th2 responses by specialized Treg subpopulation, expression of the Th17 transcription factor STAT3 is essential for controlling Th17 responses [209]. Deficiency in STAT3 in Treg cells causes spontaneous intestinal inflammation with increased Th17 cells, demonstrating their selective failure in the control of Th17 response. In addition to STAT3, another Th17 transcription factor RORγt is also expressed in Treg cells [210, 211].

Functional and phenotypic heterogeneity of Treg cells still remains a key issue in Treg cell research. In particular, many studies used ICOS as one of the activation markers, but it remains to be seen if ICOS is required for the formation of some of the Treg subsets.

1.3 The role of ICOS in Treg

Among T cells in peripheral blood, $ICOS^+$ T cell is a small subset of $CD4^+$ T cells. But they are enriched in Treg cells, as approximately 20% of Treg population are $ICOS^+$ Treg cells [212]. In addition, the ICOS expression level in Treg cells is higher than that of non-Treg $CD4^+$ T cell subsets. ICOS signaling is closely related to an anti-inflammatory response which is mediated by Treg cells. Many studies have reported that $ICOS^+$ Treg cells have an inhibitory capacity to prevent the development of autoimmune diseases [127, 212, 213] . These observations raised the shared question to me and other investigators. What is the role of ICOS signaling in Tregs cells? Elucidating the

complicated relationship between ICOS and Treg cells to control excessive immune response is an important topic. However, the dual role of ICOS in promoting Tcons and Treg cells has generated conflicting results from some disease models. Thus, numerous studies have tried to reveal the role of ICOS in Treg generation, Treg homeostasis and Treg suppressive functions. In this section, I will review what is known regarding the role of ICOS in Treg cells.

1.3.1 ICOS and thymic selection

TCR rearrangement occurs in the thymus as a process of T cell maturation, leading to a vast diversity of TCR repertoire. Clonal deletion removes autoreactive T cells in the thymus to prevent them from migrating to the periphery [214, 215]. Thymocytes expressing TCR with low to moderate affinity to self-antigens are positively selected and receive survival signals for the next step. Thymocytes containing high affinity TCR to self antigens undergo apoptosis [7] (Figure 1.3). Some intermediate level of affinity T cell clones can survive to be immuno- suppressive Treg cells [7, 216]. Thymic negative selection occurs at $CD4^+CD8^+$ double positive (DP) or $CD4^+$ or $CD8^+$ single positive (SP) stage of T cell development [217, 218]. Mature $CD4^+$ or $CD8^+$ SP thymocytes encounter tissue-specific antigens in the thymic medulla. This thymic medulla has mTEC, thymic DCs and B cells expressing costimulatory receptors, which are critical for negative selection [219-223]. Costimulatory receptor, CD28 binds CD80 or CD86 on the thymic APCs, which plays a role in negative selection [224-226]. However, CD28 interaction with CD80/CD86 is not the only costimulatory signal for negative selection. ICOS is expressed on some thymocytes. Additionally, mTECs expressed ICOSL [227], suggesting that ICOS signal could be implicated in T cell development.

1.3.1.1 ICOS and tTreg generation

Treg cells are differentiated as a T cell lineage from thymocytes [228]. The development of Treg cells depends on TCR signaling with a medium affinity for self-antigens, IL-2 and CD28 costimulatory signaling. An interesting study was reported in 2008. Liu and colleagues identified two subsets of $FOXP3^+$ tTreg cells defined by the expression of ICOS in the human thymus and

periphery. ICOS$^+$FOXP3$^+$ Treg cells used IL-10 for suppressing DC function, whereas ICOS$^-$ FOXP3$^+$ Treg cells used TGF-β mainly. They also observed that the newborn thymus has ICOS$^+$FOXP3$^+$ Treg cells, and that the thymus contained more ICOS$^+$ Treg cells than adult peripheral blood. These data suggested that ICOS$^+$ Treg cells are generated from thymus, and not by the differentiation of ICOS$^-$ Treg cells in the periphery to ICOS$^+$ Treg cells [228]. A few years later, another group reported that ICOSL on human mTECs induces tTreg via IL-2 secreted by CD4$^+$ SP thymocyte [229]. These results suggest that ICOS-ICOSL interaction may be associated with tTreg cell generation in the thymus.

1.3.2 ICOS and Treg homeostasis

The general mechanisms for Treg homeostasis are well established. The major signal is provided by continuous TCR signaling, which recognizes self-antigens [161, 162]. The second signal is costimulatory receptor interaction via CD28 [172]. In addition, IL-2 is a critical regulator of Treg homeostasis through its role in the development, maintenance and function of Treg cells [175, 230]. According to various studies, ICOS is involved in promoting cellular proliferation, survival and strong suppressor capability for Treg cells [125, 231-233].

Abrogation of ICOS in mice showed reduced Foxp3$^+$ Treg cells compared to the WT mice [125]. Upon blocking of ICOS-ICOSL interactions, human naïve CD4$^+$ T cells cultured with B cells showed decreased FOXP3 expression level [234]. ICOS-deficient Treg cells from mice showed slight Foxp3 instability under strong inflammatory conditions, correlated with methylation status of Foxp3 CNS2 [233]. Note that disrupting the expression of ICOSL on DCs also negatively affects the stability of Treg cells [235]. These data suggest the importance of ICOS for the transcriptional activity of *Foxp3* [232]. However, that is not always the case. ICOS$^{-/-}$ mice showed a similar or slightly enhanced frequency of Foxp3$^+$ Treg cells in the thymus compared to WT mice [125].

ICOS signaling may promote the proliferation of Treg cells. ICOS$^+$ Treg cells have superior proliferative ability than their counterpart ICOS$^-$ Treg cells with higher Ki67 expression [127, 236]. ICOSL expressing DCs promotes ICOS$^+$ Treg cells proliferation *in vitro* by interacting with ICOS on Treg cells [228]. Likewise, ICOS$^{-/-}$ mice showed a delayed and insufficient expansion of Treg under helminth infection [237]. When ICOS$^{-/-}$ mice were tolerated with intranasal OVA (ovalbumin), they showed significantly decreased splenic and lung-resident Treg cells compared to WT mice

[238]. However, BrdU assay for Treg proliferation showed that there is no difference in BrdU uptake in $ICOS^{-/-}$ Treg cells upon infection compared to WT mice [237]. In summary, ICOS appears to be associated with proliferation of Treg cells, however, it is not clearly elucidated yet.

ICOS signaling is involved in Treg survival. Murine $ICOS^{-/-}$ Tregs stimulated with anti-CD3 for 2 days died within a few hours, whereas $ICOS^{+}$ Treg cells became hyperproliferative and lived longer [236]. ICOS and ICOSL interaction could induce PI3K activation, leading to PI3K-Akt downstream signals, which were suggested to mediate anti-apoptotic effects [239]. Another *in vitro* culture data also shows that $ICOS^{+}$ Treg cells in the presence of IL-2 expressed a higher level of Bcl-2, an anti-apoptotic molecule, than $ICOS^{-}$Treg cells [127]. It should be noted, however, that Bcl-2 is not exclusively important for assessing Treg survival. Other anti-apoptotic proteins including Mcl-1and Bcl-xl demonstrated different results in mice of different ages. $CD44^{hi}CD62L^{lo}$ eTreg cells express high a level of ICOS, but exhibit low Bcl-2 expression under steady state [181]. In summary, ICOS signaling may be associated with Treg survival by mediating different anti-apoptotic molecules.

The cytokine IL-2 is regarded as an essential one for the expansion and survival of Treg cells. During tTreg development in the thymus, IL-2 signaling induced Foxp3 expression [198]. In addition, IL-2 signaling has an essential role in the phosphorylation of transcription factor STAT5, which is downstream of the JAK kinase, and indispensable for Foxp3 expression. Addition of IL-2 increased the expression of Mcl-1, an anti-apoptotic molecule, *in vitro* and *in vivo* [240]. $ICOS^{+}$ Treg cells from BDC2.5-NOD mice, a mouse model for type 1 diabetes, showed that $ICOS^{+}$ Treg cells are more sensitive and dependent on IL-2 for their survival than $ICOS^{-}$ Treg cells [127]. Abrogation of ICOS signaling reduced the suppressive function without influencing their reactivity to IL-2 [127]. However, eTreg cells, which showed relatively higher levels of ICOS expression compared to cTreg cells, do not actively respond to IL-2 *in vivo*, and lack of IL-2 does not impair eTreg cell homeostasis [181].

1.3.3 ICOS and T follicular regulatory cells

Tfr cells are one of $CD4^{+}$ T cell subsets which migrate into the GC to control antibody responses [241, 242]. They express Tfh cell markers (CXCR5, PD-1, Bcl6 and ICOS) and Foxp3, but Tfr cells lack the expression of IL-4, IL-21 and CD40L. Interestingly, they also express high

levels of Blimp-1, which is antagonistic to Bcl6 expression [243]. Many studies support that Tfr cells are differentiated from thymic Tregs interacting with DCs [128, 129, 241, 244].

Tfr cells inhibit humoral immunity mediated by Tfh cells. Some in vivo and in vitro data demonstrated that Tfr cells suppressed Tfh cell activation, IL-21 production, and inhibited class switch recombination and B cell activation [244, 245]. Another group also confirmed this result. They used SLAM-associated protein (SAP) deficient mice (*Sh2d1a*$^{-/-}$), as Tfr cells are dependent on SAP for their formation. These mice showed increased Tfh and GC B cell response [129]. However, the exact role of Tfr cells in the germinal center is controversial. The Tfr depletion model using Treg-specific Bcl6 knockout mice, and augmented Tfr development with Treg-specific Blimp1 knockout mice unexpectedly showed that GC B cell levels are positively correlated or irrelevant to Tfr levels [246-248]. Another study using a different Tfr deletion model suggested that Tfr cells control early, but not late, germinal center responses to modulate antigen-specific antibody and B cell memory [249].

Although Tfr cells have high level of ICOS expression on the cell surface [242, 250], little is known about the role of ICOS in Tfr cell differentiation and function. There is some literature that describes ICOS as being required for the formation of Tfr cells under chronic GVHD and influenza infection [251, 252]. There also appears to be a link between ICOS dependent signaling pathways and Tfr cells, as a study has shown that PI3K and intracellular calcium flux influence Tfr cells [253-255]. Therefore, ICOS signaling could be associated with Tfr differentiation via PI3K and intracellular calcium flux pathways.

1.3.4 ICOS and suppressive function of Treg

ICOS expression may provide Tregs with enhanced suppressive functions. Various studies have investigated the significance of ICOS and ICOSL interaction for self-tolerance mediated by Treg cells. However, the mechanisms for ICOS-associated suppressive function of Treg cells are not clearly identified yet. Under certain inflammatory models, ICOS^{+} Treg cells have stronger inhibitory capacity than ICOS^{-} Treg cells, likely a result of IL-10 expression in ICOS^{+} Treg cells [127, 238]. Moreover, numerous studies have revealed the correlation between the expression of ICOS and suppressive markers, such as CTLA-4 and the higher secretion of IL-10 [228, 256, 257].

1.3.4.1 ICOS and suppressive marker, CTLA-4 on Treg

Different from constitutively expressing CD28, CTLA-4 is rarely detected on the surface of resting T cells. CTLA-4 primarily can be found in the intracellular compartment of Treg cells and activated T cells [258]. However, surface expression of CTLA-4 is tightly controlled by restricted trafficking to the cell surface and internalization. CTLA-4 interacts with CD80/CD86 with higher affinity than CD28. This interaction plays an opposing role to CD28. Not only interrupting CD28 engagement by higher affinity interactions, CTLA-4 can effectively eliminate the costimulatory ligands from the surface of APCs [259]. CTLA-4 is essential to prevent deviant T cell response against self-proteins. CTLA-4 deficient mice develop a lymphoproliferative syndrome with T cell response to self-antigens. [260, 261]. Patients with CTLA-4 mutations also showed severe immune dysregulation, suggesting the importance of this receptor for regulating T cell response [262]. However, previous studies showed that Treg cells from CTLA-4 deficient mice still have uncompromised suppressive activity *in vitro*, and these CTLA-4 deficient Treg cells expressed increased levels of IL-10 and TGF-β [263, 264]. Nevertheless, how self-antigen recognition initiates CTLA-4 dependent suppressive function in Treg cells is not known yet.

Interestingly, relatively higher expression level of CTLA-4 was observed on the $ICOS^+$ Treg cells than $ICOS^-$ Treg cells [228]. In vitro stimulation of the $ICOS^+$ Treg cells could maintain their high expression of CTLA-4. These results suggested that there are some connections between these two cosignaling receptors [228]. A study showed that blockade of the ICOS signaling in the human sample decreased suppressive capacity of Treg cells, due to down-regulated expression of CTLA-4 of Treg surface. They also demonstrated that the blockade of ICOS signaling leads to decreased surface CTLA-4 expression in Treg cells without affecting its whole cell expression level [234]. Additionally, murine data confirmed that there is a positive relationship between ICOS and CTLA-4 expression in Treg cells. They demonstrated that $ICOS^+$ Treg cells mediate their suppressive function in a CTLA-4 dependent manner [257]. Higher suppressive capacities of $ICOS^+$ Tregs may be positively related with CTLA-4 expression [212] . These data suggest that ICOS expression plays a synergistic role with CTLA-4 to enhance the suppressive function of Treg cells.

1.3.4.2 ICOS and suppressive cytokines on Treg

Treg's suppressive function appears to be greatly correlated with the production of immunoregulatory cytokines. $ICOS^+$ Treg and $ICOS^-$ Tregs use different molecular mechanisms for their suppressive function. A human study showed that $ICOS^+$ Treg cells secret a higher level of IL-10, but a lower amount of TGF-β than $ICOS^-$ Treg cells [228]. $ICOS^+$ Treg cells secrete both IL-10 and membrane TGF-β, whereas $ICOS^-$ Treg cells only use membrane TGF-β [228]. In the murine asthma model by Th2-driven immune response, the suppressive function of Treg cells in an inflammatory environment was dependent on IL-10 and ICOS-ICOSL interaction [265]. The suppressive capacity of Treg cells was abrogated by anti-IL-10 or anti-ICOSL. They demonstrated the importance of IL-10 and ICOS signaling to protect against asthma [265]. However, another study for Th17-dependent airway hyperresponsiveness (AHR) mouse model suggested IL-10 and TGF-β were dispensable for the suppression of AHR, but IL-35 secretion by $ICOS^+$ Treg cells were required to suppress this allergic airway disease [266].Under the prediabetes status in the BDC2.5-NOD T1D mouse model, Treg cells express high levels of IL-10 in the pancreas [267]. Blockade of ICOS leads to the loss of this balance, developing rapid onset of diabetes [267]. Along with these results, ICOS blockade during antigen priming produced low amounts of IL-10 and increased IFN-γ production, leading to exacerbated EAE [117]. Maynard group also reported that ICOS deficiency led to a significant decreased in the IL-10 producing colonic Treg cells. However. this was counterbalanced by significant enhancement of IL-10 production in non-Treg CD4+ T cells [233]. In a model of murine contact hypersensitivity, it is interesting that $ICOS^+$ Treg cells produce IFN-γ and IL-17 as well as transcription factor T-bet (T-box expressed in T cells) and RORγt (RAR-related orphan receptor gamma) respectively regardless of IL-10 secretion [256]. These cytokines-producing $ICOS^+$ Treg cells could regulate activation of IFN-γ and IL-17-secreting effector $CD8^+$ T cells [256]. Therefore, although many studies reported $ICOS^+$ Treg cells are activated suppressive Tregs with IL-10 secretion, it may be context-dependent.

1.3.5 $ICOS^+$ Treg cells in disease

Upregulation of ICOS expression has been observed in many autoimmune diseases, allergic diseases, and different types of cancer. Modulation of ICOS signaling could be one of the ways to

mitigate disease severity for some diseases. Under some pathogenic conditions, Treg cells have exhibited increased levels of ICOS expression, displaying a critical function to inhibit diseases progression [212] . In this section, I will discuss the cellular and molecular mechanisms underlying ICOS mediated regulation of Treg cells in disease models.

1.3.5.1 ICOS$^+$ Treg cells and atopic dermatitis

Atopic dermatitis (AD) is a chronic pruritic skin inflammatory disease. In humans, it usually presents at around two years of age and then the disease regresses. In most cases, it disappears before adolescence. AD is prevalent in worldwide in up to 30% of children and 10% of adults [268, 269]. Due to a higher pollution burden in recent decades, AD prevalence has increased [270, 271]. Although the pathogenesis of AD is not fully elucidated, immune hyper-responsiveness is the main feature of AD. This hyper-responsiveness is caused by Th2 immune response in the acute phase followed by a Th1 response in the chronic phase [272, 273]. Similar to other inflammatory mouse models, tTreg are increased in a murine model of AD [274]. Interestingly, these tTreg cells can be differentiated into Th-like cells, as CCR6$^-$ Treg cells from AD patients showed increased IL-5 and diminished IL-10 production [275]. Thus, these CCR6$^-$ Treg cells can be regarded as Th2-like Tregs cells to promote AD [276]. Therefore, immunosuppressive Treg cells and pro-inflammatory Treg cells co-exist in AD patients. A recent study showed that the ICOS$^+$ Treg cells from AD patients have decreased ability to produce IL-10 compared to Treg cells from healthy controls. This was because ICOS$^+$ Treg cells from AD patients are more susceptible to cell death than healthy controls [277]. Thus, this ICOS$^+$ Treg cells from AD might have impaired efficacy of immunosuppression due to reduced viability, leading to decreased IL-10 production.

Contact hypersensitivity (CHS) is also considered as a type of allergic contact dermatitis, and it is one of the most common occupational diseases in western countries [278]. CHS is a T cell-mediated cutaneous inflammatory reaction, which is caused by repeated skin exposure to allergens. Well-established murine models of CHS use experimental hapten such as 2,4-dinitro-fluorobenzene (DNFB) or Oxazolone to induce CHS. Increasing evidence suggests that the severity of inflammatory skin disease might result from a deficiency of regulatory mechanisms [279, 280]. In mice under CHS condition, ICOS$^+$ Treg cells proliferate in antigen dependent manner after DNFB

sensitization [256]. Furthermore, $ICOS^+$ Treg cells are highly suppressive Treg population, producing IL-10 but also IL-17 and IFN-γ [256]. This IL-17/IFN-γ secreting $ICOS^+$ Treg cells regulate in particular the activation of DNFB-specific IFN-γ and IL-17 producing effector $CD8^+$ T cells, although it is not clear yet whether those cytokines are produced by the same cells or not. [256].

1.3.5.2 $ICOS^+$ Treg cells and type 1 diabetes

Type 1 diabetes (T1D) is a chronic autoimmune disease, caused by T cell dependent destruction of the insulin-producing β-cells [281]. NOD mouse strain spontaneously develops T1D and showed many features of human T1D [281]. These mice have a loss in immunoregulatory mechanisms, leading to profound dysregulated immune responses. Some evidence suggested that a progressive waning in Treg suppressive functions trigger T1D onset. Impaired function of Treg cells in NOD mice resulted in insufficient ability to control pathogenic effector T cells infiltration in pancreatic site, and led to diabetogenic insulitis [282-284]. Besides, loss in Treg expansion or survival in islets is associated with T1D progression by impaired balance of Treg and effector T cells [284, 285]. IL-2 deficiency also triggers impaired Treg function in islets, provoking the imbalance of those T cells [285, 286].

Since ICOS has a dual role in effector T cells and Treg cells, conflicting results have been observed in NOD mice. ICOS or ICOSL knockout mice showed reduced diabetes incidence with milder inflammation in pancreatic islets, suggesting the crucial role of ICOS in the activation of autoreactive pathogenic T cells [287, 288]. It should be noted, however, most T1D free ICOS and ICOSL deficient NOD mice showed hind leg paralysis, because there is a shift of autoimmune targets from islets to neuromuscular tissues [288, 289]. TCR transgenic NOD mice (specific for a islets autoantigen, BDC2.5-NOD mice) showed different results when ICOS is deficient. Blockade or genetic ablation of ICOS or ICOSL in this model increased disease progression [127, 267]. This result fits well with the report that $ICOS^{hi}$ Treg cells readily migrate to draining lymph nodes (LN) to inhibit autoimmune disease [127, 290]. Thus, T1D progression is correlated with the decrease in ICOS expression in islet Treg cells [127]. They observed that $ICOS^+$ Treg cells showed more proliferative and suppressive capacity with higher production of IL-10 than $ICOS^-$ Treg cells. $ICOS^+$ Treg cells protected BDC2.5-NOD mice from the onset of T1D [127, 267]. Furthermore, these $ICOS^+$ Treg cells expressed CXCR3 in the pancreatic lymph nodes in BDC2.5-NOD mice, which confers

better migratory capacity to islets on $ICOS^+$ Treg cells [290]. This result was supported by the study that $CXCR3^{-/-}$ NOD mice developed diabetes earlier than WT mice due to the lack of migratory ability in $CXCR3^-$ Treg cells [291]. ICOS expression is required for CXCR3 expression on Treg cells, and these $CXCR3^+$ $ICOS^+$ Treg cells showed a Th1-like phenotype with T-bet and IFN-γ expression [290]. These $ICOS^+$ Treg cells can suppress IFN-γ secreting effector T cells in the T1D murine model [290]. Thus, it is clear that $ICOS^+$ Treg cells have protective function in the T1D murine model.

1.3.5.3 $ICOS^+$ Treg cells and GVHD

Graft-versus-host disease (GVHD) is a major obstacle to patient care during allogeneic hematopoietic stem cell transplantation (HSCT), conferring high mortality and morbidity [292]. GVHD occurs when the healthy transplanted (graft) stem cells consider the recipient's (host) cells as foreign pathogens and destroy them. The development of GVHD is caused by aberrant effector T cells and B cells, and decreased Treg generation [293-295]. IFN-γ secretion by Th1 cells is increased in early stages post HSCT. Th17 cells produce IL-17 and IL-21 to induce fibrosis [296-298]. Thymic damage in GVHD patients causes impaired Treg development and suppressive function [299, 300]. B cells and pathogenic antibody production are also involved in GVHD. GVHD patients showed elevated soluble B cell activating factor and pre-GC B cells and post-GC plasmablasts [301].

Some studies reported that $ICOS^{-/-}$ T cells decreased IFN-γ production, which relieved acute GVHD [302]. Abrogation of ICOS signal also show reduced GVHD severity [303]. Antibody blockade of ICOS in GVHD mice improved pulmonary function by decreasing Tfh cells and GC response [304]. Our collaborator, Yu's group investigated the role of ICOS in chronic GVHD pathogenesis [251]. ICOS-deficient donor grafts resulted in reduced occurrence of chronic GVHD compared to WT control. The recipient mice receiving $ICOS^{-/-}$ donor grafts showed a significant reduction in donor Tfh, Th17, GC B cell and impaired Tfr and Treg cells. In summary, ICOS promotes T and B cell activation and differentiation for the GVHD development. These data suggest that ICOS blockade may be more promising approach to ameliorate GVHD. It should be noted, however, that ICOS is critical for the Treg and Tfr cells to suppress GVHD development. Hence,

these results indicate that the selective manipulation of ICOS-expressing T cells subsets is crucial for the treatment of chronic GVHD.

1.4 Rationale and hypothesis

Activation of naive T cells requires three signals while they interact with antigen presenting cells: T cell receptor signal, cosignaling and cytokines [2]. TCR ligation play an important role for controlling T cell clonal expansion, survival and differentiation into several T cell subsets [4]. Cosignaling receptors amplify or inhibit TCR mediated signaling and, they also play crucial roles in T cell thymic selection [6, 7]. ICOS is a member of the CD28 family of cosignaling receptors, which can augment cytokine expression in inflammatory T cells [15-17]. Although many studies indicated that ICOS is necessary for the activation and differentiation of conventional T cells, it was also reported that ICOS-expressing Treg cells have better suppressive and migratory capacities [127, 256].

Due to the dual roles of ICOS in promoting effector T cells and Treg cells, Treg-intrinsic roles in ICOS in the homeostasis and anti-inflammatory functions of Treg cells have not been completely understood. Most of the studies conducted used germline ICOS or ICOSL knockout mice to show the reduced frequency and suppressive function of total Treg cells [305]. However, it is unclear if the abrogation of ICOS in conventional T cells could affect the ICOS deficient Treg's homeostasis and suppressive function. Thus, we generated Treg-specific ICOS knockout (ICOS floxed mice bred with Foxp3-YFP-Cre line) mice to characterize the intrinsic role of ICOS in Treg cells.

We hypothesize that ICOS may control Treg homeostasis and function in Treg-intrinsic manners.

Because of the dual roles of ICOS in conventional T cells and Treg cells, conflicting results have been reported in NOD background mouse models. ICOS deficient pure NOD mice were protected from type 1 diabetes (T1D), and this correlated well with milder inflammation in pancreatic islets indicating the essential role of ICOS signaling in supporting autoreactive pathogenic T cells

[288]. However, most T1D-free ICOS and ICOSL deficient NOD mice develop neuromuscular inflammation due to a shift of autoimmune targets from islets to neuromuscular tissues [288, 289]. This observation suggests a potential alteration of TCR repertoire due to the ICOS-deficiency. In BDC2.5-NOD mice, autoreactive effector T cells cause insulitis but Treg cells suppress the progression of insulitis to overt diabetes. As a result, the incidence of T1D is much lower compared to pure NOD mice. Importantly, abrogation of ICOS and ICOSL interaction in BDC2.5-NOD mice accelerates T1D progression [127, 267, 288]. Therefore, it seems that the dominant role of ICOS in BDC2.5-NOD mice is to support Treg function. However, the relative contribution of ICOS costimulation to thymic selection vs peripheral T cell activation remains unclear.

We will delete the Icos gene in adult NOD mice and assess the impact on T1D to estimate the contribution of the peripheral T cell compartment.

1.5 Aims and objectives

Our aims are to investigate the role of ICOS in Treg homeostasis and function in steady state and inflammation conditions.

1.5.1 Aim 1

Aim 1 is to study the role of ICOS in Treg homeostasis and function in steady state and acute skin inflammatory conditions. This will be done through the following objectives.

1. Investigate the impact of Treg-specific ICOS-deficiency on peripheral Treg homeostasis and function at steady state.
2. Characterize Treg cells in skin inflammation.

1.5.2 Aim 2

The focus of Aim2 is to characterize the role of ICOS in NOD type 1 diabetes.

1. Establish an inducible ICOS genetic deletion model to determine the role of ICOS in adult mice.
2. Evaluate the role of ICOS-expressing T cells in T1D in pure NOD and BDC2.5-NOD mice.

Chapter 2. The role of ICOS in Treg's homeostasis and function in steady state and acute skin inflammation conditions

ICOS-deficient Treg cells can prevent spontaneous autoimmunity but are impaired in controlling acute inflammation

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

2.1 Summary

Inducible T-cell co-stimulator (ICOS) is induced in activated T cells and its main role is to boost differentiation and function of effector T cells. ICOS is also constitutively expressed in a subpopulation of Foxp3$^+$ regulatory T cells under steady state condition. Studies using ICOS germline knockout mice or ICOS blocking reagents suggested that ICOS has supportive roles in Treg homeostasis, migration, and function. To avoid any compounding effects that may arise from ICOS-deficient non-Treg cells, we generated a conditional knockout system in which ICOS expression is selectively abrogated in Foxp3-expressing cells (ICOS FC mice). Compared to Foxp3-Cre control mice, ICOS FC mice showed a minor numerical deficit of steady-state Treg cell numbers but did not show any signs of spontaneous autoimmunity, indicating that tissue protective Treg populations do not heavily rely on ICOS costimulation. However, ICOS FC mice showed more severe inflammation in oxazolone-induced contact hypersensitivity, a model of atopic dermatitis. This correlated with elevated numbers of inflammatory T cells expressing IFN-γ and/or TNF-α in ICOS FC mice compared to the control group. In contrast, the elimination of ICOS in all T cell compartments negated the differences confirming that ICOS has a dual positive role in effector and regulatory T cells. Single-cell transcriptome analysis suggested that ICOS-deficient Treg cells fail to mature into T-bet$^+$ CXCR3$^+$ "Th1-Treg" cells in the draining lymph node. Our results suggest that regiments that preferentially stimulate ICOS pathways in Treg cells might be beneficial for the treatment of Th1-driven inflammation.

2.2 Introduction

Inducible T-cell Co-stimulator (ICOS) is a member of the CD28 family of T cell costimulatory receptors [11]. Engagement of ICOS with its unique ligand ICOS ligand (ICOSL) enhances TCR-mediated expansion of T cells and their differentiation into effector helper T cells [6, 7]. Despite similarities in structure between CD28 and ICOS, ICOS costimulation has indispensable roles in T cell immunity, in part, due to the expression patterns of ICOS/ICOSL and the signal transduction pathways it can evoke [11, 24, 27, 28, 32]. The most remarkable role of ICOS is its ability to control germinal center reactions by promoting the differentiation of T follicular helper (Tfh) cells [18, 19].

Thus, both in human patients and in mice, germline ICOS deficiency leads to reduced Tfh generation, germinal center reaction, and reduced antibody titers [18, 19].

Foxp3$^+$ regulatory T (Treg) cells develop from CD4$^+$ T cells in the thymus or in the periphery and suppress autoreactive T cells and inflammatory T cells [130]. Thus, Treg cells play key roles in preventing autoimmunity and downregulating inflammation. Genetic modifications that damage the generation and/or suppressive function of Treg cells leads to spontaneous autoimmune symptoms, including multiorgan inflammation in humans and animal models. CD28 has been shown to be critical for the development of Treg cells from the thymus [6, 306], its homeostasis in the periphery [171, 172], and conversion of conventional CD4 T cells (Tcon) into Foxp3$^+$ Treg cells [126, 136, 137]. However, the role of ICOS in Treg cell development, homeostasis, and function remains less clear.

Although ICOS was originally described as a costimulatory receptor acutely upregulated in effector T cells after activation [8], a portion of regulatory T cells constitutively express ICOS [11]. Since ICOS is preferentially expressed in antigen experienced Treg cells such as CD44$^+$ CD62L$^-$ subset, many studies have been using ICOS as one of the activation markers that are upregulated in activated Treg cells [181, 199, 204]. Further, several studies also suggested positive roles of ICOS in Treg proliferation, survival, migration, and suppressive functions [127, 181, 236-238]. However, these studies utilized either purified Treg cells stimulated *in vitro* [127, 236], antibodies or recombinant proteins injected into mice to manipulate ICOS-ICOSL interactions [181], or mice lacking ICOS or ICOSL expression in all the cells in the body [237, 238]. Since each approach has its own limitations, the results need to be complemented with more precise experimental systems.

In order to assess the role of ICOS costimulation in Treg homeostasis and function without the interference of ICOS-deficiency in other cells, we generated a mouse model in which ICOS expression is specifically abrogated in Foxp3$^+$ Treg cells. Through flow cytometric and single cell transcriptome analyses, we demonstrate that the absence of ICOS in Foxp3$^+$ Treg cells does not heavily affect the activation status, suppressive capacity, and maintenance of Treg subpopulations. Consistently, mice born with ICOS-deficient Treg cells did not show any spontaneous autoimmune symptoms upon aging. In contrast, mice lacking ICOS-expressing Treg cells had more severe skin inflammation in an experimental model of atopic dermatitis. Single-cell transcriptome analysis of Treg cells in the draining lymph node (LN) suggests an impaired differentiation of a Treg subset equipped with highly effective anti-inflammatory functions.

2.3 Materials and method

2.3.1 Mice

Foxp3$^{YFP\text{-}Cre}$ (Jax 016959), Foxp3$^{EGFP\text{-}Cre\ ERT2}$ (Jax 016961) and C57BL/6 (Jax 00664) mice were purchased from the Jackson Laboratory and bred with other lines of mice to make composite mouse lines. ICOS conditional knockout mice were generated in C57BL/6 background as described [101]. ICOS germline knockout mice were backcrossed on the C57BL/6 background [18]. All mouse lines had been backcrossed on the C57BL/6 background for more than 10 generations before breeding with other lines. We used male ICOS WT and ICOS FC mice at 8-12 weeks of age. All mice were housed in the animal care facility at the Institut de Recherches Cliniques de Montréal (IRCM) in specific pathogen-free conditions, and all the experiments were performed in compliance with animal use protocols approved by the IRCM Animal Care Committee.

2.3.2 Flow cytometry

For flow cytometry, single cell suspension of lymph nodes and spleen were prepared by mechanical disruption via a 70 μm mesh filter (BD Biosciences), in PBS or staining buffer (PBS containing 1% bovine serum albumin (Wisent)). To prevent non-specific blinding, anti-CD16/32 (BioXCell) was used before surface staining. For intracellular staining, cells were fixed and permeabilized with Fix/Perm buffer (ThermoFisher), according to the manufacture's instruction. Antibodies for flow cytometry are anti-TCRβ (H57-597, BioLegend), Anti-CD4 (RM4-5, Invitrogen), anti-CD8 (53-6.7, Invitrogen), anti-CD25 (PC61.5, Invitrogen), anti-CTLA4 (UC10-4F10-11, BD Bioscience), anti-Foxp3 (FJK-16s, Invitrogen), anti-ICOS (C398.4A, BioLegend), anti-CD103 (2E7, BioLegend), anti-CD44 (IM7, BD Bioscience), anti-CD62L (MEL-14, BioLegend), anti-Ki67 (16A8, BioLegend), anti-Bcl2 (3F11, BD Pharmingen), anti-IFNγ (XMG1.2, Invitrogen), anti- TNFα (MP6-XT22, Invitrogen), and viability dye was from ThermoFisher. Data were acquired using LSR Fortessa (BD Biosciences) and analyzed using Flowjo V10 (BD Biosciences).

2.3.3 Contact hypersensitivity

Mice were anesthetized by isoflurane (CDMV) and sensitized by topical application of 1% oxazolone (4-Ethoxymethylene-2-phenyl-2-oxazoline-5-1, Sigma-Aldrich) in acetone / olive oil (4:1 vol/vol) on the shaved abdomen (50 µl). Five days later, the mice were challenged by topical application of 1% oxazolone on the left ear (20 µl). Mice were sacrificed at day 1 or 3 post-challenge for analysis. In some experiments, the severity of contact hypersensitivity was monitored for more than 16 days by measuring ear thickness. For flow cytometric analysis of T cells, mouse ears were separated into dorsal and ventral halves, then minced and incubated in 1.5 ml of digestion solution (RPMI 1640 supplemented with 10% FBS, 200 µg/ml of Liberase TM (Roche), and 40 µg/ml of DNAse I (Sigma-Aldrich). The samples were incubated at 37°C for 45 minutes with agitation (270 rpm). The digested samples were strained through a 40 µm filter (Falcon) and washed with PBS for staining.

2.3.4 Treg suppression assay

In vitro Treg suppression assays were performed by stimulating CFSE-labelled responder cells in the presence of Treg cells. $CD4^+$ responder T cells were FACS-sorted from *Foxp3*$^{YFP\text{-}Cre}$ *Icos*$^{+/+}$ (ICOS WT) mice based on YFP signal (YFP^-), YFP^+ Treg cells were prepared either from ICOS WT mice or *Foxp3*$^{YFP\text{-}Cre}$ *Icos*$^{F/F}$ (ICOS FC) mice. After CFSE labelling, responder cells (5 X 10^4) were seeded on 96 well plates mixed with titrated numbers of ICOS-deficient or wildtype Treg cells. T cell stimulation was achieved by adding anti-CD3 (1 µg/ml) in the presence of irradiated (900 cGY) splenic cells (2 X 10^5) or isolated dendritic cells (5 X 10^4). Dendritic cells were prepared using Easysep mouse CD11c positive selection kit (Stemcell Technologies). Proliferation of responder cells was assessed by flow cytometric analysis of CFSE dilution after 3 days of culture.

2.3.5 Intracellular cytokine staining

In order to measure cytokines-expressing cells, cells (3 X 10^6) were seeded in 24-well plates in 1 ml of culture media (RPMI 1640 medium, 10% FBS, 2 mM L-glutamine, 0.05 mM β-mercaptoethanol, 10 mM HEPES) and stimulated with PMA (50 ng/ml) and ionomycin (1 µg/ml)

for 4 hours at 37°C incubator in the presence of GolgiPlug (1 ul/ml, BD Bioscience). After stimulation, the cells were collected and analyzed by flow cytometry according to the standard intracellular staining protocol (Section 2.3.2).

2.3.6 Histology

Organs were fixed in 10% neutral buffered formalin for 24 hrs at 4°C. The samples were washed in PBS, embedded in paraffin, and cut in 5 μm sections. Slides were stained with H&E and examined under microscope to assess immune cell infiltration.

2.3.7 Single cell RNA sequencing and analysis

Single cell suspensions were prepared from either spleens (for Steady state) or cervical draining LNs (for Oxazolone challenge day 1) from ICOS WT and ICOS FC mice. Cells were stained with viability dye, anti-CD4 and anti-TCRβ. Live Treg ($CD4^+TCR\beta^+$ YFP^+) cells were sorted using a BD FACSAria (BD Biosciences) with more than 95% purity. A total of 12,000 cells from ICOS WT and ICOS FC mice were prepared for the library. Libraries were generated at the IRCM Molecular Biology and Functional Genomics Core Facility 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, and Chromium i7 Multiplex kit. Sequencing was performed by Genome Québec using a NovaSeq 6000 (Illumina) with a flow cell SP PE28x91.

The alignment of the reads was performed using *Cellranger* 4.0.0 (from 10x Genomics) with GRCm38.p6 (mm10) reference genome procured from *Ensembl*. The resulting expression matrix was loaded into *R* version 3.6.1 (from the R Foundation for Statistical Computing) to conduct analysis.

The expression matrices were stored into an R Seurat object available in the package *Seurat* version 3.0 [307]. For each condition (Steady state and Oxazolone), we filtered ICOS WT and ICOS FC samples by eliminating any cell that presented more than 10% mitochondrial RNA contamination as well as any cell with less than 200 unique genes expressed and we merged the ICOS FC and WT samples in each condition. The expression matrix was then log normalized and scaled. The most differentially expressed genes within the samples were identified. As several cell

cycle-related genes were identified, we used *CellCycleScoring* function available in *Seurat* to score cell cycle phases. After, we scaled the data by regressing cell cycle score variables (Phases S and G2/M) to minimize the importance of cell cycle genes. We then 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 and constructed a *Shared Nearest Neighbor* (SNN) graph and we used *Modularity Optimizer version 1.3.0* [308] to identify 7 clusters for Steady state condition and 8 clusters for Oxazolone condition. The cells were projected on a 2D space using a *Uniform Manifold Approximation and Projection UMAP* method [309]. We then analyzed gene expression patterns across samples and conditions using *Seurat* functions *FeaturePlot, VlnPlot,* and *DoHeatmap*.

2.3.8 Statistical analysis

Statistical analyses were performed using Prism 9.0 (GraphPad Software). All data are presented as mean ± SEM. Student's *t*-test or two-way ANOVA with a Sidak's multiple comparisons were used to judge the statistical significance of the difference of experiments groups. A p value < 0.05 was considered as statistically significant.

2.4 Results

2.4.1 Treg-specific ICOS deficiency does not lead to spontaneous autoimmunity

In order to assess the role of ICOS in Treg cell homeostasis and function, we created mice that specifically lack ICOS expression in Foxp3$^+$ Treg cells by generating *Foxp3*$^{YFP\text{-}Cre}$ *Icos*$^{F/F}$ (ICOS FC) mice. We confirmed that ICOS expression is selectively abrogated in CD4$^+$ Foxp3$^+$ T cells (Figure 2.1A). In the control *Foxp3*$^{YFP\text{-}Cre}$ *Icos*$^{+/+}$ (ICOS WT) mice, a small portion of non-Treg CD4$^+$ conventional T cells (Tcons) expressed a low level of ICOS, whereas Treg cells expressed elevated levels of ICOS in steady state. In ICOS FC mice, the pattern of ICOS expression in Tcons remained unaltered, yet Foxp3$^+$ Treg cells completely lost ICOS expression.

We observed a reduction in the frequency of Treg cells in ICOS FC mice with normal levels of Foxp3, Ki67, and Bcl2 (Figure 2.1B-D). Importantly, there was no indication that Tcons were highly activated in ICOS FC mice at 8 to 10 weeks of age (Figure 2.1E). We reasoned that a deficit in Treg cell frequency combined with some potential functional defects of Treg cells in ICOS FC mice may lead to a breach of tolerance upon aging. However, we have not detected any visible sickness in ICOS FC mice until one year of age. Also, there was no immune cell infiltration observed in typical autoimmune targets such as liver, lung, pancreas, brain, eyes, and stomach at the age of 8 months (Figure 2.1F). These results indicate that ICOS-deficient Treg cells are capable of suppressing spontaneous activation of autoreactive T cells in ICOS FC mice.

2.4.2 ICOS-deficient Treg cells maintain normal activation status and suppressive capacity

ICOS was shown to be highly expressed in the $CD44^{hi}CD62L^{lo}$ "effector Treg (eTreg)" population and possibly involved in maintaining eTreg population [181]. Compared to $CD44^{lo}$ $CD62L^{hi}$ "central Treg (cTreg)" population, eTreg cells express higher levels of CD103 and spend more time in the peripheral tissues. Also, eTreg cells express lower levels of CD25 compared to cTreg cells and manipulation of ICOS-ICOSL interaction by putative ICOSL blocking antibody reduced the pool size of eTreg cells. However, it remains unclear whether altered ICOS signaling changed the expression levels of markers of eTreg cells or induced death of some of the eTreg population. In order to address the role of ICOS in the eTreg-cTreg balance *in vivo*, we examined the ratio of eTreg vs cTreg in ICOS FC mice. Compared to ICOS WT mice, there was no alteration in eTreg-to-cTreg ratio (Figure 2.2A). These results indicate that Treg-specific loss of ICOS expression does not drastically change the composition of the eTreg population.

Next, we examined the expression levels of Treg markers that have been shown to be important for Treg fitness and suppressive functions. As shown in Figure 2.2B, ICOS-deficient Treg cells expressed normal levels of CD103 and Helios. Notably, ICOS-deficient Treg cells expressed slightly elevated levels of CD25 and CTLA-4 that are known to be important for Treg suppressive functions [131, 184]. Despite these minor differences in Treg characteristics, ICOS-deficient Treg cells were equally good at suppressing polyclonal expansion of $CD4^+$ Tcons *in vitro* (Figure 2.2C).

Collectively, these data indicate that under steady state, ICOS-deficient Treg cells can be activated to form eTreg populations and are capable of suppressing conventional T cell expansion.

2.4.3 Treg-specific ICOS deficiency reduced the proportion of CD25$^-$ Treg population

It has been shown that constitutive expression of CD25 is one of the key features of healthy Foxp3$^+$ Treg cells [199, 310]. High level of CD25 appears to enhance their sensitivity to low concentration of IL-2 facilitating pSTAT5-mediated Foxp3 expression [177]. However, CD25$^-$ Foxp3$^+$ Treg populations do exist both in lymphoid and non-lymphoid tissues [311]. Since we saw increase of CD25$^+$ Treg cells in ICOS FC mice (Figure 2.2B), we wondered if there is negative correlation between CD25 and ICOS expression levels in Treg cells. Indeed, CD25$^-$ Treg cells expressed a two-fold higher level of ICOS compared to their CD25$^+$ Treg counterparts (Figure 2.3A). We then asked if elimination of ICOS in Treg cells has any impact on the CD25$^-$ Treg population. Importantly, the ratios between CD25$^-$ Treg to CD25$^+$ Treg were reduced 2-4 times in the spleen and lymph node of ICOS FC mice compared with ICOS WT control mice (Figure 2.3B). These results raise two possibilities. First, ICOS costimulation suppresses CD25 expression itself directly or indirectly. If this is the case, ICOS-deficient Treg cells would quickly upregulate CD25. Alternatively, there might be a CD25$^-$ Treg subset whose maintenance is highly dependent on ICOS costimulation. In this scenario, a group of cells sharing gene signatures may disappear in ICOS FC mice.

2.4.4 Minimal alterations in single-cell transcriptome in ICOS-deficient Treg cells

Flow cytometric analysis of ICOS-deficient Treg cells identified only a few minor changes in the Treg characteristics. In order to confirm these findings and further explore potential homeostatic changes established at the level of Treg subpopulations, we analyzed single cell transcriptomes using splenic Treg cells sorted from an ICOS WT and ICOS FC mouse.

All the cells (12,000 cells with 6,000 per genotype) were split into seven clusters based on the similarity of gene expression patterns (Figure 2.4A). Importantly, none of the clusters were unique to ICOS WT or ICOS FC mice (Figure 2.4A). We further compared the relative expression levels of key signature genes that are differentially expressed in each cluster using a heat map. Although each cluster had 2-5 signature genes that distinguished them from other clusters, the expression levels of each markers had only marginal differences between genotypes (Figure 2.4B). These results suggest

that ICOS does not provide any indispensable function to form and maintain a particular subset of Treg cells.

The *Icos* gene is highly expressed in cluster 1 and 2 (Figure 2.4C). These clusters express low levels of *Sell* (CD62L) and high levels of *Cd44*, indicating that ICOS expression is associated with the expression of activation markers for Treg cells. However, absence of ICOS did not drastically change *Sell* and *Cd44* expression levels, consistent with our FACS data (Figure 2.2A). Also consistent with FACS data, cluster 1 and 2 had higher *Ctla4* in ICOS FC mice. Lastly, cluster 1 and 2 had lower *Il2ra* expression compared to $ICOS^{lo}$ clusters. Despite these correlations, ICOS-deficiency did not abrogate any clusters seen in ICOS WT control, nor did it generate new clusters.

Overall, these single-cell transcriptome analyses confirmed only subtle changes in Treg characteristics in steady state splenic Treg population and support the view that ICOS-deficient Treg cells preserve intact capacities to prevent spontaneous autoimmunity.

2.4.5 Augmented skin inflammation in ICOS FC mice

Most in-depth studies regarding the role of ICOS in Treg cells have been performed in BDC2.5 TCR transgenic mice in NOD background [127, 267, 290]. In BDC2.5-NOD model, autoreactive pathogenic T cells accumulate in prediabetic insulitis lesion, but ICOS-expressing Treg cells can delay onset of overt diabetes [127, 267]. One of the roles of ICOS in early phase of T cell priming in the draining lymph nodes is to promote maturation of a Th1-like Treg population. This unique population expressed high level of T-bet and CXCR3 in response to IFN-γ produced by inflammatory Th1 cells [290]. To test if Treg-specific ICOS deficiency hinders generation of this Th1-Treg population, we decide to use oxazolone-mediated contact hypersensitivity model, a mouse model of atopic dermatitis driven by IFN-γ [312]. In this context, an early study showed that a putative ICOS agonist (B7RP1-Fc, soluble recombinant ICOSL fused with human Fc fragment) increased skin inflammation in this model although the authors have not considered the impact of B7RP1-Fc on Treg cells [313].

Our data showed that ICOS FC mice had more severe skin inflammation as shown by the increase in ear thickness (Figure 2.5A) and immune cell infiltration in histological images (Figure 2.5B). This elevated skin inflammation was highly correlated with reduced a number of Treg cells in the skin and an increased number of inflammatory $CD4^+$ cells (and to some extent $CD8^+$ T cells)

that express IFN-γ or TNF-α at day 1 post challenge (Figure 2.5C and D). These skin phenotypes are well reflected in the reduced number of Treg cells and elevated inflammatory T cells in the draining LN (Figure 2.5E and F). We tried to detect T-bet$^+$ CXCR3$^+$ Th1-Treg cells under this condition. However, the number of cells in this population was too small to allow any meaningful comparisons between genotypes. We presume that Th1-Treg cells continuously differentiate and migrate to the inflamed skin. In germline ICOS KO mice, we did not find significant differences in ear swelling compared to wild type mice (Sup. 2.1A). This is presumably due to reduced activations of both in inflammatory CD4$^+$ T cells and Treg cells (Sup. 2.1B-E).

Taken together, ICOS FC mice had reduced ability to control Th1-mediated skin inflammation with reduced Treg cell numbers in the draining LN and inflamed tissue.

2.4.6 Reduced Th1-Treg-like cluster in ICOS FC mice during skin inflammation

In order to gain more insights into dynamic changes in the gene expression profile of Treg cells responding to oxazolone-induced inflammation, we performed a single-cell transcriptome analysis. The collection of ~12,000 cells was split into eight clusters and there were no unique clusters appearing or disappearing in ICOS FC Treg cells (Figure 2.6A). The most drastic changes in the gene expression profile happened in cells belonging to cluster 0 as shown in the heat map (Figure 2.6B). ICOS FC Treg cells had reduced levels of *Cd44*, *Gzmb*, and *S100a4*, but increased levels of *Sell* suggesting reduced activation/maturation levels (Figure 2.6C and D). Notably, we found a small number of Treg cells belonging to cluster 0 that coexpress genes representing Th1-Treg: *Tbx21* (T-bet) and *Cxcr3* (Figure 6E, Red arrows). These cells also expressed IL-10 and CTLA-4, known effector molecules with suppressive functions (Figure 2.6E). Importantly, the ICOS FC Treg population had fewer number of cells in this specific UMAP area, suggesting there could be a defect leading to delayed maturation of Th1-Treg like cells in the absence of ICOS costimulation.

Thus, our data suggest that ICOS promotes the maturation of a T-bet$^+$ CXCR3$^+$ Th1-Treg subset that has the capacity to migrate into inflamed tissues and suppress inflammatory T cells.

2.5 Discussion

In this study, we developed ICOS FC mouse model in order to most precisely address *in vivo* function of ICOS costimulation with minimal complications that may arise from ICOS-deficiency in other immune cells. Contrasting some of the data in the literature, we could not detect any major differences in terms of subset composition, and suppressive function of Treg cells in C57BL/6 background at homeostasis. Consequently, we could not detect any spontaneous autoimmune manifestations in ICOS FC mice. However, ICOS FC mice responded more vigorously to experimentally induced skin inflammation. We provide evidence that this is probably due to an impaired maturation of Treg cells in the draining LN hampering their migration to the inflammatory sites.

Minimal alterations in Treg behavior seen in ICOS FC mice in homeostasis contrast some of the existing data in the literature. First, it has been shown that $ICOS^{hi}$ Treg cells are more proliferative and more resistant to cell death *in vitro* [181]. Perhaps this is due to supraphysiological TCR stimulation used in the experiments. Second, injection of recombinant ICOSL-human Fc fusion protein (B7RP1-Fc) in C57BL/6 mice undergoing oxazolone-mediated skin inflammation worsened the disease [313]. The authors proposed that this is due to putative agonistic actions of B7RP1-Fc upon ligation of ICOS on effector T cells. However, our data suggest another possibility: B7RP1-Fc may have interfered with ICOS-ICOSL interaction in an antagonistic way, and the main target of B7RP-Fc could have been Treg cells. Lastly, eTreg cells expressing high level of ICOS were diminished in mice that have been treated with multiple rounds of ICOSL blocking antibody [181]. The authors provided evidence that eTreg cells are more prone to apoptosis and highly dependent on ongoing ICOS signaling for survival. However, our data shows that ICOS-deficient Treg cells maintain a normal eTreg compartment. Thus, it appears that the chronic absence of ICOS in eTreg population can be compensated by other mechanisms.

Although CD28 and ICOS belong to the same family of T cell costimulatory, they have several differences including in their signal transduction mechanisms [11]. In terms of Treg homeostasis, CD28 appears to play a more dominant role than ICOS. CD28-deficiency leads to a drastic numerical deficit in the peripheral Treg population [172]. In contrast, we detected only marginal defect in ICOS FC mice. Considering that ICOS has an limited role in the maturation of Treg cells during inflammation, combined treatment of CD28 and ICOS may confer synergistic effects.

It is important to note that ICOS has dual stimulatory roles for effector (or pathological) T cells and Treg cells. For example, in our contact hypersensitivity model, Treg-specific ICOS deficiency worsened the disease whereas germline ICOS deficiency did not alter the disease outcome. Similar observations were made in autoimmune diabetes models in NOD background [287, 288]. This dual impact of ICOS signaling in effector and regulatory T cell populations should be considered for clinical application of agonists or antagonists of ICOS-ICOSL pathways.

Since Foxp3 expression starts early during thymic development, the *Icos* gene is lost in Treg cells during its early stage of Treg development. However, in a separate work, we found that ICOS is not highly expressed in *de novo* thymic Treg cells in wildtype mice (Dong M et al.; Manuscript submitted to *Immunology & Cell Biology*). The absence of spontaneous autoimmunity in ICOS FC mice also supports the view that ICOS does not play a critical role in the thymic selection of Treg cells. On the other hand, lack of ICOS signaling partially impaired the negative selection of $CD8^+$ thymocytes (Dong M et al.; Manuscript submitted to *Immunology & Cell Biology*).

In summary, we confirmed that the main role of ICOS-expressing Treg cells is to fine-tune the maturation of peripheral Treg cells during inflammation. This implies that, if applied at the right timing with preferential delivery to Treg cells, ICOS agonists could be beneficial to control Th1-driven inflammation.

2.6 Figure and figure legends

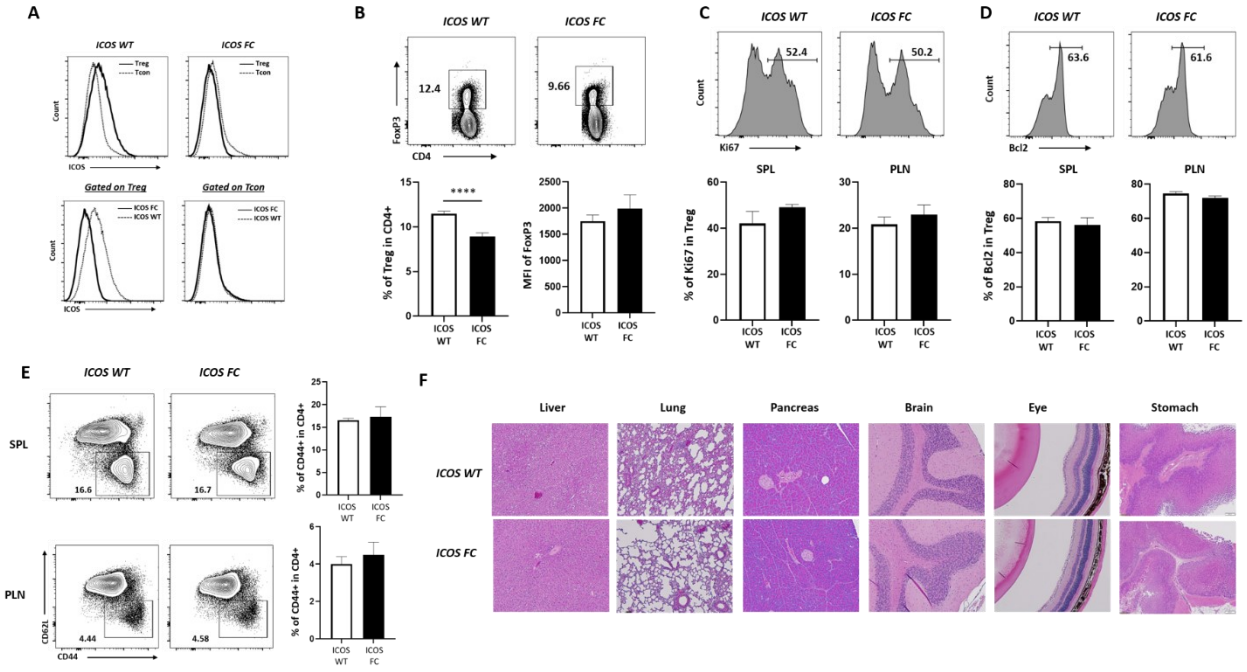


Figure 2.1 Treg-specific ICOS deficiency does not lead to spontaneous autoimmunity. (A) Assessment of ICOS expression in Treg vs Non-Treg $CD4^+$ T cells (Tcons). Total live splenocytes from ICOS WT vs ICOS FC mice were analyzed by flow cytometry to assess the level of ICOS in $CD4^+$ $Foxp3^-$ Tcons or $CD4^+$ $Foxp3^+$ Treg cells. FACS data shown are representative of 11 mice each group. **(B)** Assessment of $Foxp3^+$ Treg cells among the total $CD4^+$ T cells and frequency and MFI of Foxp3 in ICOS WT vs ICOS FC splenocytes data shown are representative of 11 mice each group. **(C-D)** The percentages of $Ki67^+$ cells **(C)** or $Bcl2^+$ cells **(D)** among the $CD4^+$ $Foxp3^+$ Treg cell population from spleen (SPL) and peripheral LN (PLN) were assessed by flow cytometry. FACS data shown are representative of 4 mice per each group. **(E)** The portion of effector/memory T cells among $CD4^+$ Tcons was analyzed using antibodies against CD44 and CD62L in 8- to 10-week-old ICOS WT and ICOS FC mice. Data shown are representative of 7 mice per each group. All the summary plots represent mean ± SEM and the statistical significance was tested by Student's *t*-test. **(F)** The presence of lymphocytic infiltration in multiple organs was examined by H&E staining from ICOS WT and ICOS FC mice.

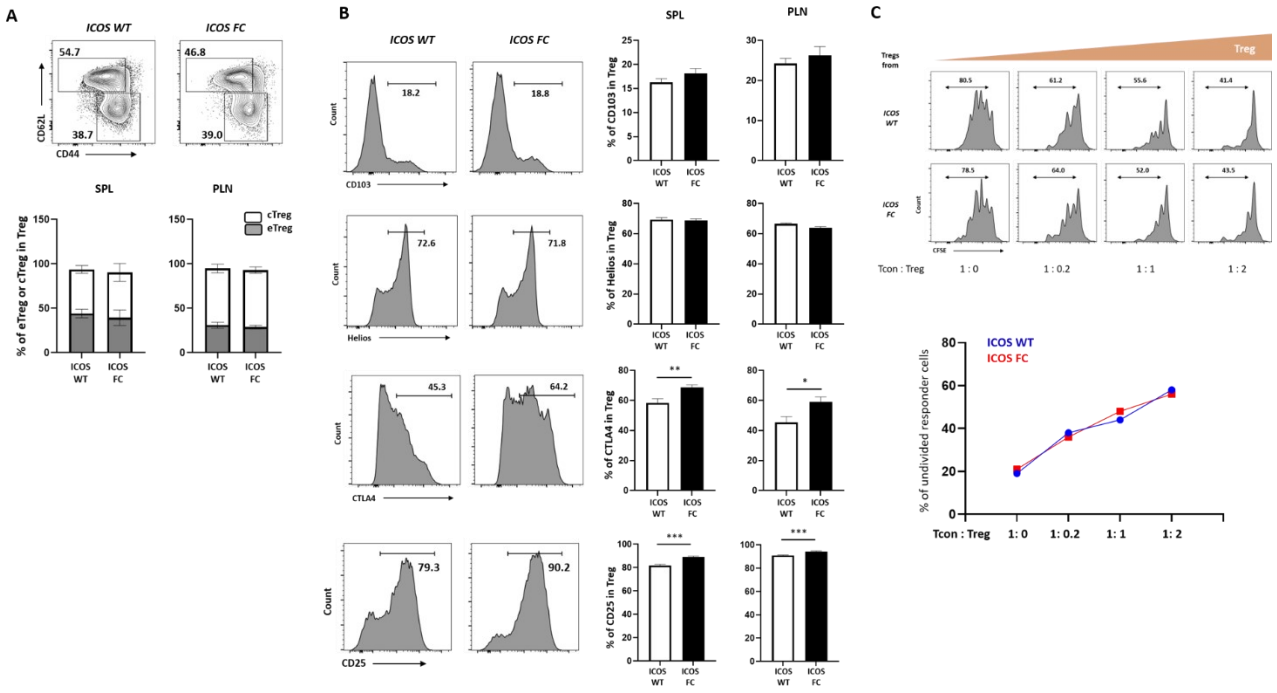


Figure 2.2 ICOS-deficient Treg cells maintain normal activation status and suppressive capacity. (A) Intact eTreg compartment in ICOS FC mice. Total live splenocytes and peripheral LN cells from ICOS WT vs ICOS FC mice were analyzed by flow cytometry to assess the levels of CD44 and CD62L expression levels. FACS plot shown are representative data from spleen and the percentages of cTreg and eTreg cells are depicted in the summary plot (7 mice each group). **(B)** Minimal alterations of Treg functional markers. The percentages of Treg cells expressing the indicated markers were assessed by flow cytometry in spleens and peripheral lymph nodes of ICOS WT vs ICOS FC mice. FACS plot shown are representative data from spleen and the percentages of Treg cells expressing each marker are summarized in the plots (7 mice each group). **(C)** Intact suppressive capacity of ICOS-deficient Treg cells. *In vitro* Treg suppression assays were set up as described in *Materials and Method*. The intensity of CFSE was used to assess proliferation of responder T cells. The percentage of undivided cells were used as indication of Treg mediated suppression. Data shown are representative of two independent experiments.

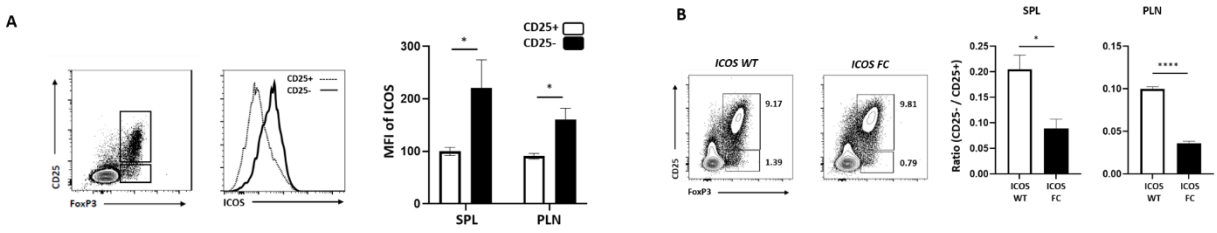


Figure 2.3 Treg-specific ICOS deficiency reduced the proportion of CD25$^-$ Treg population. (A) CD25$^-$ Treg cells express higher levels of ICOS compared to CD25$^+$ counterpart. Total live CD4$^+$ splenocytes and peripheral LN cells from ICOS WT mice were analyzed by flow cytometry to assess the relationship between CD25 and ICOS. FACS plots are representative of spleen data. ICOS MFI was calculated from spleen and peripheral LNs (n=3 for each group). **(B)** ICOS-deficient Treg cells had reduced number of CD25$^-$ Treg population. The ratios between CD25$^-$ and CD25$^+$ Treg cells were measured based on flow cytometric analysis using spleens and LNs isolated from ICOS WT and ICOS FC mice. FACS plots are representative of spleen data. ICOS MFI was calculated from spleen and peripheral LNs (n=3 for each group). All the summary plots represent mean ± SEM and the statistical significance was tested by Student's *t*-test.

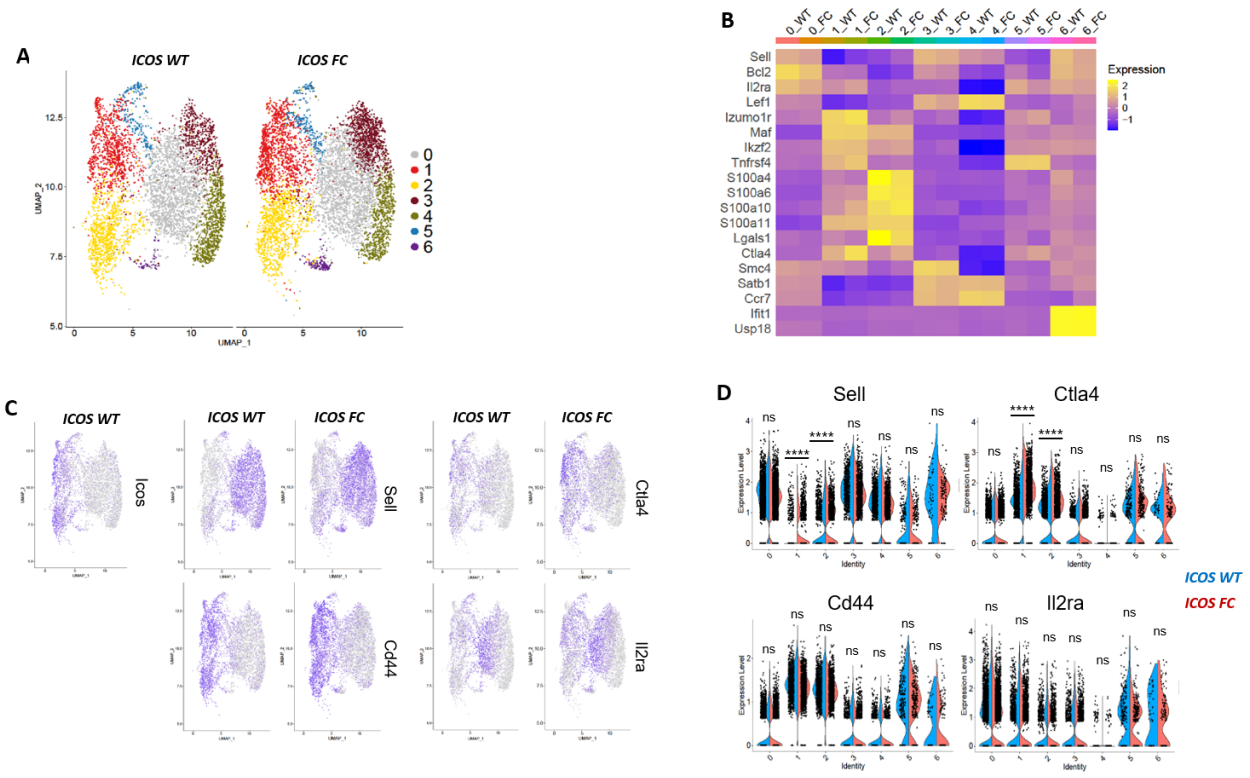


Figure 2.4 Minimal alterations in single-cell transcriptome of ICOS-deficient Treg cells. Live $CD4^+$ $Foxp3^+$ splenic Treg cells were sorted from ICOS WT and ICOS FC mice and were subjected to single cell transcriptome analysis as described in *Materials and Methods*. **(A)** UMAP projections of Treg cells. Each dot represents one cell. Seven clusters were identified, and each cluster was color-coded as indicated. **(B)** Heatmap representing relative expression levels of indicated genes in ICOS WT and ICOS FC cell clusters. **(C)** Feature plots of *Icos*, *Sell*, *Cd44*, *Ctla4*, and *Il2ra*. **(D)** Violin plots showing the gene expression levels in each cluster of ICOS WT and ICOS FC mice. **p*<0.05, ***p*<0.01, ****p*<0.001

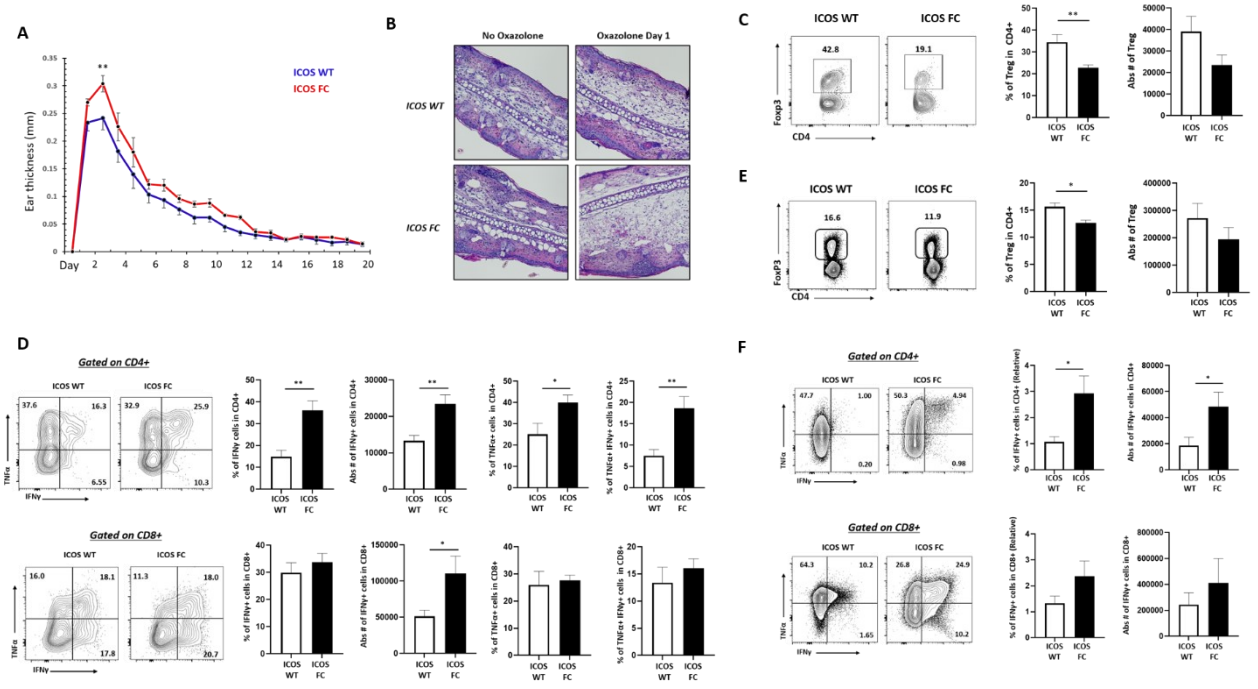


Figure 2.5 Augmented skin inflammation in ICOS FC mice. Mice were sensitized with oxazolone at the abdomen and then challenged at the ear skin 5 days later. **(A)** Ear thickness was measured daily after challenge and the increase pre-challenge measurements was calculated and plotted. The data shown are from 5 ~ 6 mice per genotype. **(B)** Inflamed ear skin samples were prepared for histology by H&E staining at day 1 post challenge. **(C)** Digested ear skin samples (day 1 post challenge) were analysed by flow cytometry to profile Treg cell. Data shown are summary of 8 mice per genotype. **(D)** Digested ear samples were acute stimulated *in vitro* to identify $CD4^+$ and $CD8^+$ T cells producing IFN-γ or TNF-α by intracellular cytokine staining. Data shown are summary of 5 mice per genotype. **(E)** Cells from draining cervical LNs (day 1 post challenge) were analyzed by flow cytometry to assess Treg contents. Data shown are summary of 4 mice per genotype. **(F)** Cells from draining cervical LNs at day 1 post challenge were acutely stimulated *in vitro* to identify $CD4^+$ and $CD8^+$ T cells producing IFN-γ or TNF-α by intracellular cytokine staining. Data shown are summary of 5 mice per genotype. Statistical analysis was performed using two-way ANOVA with Sidak's multiple comparisons (A) or Student's *t*-test (C-F).

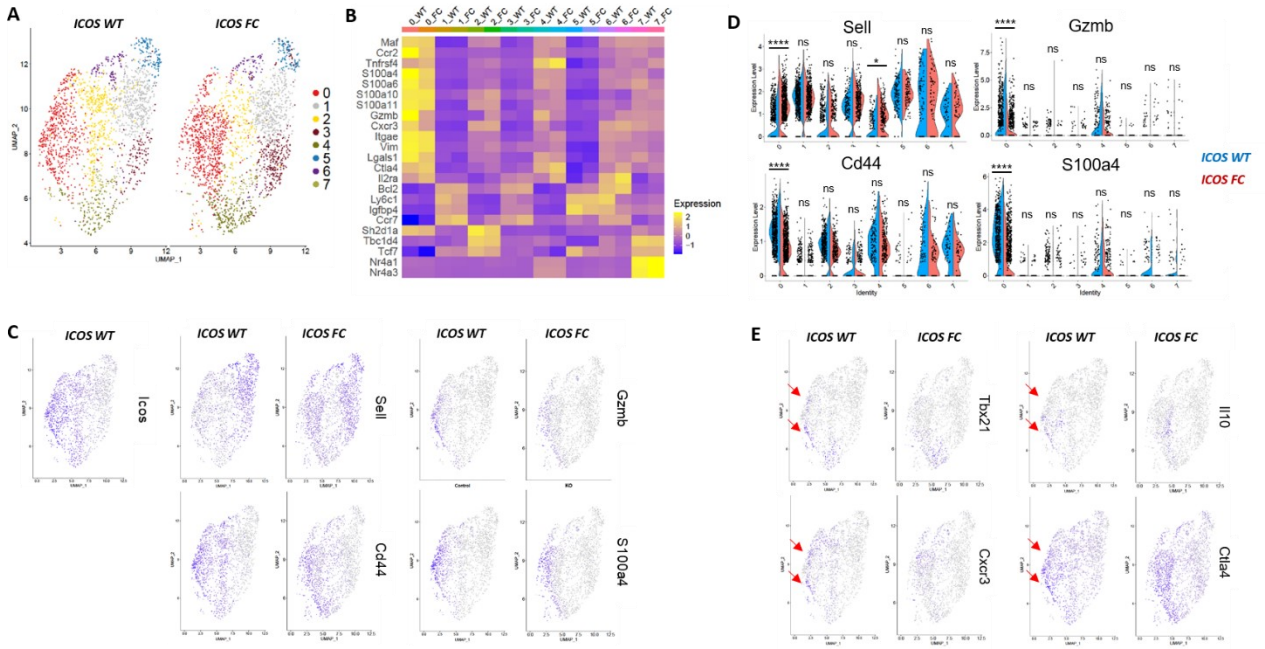
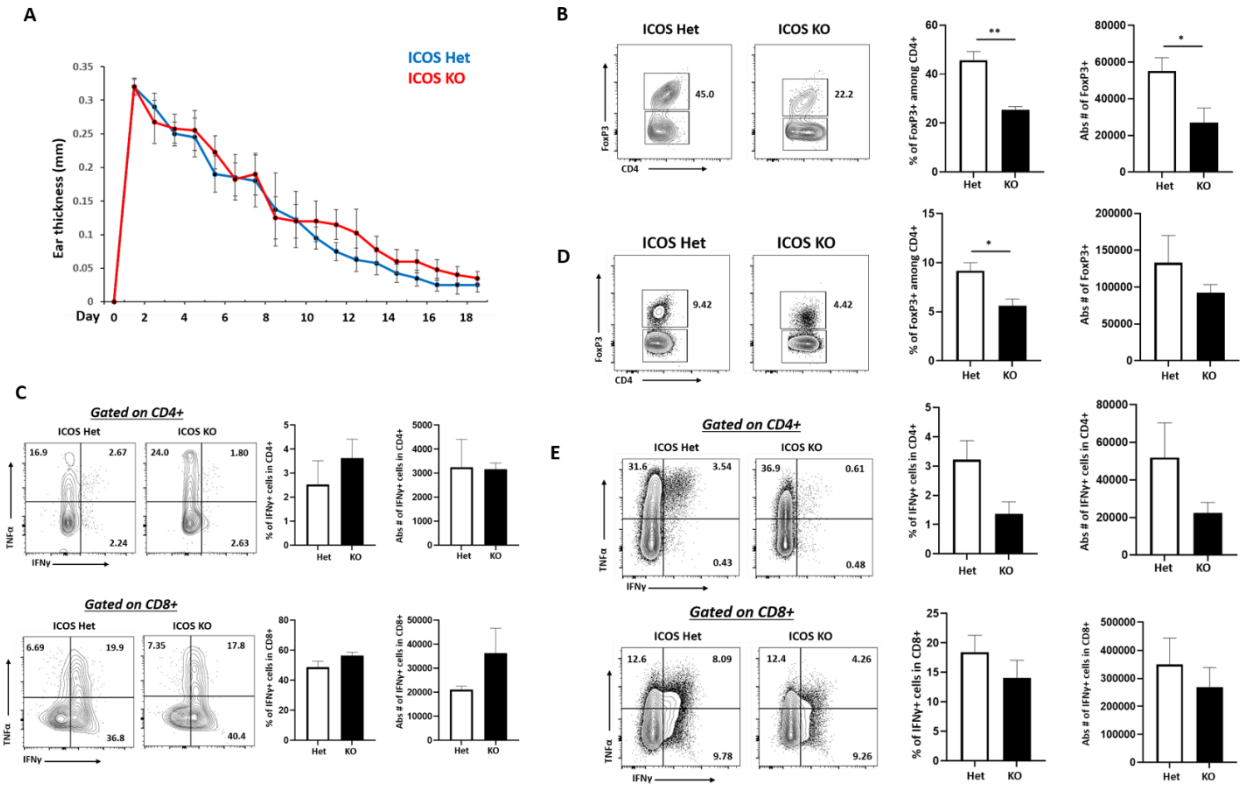


Figure 2.6 Reduced Th1-Treg-like cluster in ICOS FC mice during skin inflammation. Live $CD4^+$ $Foxp3^+$ T cells were isolated from draining cervical LNs one day after oxazolone challenge and subjected to single-cell transcriptome analysis as described in *Materials and Methods*. **(A)** UMAP projections of Treg cells. Each dot represents one cell. Eight clusters were identified, and each cluster was color-coded as indicated. **(B)** Heatmap representing relative expression levels of indicated genes in ICOS WT and ICOS FC cell clusters. **(C)** Feature plots of *Icos, Sell, Cd44, Gzmb*, and *S100a4*. **(D)** Violin plots showing the gene expression levels in each cluster and genotypes. **(E)** Feature plots of *Tbx21, Cxcr3, Il-10*, and *Ctla4.* **p*<0.05, ***p*<0.01, ****p*<0.001.



Supplementary Figure 2.1 Unaltered skin inflammation in ICOS$^{-/-}$ mice. Mice were sensitized with oxazolone at the abdomen and then challenged at the ear skin 5 days later. **(A)** Ear thickness was measured everyday after challenge and the increase above pre-challenged levels was calculated and plotted. The data shown are from 4 mice per genotype. **(B)** Digested ear skin samples (day 1 post challenge) were analysed by flow cytometry to measure Treg cell contents. Data shown are summary of 4 mice per genotype. **(C)** Digested ear samples were acute stimulated *in vitro* to identify CD4^{+} and CD8^{+} T cells producing IFN-γ or TNF-α by intracellular cytokine staining. Data shown are summary of 4 mice per genotype. **(D)** Cells from draining cervical LNs (day 1 post challenge) were analyzed by flow cytometry to assess Treg contents. Data shown are summary of 4 mice per genotype. **(E)** Cells from draining cervical LNs at day 1 post challenge were acutely stimulated *in vitro* to identify CD4^{+} and CD8^{+} T cells producing IFN-γ or TNF-α by intracellular cytokine staining. Data shown are summary of 4 mice per genotype. Statistical analysis was performed using two-way ANOVA with a Sidak's multiple comparisons (A) or Student's *t*-test (B-E).

Chapter 3. Understanding the role of ICOS in NOD mouse models of autoimmune diabetes

ICOS costimulation is required to control autoimmune diabetes in adult NOD mice

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Author contributions

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

3.1 Summary

Studies have shown that germline ICOS deficiency prevents type 1 diabetes (T1D) in pure NOD mice by reducing the activation of pathological polyclonal effector T cells. In contrast, when most T cells were designed to express TCR that recognizes self-antigens in the pancreatic islets in BDC2.5 TCR transgenic mice in NOD background, ICOS germline deficiency accelerated the onset of T1D. It is thought to be due to the loss of suppressive capacity and fitness of ICOS-deficient Treg cells within the pancreatic islets. However, it remains to be elucidated whether ICOS deficiency affects T1D through modulating T cell thymic development in these NOD models. To address this question, we created NOD mouse lines in which *Icos* gene can be acutely eliminated in adult mice through an inducible Cre system after the TCR repertoire has been established. We found that abrogation of ICOS expression in adult mice had very similar outcomes as mice with germline ICOS deficiency: reduced incidence of T1D in pure NOD mice but accelerated T1D in BDC2.5 model. Our results reinforce the existing notion that the main function of ICOS is to regulate mature effector and regulatory T cells function in NOD mouse models.

3.2 Introduction

Type 1 diabetes (T1D) is a chronic autoimmune disease caused by a T cell dependent damage of the insulin-producing β-cells of Langerhans [281, 314]. The NOD mouse strain is a well-established model of autoimmune T1D, where mice can develop diabetes spontaneously with a strong female prevalence and shares clinical features with human T1D. The enhanced Th1 immune response through the increased IL-12- STAT4 signaling by estrogen could increase the susceptibility of female NOD mice [315]. Another study showed that colonization by commensal microbes increased serum testosterone and protected male NOD mice from T1D development [316]. When T1D develops in NOD mice, mice exhibited increased levels of autoantibodies and hyperglycemia, a sign of severe dysregulated immune response [281]. Break down of the immunological tolerance is thought to be a crucial step of pathogenesis of T1D [317]. It is well known that regulatory T cells (Tregs), which express CD4 and Foxp3, are essential for the maintenance of tolerance and that impaired function of Treg cells lead to fatal autoimmune lymphoproliferative disease [318, 319].

Some authors reported that functional waning of Tregs in aged NOD mice led to impaired ability to control pathogenic effector T cells infiltration in the pancreatic site, causing diabetogenic insulitis, although the number of natural Treg cells was not changed compared to T1D-resistant mice [284]. Human data also confirmed that defective Treg function led to inability in suppressing effector T cells proliferation *in vitro*, with similar proportion of Treg cells in healthy individuals [320, 321]. Because of the reduction in quality rather than quantity of Treg cells, many groups investigated whether there are differences in Treg compositions between T1D and controls. A study reported that T1D progression in NOD mice is correlated with the decrease in ICOS expression in intra-islet Treg cells [127].

ICOS is a member of the CD28 superfamily cosignaling receptors. Similar to CD28, ICOS can induce T cell proliferation [12, 13] and augments T cell survival [127, 322, 323]. In contrast to $ICOS^-$ Treg cells, $ICOS^+$ Treg cells are more proliferative and suppressive, with a higher amount of IL-10 secretion. ICOS deficiency weakens the competitive fitness of Treg cells, resulting in the onset of T1D [127, 267]. $ICOS^+$ Treg cells showed a Th1 like phenotype with expression of T-bet and IFN-γ, which renders $ICOS^+$ Treg cells responsive to IFN-γ producing effector T cells. It provides enhanced suppressive capacity for Th1 response in T1D [290].

Interestingly, due to the dual role of ICOS in promoting conventional T cells and Treg cells, conflicting results have been reported in NOD mice. Deletion of the ICOS and ICOS ligand (ICOSL) drastically reduced diabetes incidence [287-289]. This result is correlated with slower and milder inflammation in pancreatic islets, and it indicates that ICOS signaling plays a crucial role in the activation of pathogenic T cells. However, most T1D-free ICOS deficient NOD mice showed neuromuscular inflammation instead of T1D, but the reason remains unclear [288, 289]. In contrast, autoreactive effector T cells in BDC2.5-NOD mice, which express a TCR transgene specific for a islet-specific autoantigen in the NOD background, readily cause insulitis but they are suppressed by Treg cells in pancreatic lesions. Therefore, the overall T1D development incidence is much lower than pure NOD mice. Of note, blockade or genetic abrogation of ICOS signaling pathways escalated disease progression in BDC2.5-NOD mice [127, 267]. This observation is consistent with the reports that $ICOS^{hi}$ Treg cells readily migrate to draining lymph node (LN) and have a more potent suppressive ability than $ICOS^{lo}$ Treg cells [127, 290]. Thus, in the BDC2.5-NOD T1D murine model, ICOS seems to support anti-autoimmune Treg function.

Importantly, we and others have shown that ICOS is highly expressed in thymocytes and ICOSL is expressed in antigen presenting cells in the thymic microenvironment [227, 324]. Further disruption of ICOS-ICOSL interaction in developing thymocytes partially impaired negative selection of CD8 SP thymocytes (Dong M et al, manuscript submitted). This raises the possibility that ICOS deficiency in NOD mice could have impacted T1D incidence through alteration of thymic selection processes. We began to address the relative role of ICOS in thymic selection vs peripheral control by establishing an inducible ICOS genetic deletion model in pure NOD and BDC2.5-NOD mice. We found that acute loss of ICOS in adult NOD mice led similar impact on T1D disease progression as germline ICOS-deficiency highlighting a dominant role of ICOS in the control of peripheral T cells.

3.3 Materials and method

3.3.1 Mice

NOD (Jax 001976), BDC2.5-NOD (Jax 004460) and Ubc-CreERT2 (Jax 007001) mice were obtained from The Jackson Laboratory. ICOS conditional knockout [101] and Ubc-CreERT2 were backcrossed onto NOD background for more than 15 generations. Afterwards, these lines were interbred to generate composite lines. To get inducible *Icos* gene deletion, *UBC-CreERT2 ICOS*$^{F/F}$ mice and their control mice, *UBC-CreERT2* were fed with tamoxifen. Female mice were fed with tamoxifen dissolved in corn oil (Sigma-Aldrich) daily at a dose of 200 ug/g body weight by oral gavage for 5 days. Tamoxifen treatment was initiated at the age of 8 weeks. All mice were housed in the animal facilities at the Institut de recherches cliniques de Montréal (IRCM) in a specific pathogen-free environment and all the experiments were performed in accordance with animal use protocols approved by the IRCM animal care committee.

3.3.2 Blood glucose monitoring

T1D incidence was assessed by weekly monitoring of blood glucose levels. Glycemia was determined using blood glucose strips (Miles Diagnostic). Mice were considered diabetic following two consecutive blood glucose readings above 10 mM.

3.3.3 Pancreatic tissue digestion

Single cell suspensions of the pancreatic draining lymph node were prepared by mechanical disruption via 70 μm mesh filter (BD Biosciences) in PBS or staining buffer (PBS containing 1 % bovine serum albumin (Wisent)). Pancreatic single-cell suspensions were prepared using collagenase V (GIBCO). Pancreatic tissue fragments were incubated in 1 mg/ml collagenase V and DNase I (to a final concentration of 0.1 mg/ml; SIGMA) at 37°C for 20 min. The fragments were gently ground on 70 μm mesh filter with PBS and washed two times with PBS to maximize the release of single cells. The single cells were collected for the staining.

3.3.4 Antibodies and flow cytometry

In order to prevent non-specific binding, anti-CD16/32 (BioXCell) was used prior to surface staining. For intracellular staining, surface-stained cells were fixed and permeabilized with Fix/Perm buffer (ThermoFisher). Antibodies used for flow cytometry are listed as follows: anti-CD4 (RM4-5, Invitrogen), anti-CD8 (53-6.7, Invitrogen), anti-Foxp3 (FJK-16s, invitrogen), anti-ICOS (C398.4A, BioLegend), anti CD25 (PC61.5, Invitrogen), anti-CD44 (IM7, BD Bioscience), anti-CD62L (MEL-14, BioLegend), fixable viability dye (ThermoFisher), anti-TCR Vβ4 (KT4, BD Bioscience).

3.3.5 *In vitro* T cell activation

For antigen specific activation, isolated BDC2.5 CD4 T cells (5×10^4) from peripheral lymph nodes were co-cultured for 3 days with irradiated splenocytes (2×10^5) at 37°C. Synthetic BDC2.5 mimotope peptide (RTRPLWVRME, Anaspec) or soluble anti-CD3ε (as control) was added to the co-cultures with indicated concentration.

3.3.6 Histology

Pancreas was fixed in 10% neutral buffered formalin for 2 days at 4°C. The samples were washed in PBS, embedded in paraffin, and cut in 5 μm sections. Slides were stained with H&E and examined under microscope to assess immune cell infiltration.

3.3.7 Statistical analysis

All data are presented as mean and standard error of mean (SEM). Differences between experimental groups were tested with Student's *t*-test and Fisher's exact test in Prism 9.0 (GraphPad Software). Statistical significance was judged based on the *p* value, $p < 0.05$ was considered as statistically significant.

3.4 Results

3.4.1 Ubiquitous loss of ICOS in adult NOD mice decreased the incidence of type 1 diabetes

It has been shown that germline ICOS KO reduces incidence of T1D in pure NOD mice by reducing activation of pathological polyclonal effector T cell [287-289]. To further investigate the relative role of ICOS in controlling mature T cell population vs. early thymic events, we created animal models in which ICOS can be deleted by feeding tamoxifen in adult mice. First, we utilized a mouse line that ubiquitously expresses inducible Cre (Ubc-CreERT2; hereafter UCE) in NOD background. Compared with NOD UCE^{+} $ICOS^{F/+}$ mice, acute deletion of ICOS conditional allele by tamoxifen in NOD $UCE^{+}ICOS^{F/F}$ mice substantially delayed the onset of T1D. Further, the incidence of T1D at 6 months of age was only 40% compared with 80% in the control group (Figure 3.1A). The reduction in diabetes incidence after acute deletion of ICOS in the adult NOD mice may be attributable to the decline of lymphocyte infiltration into the islets. Histological examination of mice revealed that pancreatic islets had less infiltrated lymphocyte in NOD UCE^{+} $ICOS^{F/F}$ mice than their counterparts at 24 weeks of age (Figure 3.1B). These results are consistent with the view that ubiquitous loss of ICOS in pure NOD mice mainly impairs pathological T cells.

3.4.2 Profiling of pancreatic lymph node T cells after acute deletion of *Icos* gene in NOD mice

We further investigated $CD4^+$ T cells, $CD8^+$ T cells, and Treg cells in pancreatic draining lymph nodes in NOD mice that lost the *Icos* gene at the adult stage vs. controls. The frequency and number of $CD4^+$ T cells or $CD8^+$ T cells in NOD UCE^+ $ICOS^{F/F}$ mice were comparable to NOD UCE^+ $ICOS^{F/+}$ mice in the pancreatic lymph nodes 12 to 16 weeks after tamoxifen treatment (Figure 3.2 A). CD44 and CD62L were used to identify effector/memory $CD44^+$ $CD62L^-$ T cell subsets [325, 326]. Although it was not significantly different, there was a trend of reduced effector $CD4^+$ $CD44^+$ $CD62L^-$ T cells in NOD UCE^+ $ICOS^{F/F}$ mice as compared to the control (Figure 3.2 B). Decreased T1D incidence and lower islet-infiltrating lymphocytes in NOD UCE^+ $ICOS^{F/F}$ mice could be explained by these decreased effector $CD4^+$ T cells in pancreatic lymph node. More careful analysis of T cell contents and activation status in pancreatic lymph node and pancreatic islets at early and late stage of T1D is required to ascertain this idea.

A previous study using $ICOS^{-/-}$ NOD mice showed that defective diabetogenic effector and Treg cells led to the protection of $ICOS^{-/-}$ NOD mice from diabetes development [288]. We next determined whether the decreased diabetogenicity of $CD4^+$ T cells in NOD UCE^+ $ICOS^{F/F}$ was due to altered Treg cell numbers in the pancreatic lymph node. We observed that the frequency or number of Treg cells were similar (Figure 3.2C). Treg cells can be divided into subsets based on the expression of $CD44^{lo}CD62L^{hi}$ central Treg (cTreg) or $CD44^{hi}CD62L^{lo}$ effector Treg (eTreg) [181]. It is well established that eTreg cells are highly suppressive and can regulate inflammation in nonlymphoid tissues [327]. Interestingly, there was a trend of reduced $CD44^{hi}CD62L^{lo}$ eTreg cell numbers in NOD UCE^+ $ICOS^{F/F}$ mice compared to their counterparts (Figure 3.2C).

These results suggested that ICOS costimulation promotes activation of both effector $CD4^+$ T cells and Treg cells within the draining lymph nodes.

3.4.3 Accelerated T1D incidence in ICOS-deficient BDC2.5-NOD mice

NOD mice expressing BDC2.5 TCR transgene have been used as a useful model to study the role of immune components in the pathogenesis of insulin-dependent diabetes mellitus [328]. BDC2.5 TCR gene was originally cloned from an autoreactive pathogenic $CD4^+$ T cell subset from

a diabetic NOD mouse. Paradoxically, mice expressing BDC2.5 transgene were protected from T1D due to Treg cells arising through TCRα chain editing [329]. More than 95% of mature T cells from BDC2.5-NOD mice expressed the transgene-encoded Vβ4 segment [330]. Consistent with this data, we confirmed that most of T cells from BDC2.5-NOD mice were $V\beta4^+$ (Figure 3.3 A). BDC2.5 $CD4^+$ T cells are reactive to an autoantigen in the context of the major histocompatibility complex II molecules $I\text{-}A^{g7}$ [331]. While agonistic mimotope peptide stimulated BDC2.5 $CD4^+$ T cells at 100 ng/ml concentration, $CD4^+$ T cells isolated from pure NOD mice could not be stimulated with the mimotope (Figure 3.3B).

To confirm that germline ICOS-deficiency accelerates T1D in BDC2.5-NOD mice, we generated ICOS deficient BDC2.5-NOD mice using inherent leakiness of Ubc-CreERT2 transgene. Although the recombinant CreERT2 enzyme is supposed to be inactive in the absence of tamoxifen, we found that approximately 10% of pups lost ICOS conditional allele when born from parents possessing CreERT2 transgene and ICOS conditional allele. We set up a PCR protocol to identify the disrupted ICOS allele to get ICOS-deficient BDC2.5-NOD mice (Figure 3.3 C). As shown in Figure 3.3D, diabetes developed from the age of 8 weeks in female ICOS deficient BDC2.5-NOD mice and reached 80% final prevalence of diabetes at 21 weeks. In contrast, ICOS-sufficient BDC2.5-NOD mice showed 50% of T1D incidence at the age of 22 weeks, similar to data in the literature [332]. Therefore, it seems that the dominant role of ICOS in BDC2.5-NOD mice is to support mechanisms that suppress pathogenic autoreactive T cells, possibly by promoting Treg cell differentiation and/or function.

It was reported that $ICOS^{-/-}$ NOD mice protected from T1D developed neuromuscular inflammation instead [288]. However, we could not observe any signs of neuromuscular inflammation in ICOS-deficient NOD mice in our colony (data not depicted)

3.4.4 Acute deletion of ICOS in adult BDC2.5 mice induce T1D

In contrast to pure $ICOS^{-/-}$ NOD mice, accelerated diabetes was observed in BDC2.5 transgenic $ICOS^{-/-}$ NOD mice, demonstrating that diabetogenic BDC2.5 effector T cells can cause disease in an ICOS-independent manner. It also suggested that ICOS could be important for immunosuppressive components such as Treg cells [288]. One possibility is that germline deletion of ICOS generates “holes” in the tissue protective Treg repertories in BDC2.5-NOD mice during

thymic selection. However, it is also possible that the absence of ICOS in the mature T cells in BDC2.5 mice is sufficient to tip the balance towards activation of pathogenic T cells in the periphery. As a first step to distinguish these possibilities, we generated BDC2.5-NOD mice in which *Icos* gene can be deleted in adult mice through inducible Cre activity using Ubc-CreERT2 system. We acutely abrogated ICOS expression in BDC2.5-NOD at the age of 8~10 weeks. Strikingly, this ubiquitous deletion of *Icos* gene in adult BDC2.5-NOD mice also led to an early onset of T1D (Figure 3.4A). Consistently, histological analysis revealed high levels of insulitis in BDC2.5 mice that have lost ICOS expression through tamoxifen treatment (Figure 3.4B). These results indicated that ICOS is required for the immune suppressive compartment in adult mice. It is yet to be tested if the number or suppressive functions of Treg cells in the pancreatic islets or draining lymph nodes are diminished under these conditions. Interestingly, we could not observe T1D initiation in the tamoxifen treated BDC2.5 $UCE^{+}ICOS^{F/+}$ control group after 24 weeks of monitoring. This could be due to the immunosuppressive effects of tamoxifen inhibiting pathogenic T cells [333].

3.5 Discussion

In this study, we tested the impact of *Icos* gene deletion on T1D disease progression in two NOD mouse models: plain NOD and BDC2.5 TCR transgenic NOD mice. In plain NOD mice, acute elimination of ICOS in adult mice delayed onset and reduced overall incidence of T1D; however, in BDC2.5 mice the same treatment accelerated T1D disease progression. Thus, abrogation of ICOS expression in adult mice had similar impacts to those caused by germline ICOS deficiency in both models.

The main goal of our study was to assess the relative roles of ICOS in T cell thymic development compared with peripheral T cell activation and function in the mouse models of autoimmune diabetes. There have been numerous studies demonstrating the role of ICOS in T cells subset differentiation and function in the periphery. There is also evidence that ICOS can influence T cell development in the thymus. In particular, our recent work indicates that lack of ICOS-ICOSL interaction can partially impair negative selection of thymic $CD8^{+}$ T cells in C57BL/6 mice (Dong M et al, manuscript submitted to *Immunology & Cell Biology*). Since most studies on ICOS in NOD model utilized germline knockout mice or transient interruption of ICOS-ICOSL interaction by

repeated injection of antibodies, it has been difficult to judge whether the ICOS-dependent thymic selection process altered T1D progression. Our study showed that acute deletion of ICOS in adult NOD mice had very similar outcome as ICOS germline deficiency supporting the crucial role of ICOS in mature T cell function. Therefore, manipulation of ICOS-ICOSL interaction in the mature T cell stage is likely to produce beneficial effects in autoimmune disease treatment.

Nevertheless, our data cannot rule out the possibility that ICOS-ICOSL interaction in the thymus influence T1D disease progression by altering TCR repertoire in NOD mouse models. For example, subtle changes in the efficiency of negative selection may give rise to more potent autoimmune T cell expansion in the pancreas or change the target organs. On the other hand, ICOS-deficiency may alter the specificity and/or affinity of Treg TCR, thereby influencing the disease progression in BDC2.5 mice. This possibility could be addressed by sequencing TCR genes isolated from T cells in the prediabetic islets of BDC2.5 mice to determine if dominant TCR clonotypes are altered by ICOS deficiency.

Another aim of this study was to distinguish the role of ICOS in pathogenic T cells vs Treg cells in NOD models. The prevailing view holds that the reason ICOS germline deficiency led to the opposite outcomes in plain NOD vs BDC2.5 mice is that ICOS-deficiency impairs both pathogenic T cells and Treg cells. According to this model, pathogenic T cells are critically affected in NOD mice whereas Treg cells are more severely weakened in BDC2.5 mice in the absence of ICOS. Indeed, our acute deletion fits well with this model. However, we failed to provide more compelling evidence due to technical difficulties. We attempted to create a NOD mouse model in which the *Icos* gene can be deleted only in Foxp3 expressing cells. However, available Foxp3-IRES-Cre knockin lines had reduced Foxp3 expression and this turned out to be sufficient to cause early onset of T1D in NOD mice even before elimination of the *Icos* gene. In order to avoid this problem, we purchased Foxp3-EGFP-Cre BAC transgenic line made in NOD background (Jax 008694) [334] and tried to breed with ICOS conditional mutant line. Unfortunately, the Foxp3-EGFP-Cre BAC transgene always segregated with ICOS wild type locus (presumably due to close proximity of the transgene to *Icos* locus) preventing us from generating useful mouse lines.

In summary, we confirm that elimination of the *Icos* gene in adult mice has very similar effects as germline deficient mice on disease onset and progression of T1D in two NOD models. Our results reinforce the view that the main role of ICOS is to promote the function of effector T cells and Treg cells in the periphery.

3.6 Figure and figure legends

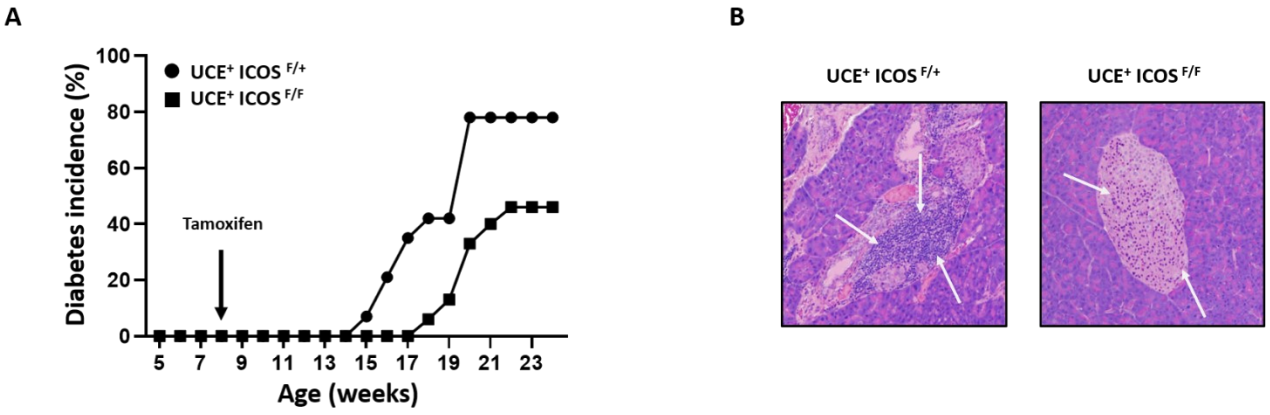

Figure 3.1 Ubiquitous loss of ICOS in adult NOD mice reduced T1D incidence. Female NOD mice with $UCE^{+}ICOS^{F/+}$ (n=14) or $UCE^{+}ICOS^{F/F}$ (n=15) genotype were treated with tamoxifen at 8 weeks of age. Blood glucose levels were monitored starting at 5 weeks of age until 24 weeks of age. Moribund mice were euthanized according to the animal care guidelines. **(A)** The incidence of T1D (blood glucose level >10 mM) were calculated as percentage. Fisher's exact test was used (p=0.1281) **(B)** Representative images of pancreatic islets from diabetic $UCE^{+}ICOS^{F/+}$ mice (n=14) and non-diabetic $UCE^{+}ICOS^{F/F}$ mice (n=15)

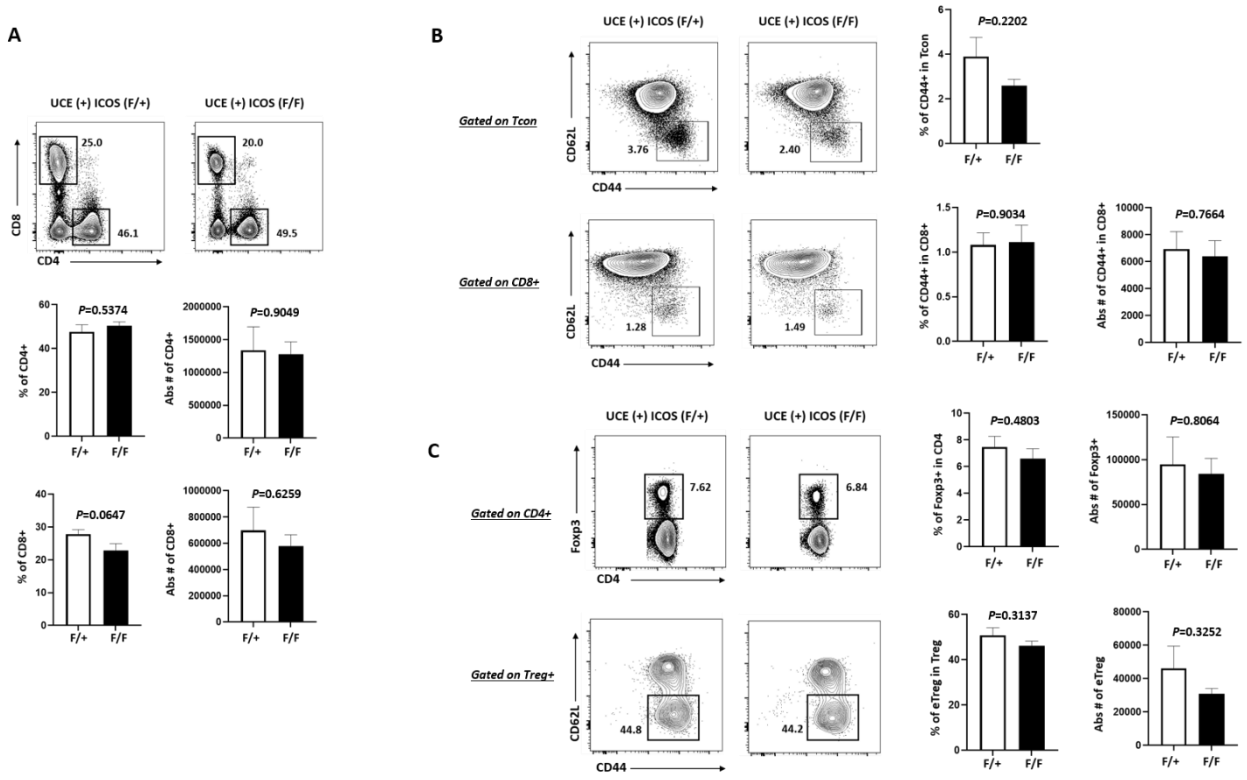

Figure 3.2 Profiling of pancreatic lymph node T cells after acute deletion of *Icos* gene in NOD mice. Female NOD mice with $UCE^{+}ICOS^{F/+}$ (n=8) or $UCE^{+}ICOS^{F/F}$ (n=5) genotype were treated with tamoxifen at 8 weeks of age. Pancreatic lymph nodes were isolated at 24 weeks of age and T cell number and activation status were analyzed by flow cytometry. **(A)** The percentage and number of $CD4^{+}$ Tcons, $CD8^{+}$ T cells. **(B)** The percentages and numbers of $CD44^{+}CD62L^{-}$ Tcons and $CD8^{+}$ T cells are presented. **(C)** The percentage and number of $CD44^{hi}CD62L^{lo}$ Treg cells are presented.

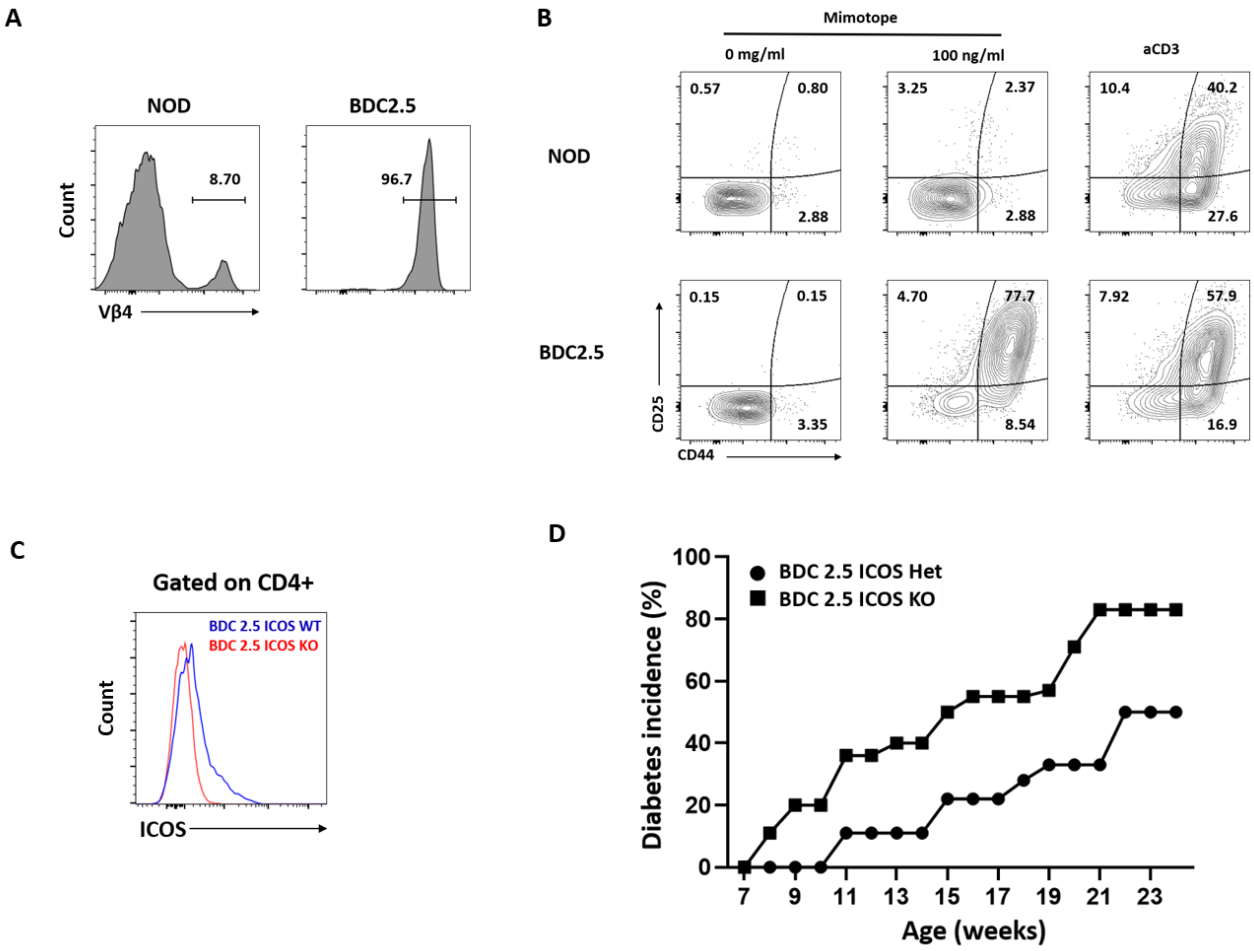

Figure 3.3 Germline deletion of *Icos* gene in BDC2.5-NOD mice accelerated T1D. (A) TCR profiling of BDC2.5 TCR transgene. Blood samples from 10~16 week-old NOD or BDC2.5-NOD mice were analyzed by flow cytometry. **(B)** Antigen-specific response of BDC2.5 T cells. Splenic CD4 T cells were stimulated *in vitro* using irradiated splenocytes loaded with BDC2.5-specific mimotope or by adding anti CD3-antibodies. After 3 days of incubation, CD4 T cells were assessed for the expression of activation markers CD25 and CD44. **(C)** Splenocytes from wildtype or ICOS KO BDC2.5 mice were analysed by flow cytometry to confirm ICOS-deficiency in the KO mice. **(D)** Female BDC2.5 ICOS KO mice (n=11) or BDC2.5 ICOS Het mice (n=12) were monitored for diabetes by measuring blood glucose levels once a week. Fisher's exact test was used (p=0.500)

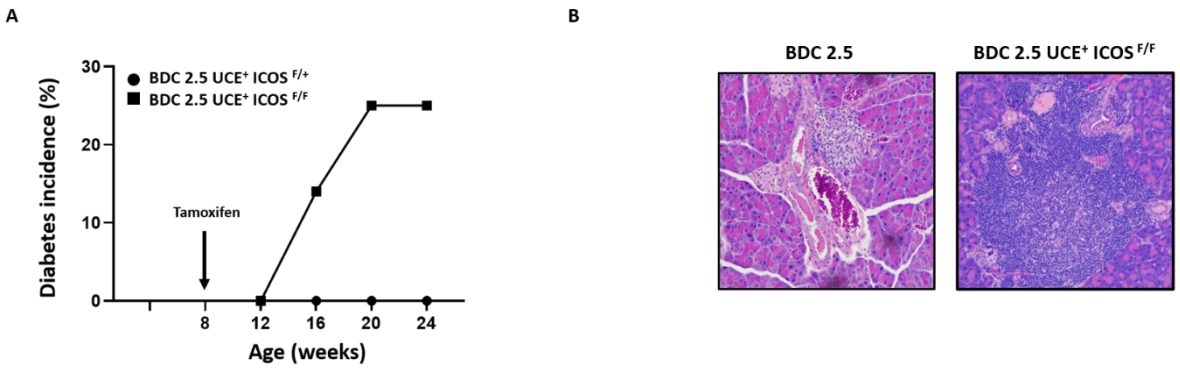

Figure 3.4 Ubiquitous loss of *Icos* gene in adult BDC2.5-NOD mice accelerated T1D. Female BDC2.5 mice with $UCE^{+}ICOS^{F/+}$ (n=10) or UCE^{+} $ICOS^{F/F}$ (n=15) were treated with tamoxifen at 8~10 weeks of age. Blood glucose levels were monitored starting at 12 weeks of age until 24 weeks of age. Moribund mice were sacrificed according to the animal care guidelines. **(A)** The incidence of T1D (blood glucose level > 10 mM) were calculated as percentage. **(B)** Representative images of pancreatic islets from diabetic BDC2.5 $UCE^{+}ICOS^{F/F}$ mice is shown along with pure BDC2.5.

Chapter 4. Discussion

4.1 Summary

The goal of the research presented in this thesis is to understand the role of ICOS in Treg homeostasis and function in steady state and inflammation condition. In Chapter 2, we used a conditional knockout system in which *Icos* gene is selectively deleted in Foxp3-expressing cells (ICOS FC mice) to investigate the role of ICOS in Treg homeostasis and function. ICOS FC mice demonstrated that ICOS-deficiency has minimal impact on the overall maintenance of steady-state Treg population. These mice also did not show any signs of spontaneous multi-organ inflammation indicating that steady state Treg cells do not heavily rely on ICOS costimulation. However, ICOS FC mice showed reduced ability to handle Th1-driven inflammation in oxazolone-induced skin inflammation. It was correlated with an elevated number of IFN-γ producing effector T cells in ICOS FC mice compared to their counterparts. Intriguingly, in germline ICOS knockout mice, we could not observe the significant differences in inflammation compared to wild type mice indicating that ICOS has a dual positive regulatory role in effector T cells and Treg compartments. In addition, single-cell transcriptome analysis suggested that ICOS-deficient Treg cells impaired the maturation into T-bet^{+} CXCR3^{+} "Th1-Treg" cells in the draining LN under skin inflammation. These results support that ICOS costimulation in Treg cells might be beneficial for the treatment of Th1-driven inflammation (Figure 4.1)

In Chapter 3, we investigate the role of ICOS in the NOD T1D mouse model. It has been known that germline ICOS knockout prevents T1D in pure NOD mice, showing the reduced activation of pathological polyclonal effect T cells [288, 289]. However, in BDC2.5 TCR transgenic mice in NOD background, in which most T cells are designed to express TCR that recognizes self-antigens in the pancreatic islets, ICOS deficiency accelerated the onset of T1D [288]. Previous studies suggested that it is because of the loss of suppressive capacity and fitness of ICOS-deficient Treg cells in pancreatic islets [127, 288]. To investigate the temporal requirement of ICOS costimulation in NOD mice, we created inducible ICOS knockout in NOD mouse models. Our preliminary data are consistent with the view that ubiquitous loss of ICOS in pure NOD mice impairs pathological T cells. In addition, germline deletion of *Icos* gene in adult BDC2.5-NOD mice also showed accelerated T1D development. These results strengthen the notion that the main function of ICOS is to regulate mature effector and regulatory T cells in NOD mouse models.

In this thesis, I have shown that ICOS FC mice have minimal alterations in the composition of Treg subpopulations in the absence of inflammation. However, ICOS FC mice were more prone to Th1-driven inflammation in the skin and pancreas. Based on flow cytometric and single-cell transcriptome analysis, I gathered evidence supporting the prevailing view that ICOS costimulation promotes the differentiation of T-bet^{+} Treg subset during Th1-driven inflammation.

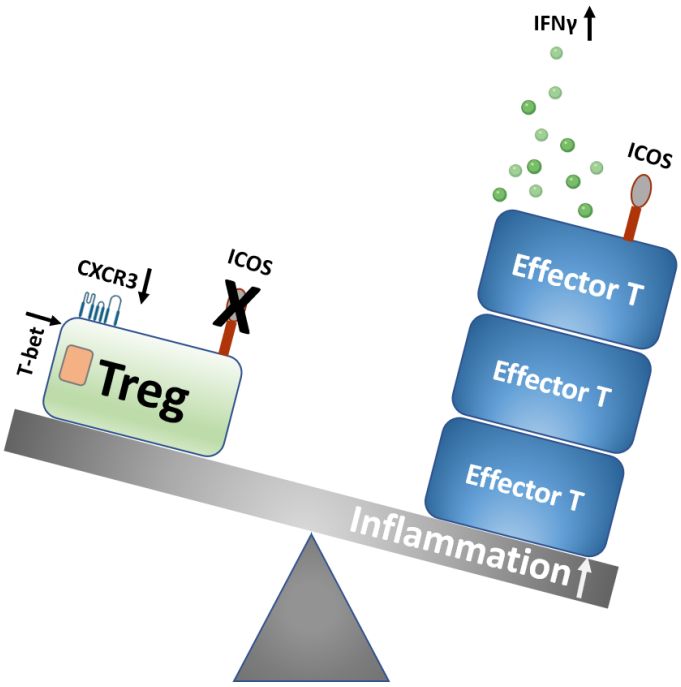

Figure 4.1 Graphical summary for ICOS$^+$ Treg functions in skin inflammation. ICOS FC mice showed more severe inflammation in oxazolone-induced skin inflammation. This was correlated with an increase of IFN-γ producing effector T cells. Single-cell transcriptome analysis suggested that ICOS-deficient Treg cells fail to mature into T-bet$^+$ CXCR3$^+$ Th1-like Treg cells in the draining LN.

4.2 Outstanding questions

4.2.1 When does ICOS become indispensable in Treg responses?

Our data indicate that the role of ICOS in Treg homeostasis is easily compensated by other mechanisms. One of the key mechanisms that compensate ICOS-deficiency is likely CD28-mediated costimulation. CD28 has been shown to be crucial for the homeostasis of Treg cells such that germline loss of CD28 or its ligands (CD80 and CD86) in NOD mice accelerates progression of T1D [171]. In contrast, ICOS germline deficiency in plain NOD slowed disease onset and severity [287, 288]. The dominant role of CD28 over ICOS in Treg biology goes beyond Treg homeostasis. CD28 deficiency greatly reduces Treg thymic output [6, 306], whereas there is no strong evidence for ICOS being indispensable for thymic Treg generation. Even for the generation of inducible Treg cells from naïve $CD4^+$ T cells, CD28 play a dominant role over ICOS [126].

On the other hand, ICOS seems to be indispensable for certain Treg subsets. First, ICOS deficiency affects differentiation of Treg cells that control Th1-mediated inflammation during contact hypersensitivity and T1D in NOD mice. Features of this Treg cell population include expression of T-bet and CXCR3. One of the reasons why ICOS-expressing Treg cells play prominent role in Th1 condition is the availability of costimulatory ligands. ICOSL can be induced in non-lymphoid tissues by inflammatory cues such as TNF-α [28] whereas CD80/CD86 are limited in antigen presenting cells [11]. Second, our group has data showing that ICOS is critical for the generation of T follicular regulatory (Tfr) cells. ICOS-expressing Tfr cells are especially important to prevent the expansion of autoreactive germinal center B cells and production of autoantibodies during the germinal center reaction (Panneton V et al., manuscript in preparation). The prominent role of ICOS in Tfr differentiation may rely on its unique ability to control expression of genes that are necessary for the germinal center T cell signatures such as Bcl6 and CXCR5 through its unique signaling capacity.

These distinct roles of CD28 and ICOS in Treg generation functions could be exploited to treat autoimmune or inflammatory diseases. For example, reagents that boost CD28 costimulation can enhance homeostasis of Treg cells or induce conversion of naïve $CD4^+$ T cells into Treg compartment whereas agents promoting ICOS-ICOSL pathway could facilitate differentiation of Th1-Treg subset. However, both CD28 and ICOS have costimulatory role in effector or

inflammatory T cells. Thus, the challenge is to deliver these agonist signals preferentially to Treg cells. Much work is needed to figure out how.

4.2.2 Does ICOS costimulation affect TCR repertoire?

Our study presented in Chapter 3 investigated the impact of the *Icos* gene on T1D disease progression in pure NOD and BDC2.5 TCR transgenic mice. It was known that the protection of diabetes in ICOS$^{-/-}$ NOD mice was unexpectedly associated with the development of a neuromuscular inflammation due to a shift of autoimmune targets from islets to neuromuscular tissues. Although intriguing, there is no clear explanation for this observation. We hypothesized that this could be due to an alteration of TCR repertoire in ICOS KO mice. Thus, we monitored whether ICOS germline-deficient NOD mice caused neuromyopathy and hind leg paralysis. However, we could not observe any signs of neuromuscular inflammation in our colony. This discrepancy may have risen from different extents of C57BL/6 genes still remaining in the background NOD lines or differences in housing conditions. Regardless, we could not confirm the deviation of autoimmune targets in ICOS-deficient NOD mice.

In C57BL/6 background, we studied the role of ICOS in T cell thymic selection with a hypothesis that ICOS signaling promotes the selection of thymic Treg cells specific to tissue-restricted antigens to protect the autoimmune attack. In collaboration with Dr. Heather Melichar's team, we confirmed that ICOS is highly expressed in CD4 SP and CD8 SP thymocytes, and ICOSL is also expressed in mTEC, DCs and B cells. In contrast, ICOS expression in de novo thymic Treg cells was low. Consistent with these expression pattern, Melichar group observed impaired negative selection of CD8^{+} T cells on ICOSL deficient thymic slices (Dong M et al, manuscript submitted to *Immunology & Cell Biology*). These results suggest ICOS signaling promotes thymic negative selection and ICOS germline deficiency in NOD may give rise more potent autoimmune T cell expansion in the pancreas or change the target organs. However, data from ICOS deficient NOD mice disputed this idea by showing the delayed onset of T1D. In addition, we cannot rule out the possibility that deficiency of *Icos* gene may change the specificity or affinity of Treg TCR, accelerating disease progression in BDC2.5 mice. If ICOS-deficiency generates "holes" in the tissue protective Treg repertoire, TCR sequencing will reveal the changes in the diversity of the TCR

clones. If the TCRβ chain diversity is not enough to distinguish the subtle differences in Treg diversity, additionally we can compare TCRα chain clonotypes in ICOS deficient BDC2.5-NOD mice compared with its wild type control mice. Nevertheless, our data show that acute deletion of ICOS in adult NOD mice has similar effects as germline ICOS deficiency. Thus, regardless of potential contributions from thymic selection, our data do not change the prevailing view that ICOS is preferentially involved in the regulation of T cells in the periphery under inflammatory conditions.

We tried to characterize the role of ICOS in Treg cells in NOD mouse model as we had in skin inflammation model. We tried to generate Treg specific ICOS deficient NOD mouse models using our available Foxp3-IRES-EGFP-Cre ERT2 (JAX 016961) knockin lines. These mice were backcrossed onto NOD background, but before the ICOS deletion, they showed early onset of T1D due to the low expression level of Foxp3. In order to avoid this problem, we purchased Foxp3-EGFP-Cre BAC transgenic line made in NOD background (Jax 008694) [334] and tried to breed with ICOS conditional mutant line. Unfortunately, we could not generate useful mouse lines from this BAC transgenic line again because the Foxp3-EGFP-Cre BAC transgene always segregated with ICOS wildtype locus. Thus, we could not distinguish the orle of ICOS in pathogenic T cells and Treg cells in NOD mice.

Lastly, we did not investigate the relative contribution of acute deletion of ICOS in T cell profile such as Th1 $CD4^+$ T cells and Treg cells in BDC2.5-NOD mouse. Kornete *et al.* reported that the expression of chemokine receptor CXCR3 for Treg's migration to the inflamed region is ICOS dependent in pancreatic LN [290]. Moreover, this Th1-like Treg cells are characterized by the production of IFN-γ, and the expression of T-bet and IFN-γ receptor to suppress the IFN-γ producing effector T cells. This data was generated from ICOS germline knockout BDC2.5-NOD mice. Although our acute deletion fits well with the ICOS deficient BDC2.5-NOD mice, demonstrating that Treg cells are more severely weakened in BDC2.5-NOD mice in the absence of ICOS, it is necessary whether acute deletion of ICOS in adult BDC2.5-NOD mice still have lower migration capacity compared to their control counterpart. We will examine the kinetics of Treg profile under different time point of T1D development in ICOS deficient BDC 2.5-NOD mice. However, it is most necessary to elucidate the role of ICOS in Treg cells under T1D inflammation when the Treg specific ICOS deficient NOD mice are available.

4.3 Potential clinical implications

The ICOS-ICOSL interaction has been a potential therapeutic target in multiple clinical settings such as antibody-driven autoimmune diseases, vaccination and cancer immunotherapies [108, 335]. For example, a humanized monoclonal antibody against ICOSL has been shown to reduce IgG antibody titers without changing overall serum antibody titers [336] and showed some potential efficacy for systemic lupus erythematosus (SLE) [337]. However, the most promising ICOS clinical trials are taking place in the filed of cancer immunotherapy. Importantly, initial mechanistic insights gathered from these trials are in line with our findings that ICOS costimulation influences both effector T cells and Treg compartments.

In recent years, immune checkpoint blockade became a promising cancer treatment option [338, 339]. A combination of existing therapies such as CTLA-4 blockade with another T cell co-stimulatory pathway is one way of improving the efficacy. However, since ICOS is expressed in multiple anti- or pro-tumor immune cells, the compounding effects of agonistic or antagonistic reagents remain as concerns. As such, results from pre-clinical and clinical studies often contradict each other. Some studies support the notion that activation of ICOS pathway improves checkpoint blockade regimens [338, 340]. Patients who respond to anti-CTLA-4 blockade showed increased $CD4^+$ $ICOS^+$ IFN-γ^+ T cells [341, 342], suggesting the importance of $ICOS^+$ effector T cells. In addition, ICOSL-overexpressing tumor vaccine along with CTLA-4 blockade showed improved anti-tumor T cell responses in mice [343]. In contrast, other studies have shown that ICOS may play dominant pro-tumor roles in certain tumor environments by supporting Treg cells. Some human melanoma cells express high levels of ICOSL which promote Treg expansion [344], which is highly associated with poor prognosis [345]. Therefore, one should be aware of the potential danger of utilizing ICOS agonist antibodies in cancer immunotherapy.

Despite these complications, there could be a way to exploit the differential expression levels of ICOS in tumor-infiltrating T cells. Sainson *et al*. have shown that, both in mice and humans, tumor infiltrating Treg cells express much higher levels of ICOS compared with $CD8^+$ cytotoxic T cells [346]. Capitalizing on this, they invented a humanized monoclonal antibody that can deplete $ICOS^{hi}$ Treg cells while expanding $ICOS^{lo}$ anti-tumor $CD8^+$ T cells in mouse tumor models. This anti-ICOS antibody showed anti-tumor efficacy on its own and showed synergistic effects with anti-PDL1 antibody in mice.

Thus, it seems feasible to expand effector T cells while depleting Treg populations in tumor microenvironment. It is yet to be seen if there is a way to increase Treg cells while suppressing pathological effector T cells in autoimmune diseases and allergies.

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