The role of inducible T-cell costimulator (ICOS) in anti-cancer immunity

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Abstract

Inducible T-cell costimulator (ICOS) is a costimulatory receptor important for the activation and function of T cells. In the clinic, agonists for ICOS are being explored as a cancer therapeutic with the goal of stimulating T cells to eliminate cancer cells. However, conflicting findings in the field suggest that the role of ICOS in cancer has not been fully elucidated. While some reports showed ICOS enhancing the anti-tumor activity of effector T (Teff) cells, others demonstrated that ICOS facilitated pro-tumor regulatory T (Treg) cell immunosuppression. In this study, we aimed to investigate the role of ICOS in different T cell subsets using a murine model of metastatic melanoma. We first found that ICOS deletion in both Teff and Treg cells did not impact tumor burden at terminal stages of tumor progression. In contrast, selective deletion of ICOS in Treg cells reduced tumor-infiltrating Tregs and improved tumor control. This impact of ICOS on tumor-infiltrating T cells is established at early stages of tumor progression and is maintained into terminal stages of tumor growth. Single-cell transcriptomic analysis of tumor-infiltrating T cells revealed that when ICOS was deleted in all T cells, there was a trending reduction in Treg activation and expression of suppressive molecules, along with augmented proinflammatory chemokines specifically in $CD8^+$ Teff cells. Interestingly, we also discovered that ICOS deletion reduced genes associated with Th17 cell identity and effector functions. Lastly, flow cytometry revealed an increase in the number of tumor-infiltrating $\gamma\delta$ T cells but not in the number of putative NK cells when ICOS is deficient in all T cells. Our preliminary findings suggest that there are differential requirements for ICOS in Teff versus Treg cell biology, highlighting the need to further investigate the role

of ICOS in cancer to better inform the use of ICOS as a cancer therapeutic target in the clinic.

Résumé

« *Inducible T-cell costimulator* » (ICOS) est un récepteur de co-stimulation important pour l'activation et la fonction des cellules T. Dans un contexte clinique, des agonistes d'ICOS sont considérés comme traitement potentiel contre le cancer dans le but de stimuler les cellules T et d'éliminer les cellules tumorales. Cependant, des résultats de recherche contradictoires dans le domaine suggèrent que le rôle d'ICOS n'a pas été entièrement élucidé. En effet, certaines recherches ont démontré qu'ICOS augmente l'activité anti-tumorale des cellules T effectrices (Teff), alors que d'autres ont conclu qu'ICOS agit sur les cellules T régulatrices (Treg) pro-tumorales pour promouvoir l'immunosuppression. Le but de cette étude est de déterminer le rôle d'ICOS chez différents sous-types de cellules T en utilisant un modèle murin de mélanome métastatique. Nous avons premièrement élucidé que la déplétion d'ICOS à la fois chez les cellules Teff et Treg n'a pas d'influence sur la charge tumorale dans les stades terminaux de développement du cancer. Inversement, l'absence d'ICOS seulement chez les cellules Treg diminue leur infiltration dans la tumeur et augmente le contrôle de la charge tumorale. La diminution des cellules Treg infiltrant la tumeur est observée dès les stades précoces de croissance tumorale et est maintenue jusqu'aux stades terminaux. L'analyse transcriptomique unicellulaire des cellules T infiltrant la tumeur a révélé que lorsque ICOS est absent chez toutes les cellules T, il y a une tendance à la réduction de l'activation des cellules Treg et de l'expression des molécules suppressives par celles-ci, ainsi qu'une augmentation des chimiokines pro-inflammatoires chez les cellules T CD8 effectrices. Nous avons également découvert que la déplétion d'ICOS diminue l'expression des gènes associés à l'identité et à la fonction des cellules T de type Th17.

Finalement, la cytométrie en flux a révélé une augmentation du nombre de cellules T γδ infiltrant la tumeur, mais un nombre stable de cellules NK quand ICOS est supprimé de toutes les cellules T. Nos résultats préliminaires suggèrent que le rôle d'ICOS dans la biologie des cellules Teff et Treg est distinct et qu'une compréhension approfondie du rôle d'ICOS dans le contexte du cancer est cruciale pour l'utilisation efficace d'ICOS comme cible thérapeutique dans la clinique.

List of Acronyms and Abbreviations

Ag	Antigen
AML	Acute myeloid leukemia
APC	Antigen-presenting cell
B7-H6	B7-homolog 6
Bcl-2	B-cell lymphoma 2
Bcl-6	B-cell lymphoma 6
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	Cluster of differentiation
CNS2	Conserved non-coding sequence 2
CTL	Cytotoxic T-lymphocyte
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
CX3CR1	C-X3-C motif chemokine receptor 1
CXCL	C-X-C motif ligand
CXCR	C-X-C motif chemokine receptor
DC	Dendritic cell
DN	Double negative
DP	Double positive
EDTA	Ethylenediaminetetraacetic acid
EOMES	Eomesodermin
FasL	Fas ligand
FOXP3	Forkhead box P3
GC	Germinal centre
ICAM	Intercellular adhesion molecule
ICOS	Inducible T-cell costimulator
ICOSL	Inducible T-cell costimulator ligand
IFN	Interferon
IL	Interleukin
IL-2R	Interleukin-2 receptor
IL-12R	Interleukin-12 receptor
ILC	Innate lymphoid cell
ink	Invariant natural killer T cell
IPEX	Immunodysregulation polyendocrinopathy enteropathy X-linked
KIR	Killer Ig-like receptor
LAG3	Lymphocyte-activation gene 3
Lck	Lymphocyte-specific protein tyrosine kinase
LFA-1	Lymphocyte function-associated antigen 1
MDSC	Myeloid-derived suppressor cell

MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MICA	MHC class 1 polypeptide-related sequence A
MICB	MHC class 1 polypeptide-related sequence B
NCR	Natural cytotoxicity receptor
NK	Natural killer
OVA	Ovalbumin
NOD	Non-obese diabetic
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PD-1	Programmed cell death protein 1
pDC	Plasmacytoid dendritic cell
PI3K	Phosphoinositide 3-kinase
PLCγ1	Phospholipase C-gamma 1
RPM	Revolutions per minute
SHM	Somatic hypermutation
SNN	Shared nearest neighbour
TBK1	TANK (TRAF family member-associated nuclear factor-κ-B activator) binding kinase 1
Tconv	Conventional T
TCR	T cell receptor
Teff	Effector T
Tfh	T follicular helper
TGF-β	Transforming growth factor beta
Th	T helper
TME	Tumor microenvironment
TNF	Tumor necrosis factor
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
TRAIL-R	Tumor necrosis factor-related apoptosis-inducing ligand receptor
Treg	Regulatory T
UMAP	Uniform manifold approximation and projection
VEGF-A	Vascular endothelial growth factor A

Author Contributions

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

Dr. Woong-Kyung Suh conceived and supervised the study. Experiments were designed by **Joanna Li** and **Dr. Woong-Kyung Suh**. **Joanna Li** performed most of the experiments including mouse dissections, tissue preparations, cell culture, and flow cytometry. **Joanna Li**, **Vincent Panneton**, **Jinsam Chang**, **Antoine Bouchard**, **Nikoletta Diamantopoulos**, and **Saba Mohammaei** genotyped the mice used in this study. Intravenous injections were performed by animal technicians at the IRCM animal facilities. Flow cytometry data were analyzed by **Joanna Li** with input from **Dr. Woong-Kyung Suh**. Single cell RNA sequencing was performed by **Joanna Li** and the IRCM Molecular Biology platform with advice from **Vincent Panneton** and **Dr. Woong-Kyung Suh**. **Nikoletta Diamantopoulos** analyzed the single cell sequencing data with input from **Joanna Li**, **Antoine Bouchard**, and **Dr. Woong-Kyung Suh**. **Antoine Bouchard** assisted in the translation of the English abstract into French, while **Nikoletta Diamantopoulos** contributed to the Methods section for single cell transcriptome analysis. All figures were generated by **Joanna Li** with input from **Dr. Woong-Kyung Suh**, with the exception of Figure 1.1 which previously appeared in a publication co-authored by **Joanna Li**.[1]

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Chapter 1: Introduction

1.1 The cancer immune response

1.1.1 Overview

The immune system was first discovered to be involved in cancer control in the 19th century by William Coley, who is now often referred to as "the Father of Immunotherapy". Through investigating 18th century medical records and literature, he identified a correlation between patients with group A Streptococcus skin infections and better prognosis in soft tissue sarcomas.[2] He subsequently discovered the importance of immune stimulation in tumor control by injecting heat-inactivated *S. pyogenes* and *Serratia marcescens* bacteria into cancer patients, which cured multiple patients long-term.[3] Subsequent work by Paul Ehrlich culminated in the idea of cancer immunosurveillance, which hypothesized that the immune system is crucial for the repression of neoplasia.[3] Since discovering the link between the immune system and cancer in the 19th and 20th century, we now understand not only the importance of innate and adaptive immune cells in recognizing and destroying transformed cells, but also the ability of immune cell subsets that could inadvertently promote tumor growth and facilitate tumor escape.[4] The following sections will provide a succinct summary of key adaptive and innate immune cells involved in anti- and pro-tumor immunity, as well as discuss some emerging immune subsets that may play a key role in the cancer immune response.

1.1.2 CD4$^+$ T cell help in the context of anti-tumor immunity

CD4$^+$ conventional T cells are cells that express T cell receptors (TCR) with α and β chains that recognize peptide antigens (Ags) presented in the context of major histocompatibility complex class II (MHC-II) on antigen-presenting cells (APCs).[5] This subset of cells in the adaptive immune system is named for its expression of the CD4 co-receptor, which is important for stabilizing the TCR:peptide-MHC-II interaction by binding to the β2 domain of MHC-II.[5] CD4$^+$ T cells are derived from the thymus, where thymocytes with TCRs that have intermediate affinity for self-peptides are positively selected.[5] In the periphery, naïve CD4$^+$ T cells can differentiate into different specialized helper T (Th) subsets depending on the strength of the TCR and costimulatory signals, as well as the availability of cytokines unique to each subset.[6] In the context of cancer, it has been shown that human CD4$^+$ T cells are activated upon recognition of a variety of tumor Ags, including MHC-II restricted tissue-specific Ags, Ags derived from viral genes that cause tumor transformation, as well as peptides that arise as a result of gene mutations or fusion in tumor cells.[7] For example, human melanoma cells can elicit a CD4$^+$ T cell response due to the recognition of mutant neo-epitopes by human CD4$^+$ T cells.[8]

The key function of CD4$^+$ T cells in anti-tumor immunity is to provide help to CD8$^+$ cytotoxic T lymphocytes (CTLs).[9,10] Early studies where tumor antigen-specific CD4$^+$ T cells were adoptively transferred or depleted in tumor-bearing mice revealed that CD4$^+$ T cells were required for the effector phase of anti-tumor responses in tumors that do not express MHC-II, which is the case for most cancer cell types.[11-13] Subsequent studies point to a critical role of activated CD4$^+$ helper T cells in supporting the activation, differentiation, and effector functions of CTLs in cancer.[9,10] One of the mechanisms of

$CD4^+$ T cell help for CTLs is the licensing of dendritic cells (DCs) to efficiently cross-present endocytosed tumor Ags on MHC-I. This licensing process relies on the interaction of $CD4^+$ TCR with Ag-MHC-II complexes on DCs, as well as the interaction of CD40L on $CD4^+$ T cells with CD40 on DCs.[14] These interactions and associated downstream signaling then upregulate the expression of MHC-I on DCs, allowing $CD8^+$ T cells to interact with DCs.[15] This mechanism of $CD4^+$ T cell help to CTLs requires both $CD4^+$ and $CD8^+$ T cells to recognize linked epitopes, often presented by the same APC.[16] In addition to augmented cross presentation, DC interaction with $CD4^+$ T cells can also lead to increased Ag presentation, costimulatory ligands such as B7.1 and B7.2 expression, and the functional maturation of DCs.[14] These processes are dependent on the interaction between CD40L expressed on $CD4^+$ T cells with CD40 expressed on DCs, as antibody-based stimulation of CD40 could bypass the requirement of $CD4^+$ T cell help to elicit potent CTL responses.[17] Ligation of CD40:CD40L can also increase the co-stimulation of CD70 on DCs with CD27 on CTLs, an interaction essential for the survival and effector function of CTLs by upregulating IL-12R and IL-2R on CTLs.[18]

Studies have shown that CTLs that have received help are better able to migrate into the tumor and exert functional responses. For example, the interaction of $CD4^+$ T cells with DCs induces DCs to make CCL3/4 to attract $CCR5^+$ CTLs to the site of interaction.[19] CTLs that arise in the context of $CD4^+$ help also express higher levels of CXCR4 and CX_3CR1 which promotes CTL migration into the tumor.[20] Furthermore, within the tumor microenvironment (TME), $CD4^+$ T cells can release IL-2 to activate and sustain CTL proliferation.[21] Finally, helped CTLs are shown to have augmented effector cytokine production such as IFN-γ and TNF-α,[22] and they also have improved cytotoxic functions,

as evident in the higher expression level of granzyme B, as well as decreased levels of PD-1 and LAG3 co-inhibition in helped CTLs.[20]

While $CD4^+$ T cells play an important role in supporting CTLs in cancer, they also have anti-tumor impacts on other immune subsets. Th1 cells are a key $CD4^+$ Th subset involved in anti-tumor immunity via the production of type 1 cytokines. Th1 cells arise upon the reception of IL-12 from DCs, and their lineage is delineated by the expression of the transcription factor T-bet.[23] Th1 cells are an important source of IFN-γ, which has the ability to directly cause tumor senescence.[24] IFN-γ can also support natural killer (NK) cell mediated lysis of cancer cells by increasing the expression of intercellular adhesion molecule 1 (ICAM-1) on tumor cells, strengthening the interactions between NK cells and target tumor cells.[25] In addition, Th1-derived IFN-γ is shown to activate macrophages to eliminate tumor cells and enhance CTL effector functions in a melanoma model.[26]

Lastly, some studies have shown that $CD4^+$ T cells can confer cytotoxic activity by killing target cells in an MHC-dependent manner. In a model of established melanoma, $CD4^+$ T cells were able to directly recognize tumor Ags presented on the surface of tumor cells expressing MHC-II, while conferring cytolytic activity via the release of cytotoxic granules akin to CTL cytotoxicity.[27,28]

1.1.3 $CD8^+$ cytotoxic T lymphocytes (CTLs) in anti-tumor immunity

$CD8^+$ CTLs were first discovered to be a key anti-tumor cell type when researchers found a correlation of better tumor control in patients with higher number of tumor-infiltrating $CD8^+$ T cells.[29] Subsequent studies have confirmed that CTLs play an important anti-tumor role, mainly through tumor cell cytotoxicity upon recognition of tumor-

associated and tumor-specific Ags presented on MHC-I.[30] First, $CD8^+$ T cells arrive in the tumor guided by chemokine gradients such as CCL3, CCL4, as well as CXCL9 and CXCL10.[19] In the TME, $CD8^+$ T cells can interact with $CD103^+$ DCs via TCR:peptide-MHC-I interactions, as well as CD28 and CD27 on $CD8^+$ T cells with B7.1/B7.2 and CD70 on DCs, respectively.[31,32] This interaction can prime or re-stimulate $CD8^+$ T cells that may have previously encountered helped DCs in the draining lymph node.[31,32]

Upon $CD8^+$:DC interactions, the ligation of CTL adhesion molecules and integrins to tumor ligands, CTLs can direct the exocytosis of lytic granules to the site of CTL:tumor cell interaction (the immunological synapse).[33] Some key adhesion molecules and integrins involved in this process include LFA-1 and CD103, which bind to ICAMs and E-cadherin expressed on tumor cells, respectively.[34,35] The ligation of these molecules is important for polarizing pre-formed cytoplasmic granules to the membrane of CTLs.[34,35]

CTL granules are mainly composed of two main classes of proteins: perforin, which is responsible for forming pores on tumor cells,[36] and granzymes, notably granzyme A and B, which are serine proteases that enter tumor cells to initiate apoptosis.[37] Perforin is particularly critical for the anti-tumor effector activity of CTLs, as perforin-deficient mice have uncontrollable tumor growth despite having intact granzyme-mediated cytotoxicity.[38] The mechanism of granzyme cytotoxicity relies on programmed cell death initiation through the cleavage of downstream caspases and pro-apoptotic Bcl-2 family proteins.[37] It is interesting to note that the loss of specific granzymes does not necessarily lead to higher tumor incidence in mice, likely due to the redundancy in function of granzyme family proteins.[39]

In addition to granule-mediated cytotoxicity, CTLs can also eliminate tumor cells through the expression of death-associated ligands interacting with death receptors on tumor cells. Known death ligands expressed by CTLs include FasL and TRAIL, which interact with Fas and TRAIL-R1/2 expressed by tumor cells, respectively.[40] The receptor-ligand interaction initiates the downstream activation of procaspases, which activates apoptotic programs in tumor cells. Mice with deficiency in the death receptor TRAIL-R are more susceptible to tumorigenesis,[41] highlighting the key role of death ligands in CTL anti-tumor immunity.

Lastly, CTLs have been shown to have indirect anti-tumor immunity through the release of IFN-γ. Autocrine IFN-γ production is important for enhancing CTL motility, survival, as well as cytotoxic functions.[42] In addition, there is some evidence suggesting that CTL-derived IFN-γ is crucial for tumor angiostasis, as tumor-bearing mice transferred with $CD8^+$ T cells from IFN-γ-deficient mice fail to control lung metastases due to increased angiogenesis.[43]

1.1.4 NK and NKT cells as key anti-tumor innate immune cell types

Natural killer (NK) cells, often considered as the innate counterpart to CTLs due to their potent cytotoxic activity, have been known to mediate important innate type immunity against tumor cells.[44] NK cells are a subset of innate lymphoid cells (ILCs) and are classified as innate immune cells because they do not express CD3 or TCR, and they arise from common lymphoid progenitors in the bone marrow similar to other subsets of ILCs.[45] NK cells mainly rely on the cytokines IL-2 and IL-15[46] as well as transcription factors such as EOMES[47] for survival and development.

Unlike CTLs, NK cells are not antigen specific but rather rely on integrating signals from a host of activating and inhibitory receptors to identify target cells. Activating receptors from NK cells can receive signals from MHC-I related proteins such as MICA and MICB, which binds to the receptor NKG2D[48,49], as well as non-MHC-I molecules such as heparan sulfate glycosaminoglycans, which binds to natural cytotoxicity receptors (NCRs) such as NKp46, 44, and 30.[50-52] In contrast, NK inhibitory receptors such as types of killer Ig-like receptors (KIRs) that transduce inhibitory signals can interact with MHC-I to prevent NK targeting of healthy, non-transformed cells.[53]

There are two main models in which NK cells can be activated to kill tumor cells: the “missing self” and the “stress-induced self” model. In the “missing self” model, NK cells become activated due to the reception of activating signals without the delivery of an inhibitory signal because MHC-I is lost in tumor cells.[54] Since many tumor cells often lack or express low levels of MHC-I, the “missing self” model likely identifies a significant mechanism of NK cell activation in cancer.[55] Recent studies have proposed the “stress-induced self” model to describe situations where NK-mediated cytotoxicity is intact despite target tumor cells still expressing MHC-I. The “stress-induced self” model hypothesizes that NK cells can target tumor cells due to the reception of an overwhelming amount of stimulation through stress-induced ligands, which are upregulated in tumor cells. The NKG2D receptor in NK cells play a key role in this model by receiving activating signals from MICA and MICB, which are proteins that are upregulated in human tumor cells compared to normal cells.[56] Studies have observed increased tumor incidence in murine spontaneous tumor models with NKG2D deficiency, highlighting the importance of NKG2D and the “stress-induced self” model in NK tumor immunosurveillance.[57] Other

receptors in the NCR family such as NKp30 may also play an important role in the "stress-induced self" model by delivering an activating signal, as NKp30 binds specifically to B7-H6, a molecule uniquely expressed on tumor cells but is absent on non-transformed cells in humans.[58]

Upon activation, NK cells employ similar mechanisms of anti-tumor cytotoxicity as CTLs, such as the use of perforin and granzymes as well as Fas-FasL interactions.[59,60] In addition to direct cytolysis, NK cells are also potent early producers of IFN-γ, which can have apoptotic effects through caspase signaling initiation at high doses.[61,62] NK-derived IFN-γ may additionally have an anti-angiogenic effect by reducing endothelial cell numbers and interacting with stromal fibroblast to downregulate vascular endothelial growth factor A (VEGF-A), promoting tumor ischemia.[63,64] Lastly, there is evidence supporting NK-derived IFN-γ inducing M1-polarized macrophages and increasing macrophage phagocytosis, which inhibits tumor growth.[65]

In addition to NK cells, NKT cells have also increasingly been demonstrated to play key roles in anti-tumor immunity independently of NK cells. For example, in multiple cancers, the presence of NKT cells correlated with better patient prognosis.[66,67] Despite sharing part of its name with NK cells, NKT cells, the most well-characterized of which is the type 1 invariant NKT (iNKT) cells, are in fact a subset of T cells. This is because NKT cells express TCR specific to lipid antigens presented in the context of CD1d, which is an MHC-I-like molecule.[68] Upon recognition of the lipid-CD1d complex, NKT cells can exert a variety of functions against tumor cells. For one, NKT cells possess direct cytotoxic potential against tumor cells via the perforin-granzyme B pathway[69] as well as through Fas-FasL interactions.[70] Furthermore, NKT cells can modulate innate and adaptive

immune cells via rapid cytokine release. NKT cells are capable of secreting both type 1 and type 2 cytokines to recruit NK cells, macrophages, and CTLs, as demonstrated in mouse models where the injection of α-galactosylceramide, a synthetic glycolipid, led to enhanced anti-tumor immunity.[71] Lastly, NKT cells have been shown to promote the expression of MHC-II and costimulatory molecules on DCs,[72] as well as stimulate the secretion of chemokines that attract CTLs from DCs through interacting with CD1d expressed on immature DCs.[73] Taken together, this suggests that NKT cells are an important anti-tumor subset with unique mechanisms of tumor rejection.

1.1.5 Treg immunosuppression in pro-tumor immunity

The discovery of regulatory T (Treg) cells originates from its identification as a $CD4^+CD25^+$ T cell subset that plays important roles in self-tolerance and the prevention of autoimmunity.[74] Later studies were able to identify the transcription factor forkhead box P3 (Foxp3) as a master transcriptional regulator of Treg lineage. This was discovered through the identification of humans with Foxp3 mutations, who have a disease termed immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) characterized by severe autoimmune diseases.[75] In addition, mice with Foxp3 mutations (scurfy mice) are known to be Treg deficient and present with autoimmunity, suggesting that Foxp3 expression plays critical roles for Treg cells.[76,77]

There are two main types of Treg cells. Thymic Tregs (tTreg) are $CD4^+$ T cells that become a distinct T cell subset, having been positively selected in the thymus due to having moderately high affinity for self-peptide/MHC without being negatively selected.[78] On the other hand, peripheral Tregs (pTreg) are generated from $CD4^+$ Tconv cells in the

periphery, often with Ag specificities against non-self Ags such as those derived from the microbiota.[79]

Treg cells were first discovered to be involved in cancer immunity when it was discovered that tumor-bearing mice given anti-CD25 antibodies, which mostly depleted $CD25^+$ Treg cells, had a reduction in tumor burden along with improved anti-tumor immunity.[80,81] Further studies in humans confirmed that a high Treg:Teff ratio in the TME was associated with poor patient prognosis in multiple cancers.[82] In addition, Treg cells made up 10-50% of $CD4^+$ T cells in the tumor.[83] The high frequency of Tregs could be due to pTregs being induced in tumor sites, where latent TGF-β binding to tumor surface integrins such as αvβ8 can activate TGF-β expressed by pTregs. This promotes pTreg generation through *cis* interaction with the TGF-β receptor.[84] Taken together with results from early tumor studies in mice, this suggests that tumor-infiltrating Treg cells could be exerting immunosuppressive functions against anti-tumor immune cells within the TME.

Treg cells utilize a diverse variety of immunosuppressive mechanisms to exert its effector functions. For one, Treg cells express high levels of the coinhibitory receptor CTLA-4, which binds to B7.1 and B7.2 on APCs with higher affinity than CD28, effectively preventing T cell activation.[85] The CTLA-4-B7.1/B7.2 complex can also be trogocytosed by Treg cells, which limits the costimulatory signals available to Tconv cells.[86] The importance of CTLA-4 in Treg function is highlighted in mice whose Tregs lack CTLA-4, which led to impaired Treg immunosuppression accompanied with augmented anti-tumor immunity due to hyper-proliferative Tconv cells.[87]

In addition to the expression of coinhibitory receptors, Treg cells are also potent producers of the cytokines IL-10 and TGF-β, which appear to have immunosuppressive

effects. Although the mechanism of IL-10 and TGF-β immunosuppression remains unclear, the loss of IL-10$^+$ Treg cells is associated with increased disease severity in mouse models of type 1 diabetes.[88] This suggests that Treg-derived IL-10 indeed has important suppressive functions. On the other hand, TGF-β secreted by Treg cells has been demonstrated to be required for autoimmune colitis prevention.[89] And in tumor models, TGF-β receptor deficient CD8$^+$ T cells were more resistant to Treg suppression, leading to increased tumor rejection and CTL cytotoxicity in the mice.[90] This further suggests that Treg-derived TGF-β is involved in Treg immunosuppression.

Aside from adaptive Tconv cells, Treg cells also appear capable of suppressing innate immune cell types such as NK cells. Treg suppression of NK cells appears largely mediated through the TGF-β pathway. Studies have shown that adoptively transferred Treg cells in tumor-bearing mice inhibited NKG2D-mediated cytolysis by NK cells, a process that was dependent on the presence of membrane-bound TGF-β.[91] In addition, TGF-β neutralizing antibodies added *in vitro* to NK-Treg co-cultures restored NK cell cytotoxicity of tumor cells,[92] confirming the requirement of TGF-β in suppressing NK activity. Other mechanisms of NK suppression by Treg cells include direct cytolysis of NK cells via granzyme B release,[93,94] or by limiting the availability of IL-2 to NK cells and inhibit NK cell proliferation because Treg cells express high levels of the IL-2R subunit CD25.[74,95]

Recent studies have suggested that Treg cells may also be important in the suppression of pathogenic $\gamma\delta$ T cells. For example, mice with defective Treg cells had an increase in the activation of IL-17$^+$, colitis-inducing $\gamma\delta$ T cells.[96] However, adoptive transfer of healthy Treg cells was able to inhibit pathogenic $\gamma\delta$ T cell activation, proliferation, and

cytokine production.[96] Additional studies in bacterial infection models also demonstrated that $CD4^+CD25^+$ Tregs can inhibit IFN-γ production specifically in γδ T cells,[97] further solidifying the ability of Treg cells to suppress γδ T cells.

1.1.6 Controversial players in tumor immunity: Th17 cells

Th17 cells are a subset of $CD4^+$ Th cells that are induced by the polarizing cytokines IL-1, IL-6, IL-21, and TGF-β.[98-100] Th17 cells are characterized by the master transcription factor RORγt and the production of the cytokines IL-17A, IL-17F.[100] The role of Th17 cells in cancer has been controversial in recent years, with studies proposing both anti- and pro-tumor roles of Th17 that seem to depend on the type of cancer model studied.

Studies that suggest Th17 cells act as a mostly pro-tumor subset largely focus on the pro-angiogenic effects of its effector cytokine IL-17A.[101] There is evidence suggesting that IL-17A can promote tumor proliferation by stimulating fibroblasts to upregulate the expression of VEGF.[102] In addition, in a lung adenocarcinoma model, IL-17 was shown to promote the expression of IL-6, IL-8 and VEGF in the tumor tissue which enhanced tumor angiogenesis.[103] Other studies focused on immune cells that are impacted by IL-17 production. One study demonstrated that in a model of breast cancer, IL-17 could recruit pro-tumorigenic neutrophils to the TME.[104] In addition, neutralizing IL-17 by IL-17 blockade was able to slow tumor progression due to neutrophil migration being prevented.[104]

On the other hand, correlational studies examining patient prognosis with Th17 infiltration found that having higher levels of IL-17A and RORγt gene expression,

indicating activated Th17 cells, correlated with better patient prognosis and longer disease-free survival in colorectal cancer and oropharyngeal squamous carcinoma.[105,106] A proposed mechanism of action includes the ability of IL-17 to synergize with IFN-γ to induce tumor cell production of CXCL9 and CXCL10, which could enhance the migration of $CD8^+$ Teff and NK cells to the TME.[107] IL-17 also appears to modulate macrophage activation, inducing macrophages to produce IL-12 which may promote the production of anti-tumor CTLs.[108] Furthermore, IL-17F has been suggested to play an anti-angiogenic role in hepatocellular carcinoma, where IL-17F transfected tumor cells had reduced production of angiogenic factors that corresponded with decreased tumor size when tumor cells were transplanted into mice.[109] Altogether, the two lines of evidence demonstrate the controversial nature of Th17 in cancer immunity and highlight the need for more definitive studies to precisely parse out its role.

1.1.7 Controversial players in tumor immunity: γδ T cells

γδ T cells are a subset of T cells whose TCR is composed of γ and δ chains in contrast to the α and β chains found in Tconv cells.[110] Unlike Tconv cells, γδ T cells recognize Ags in a non-MHC restricted manner and are potent secretors of cytokines upon activation.[110] Due to its ability to recognize a broad spectrum of stress-induced molecules, including tumor-associated stress proteins, γδT cells have become attractive targets in adoptive cell therapies to treat cancer.[110] However, whether γδ T cells play a mainly anti- or pro-tumor role remains to be elucidated.

Studies that propose an anti-tumor role of γδ T cells tend to focus on the human Vγ9Vδ2 T subset, which has been shown *in vitro* to lyse cancer cells through granule

cytotoxicity after recognizing MICA, MICB, and phosphorylated Ags accumulated on cancer cells.[111] The Vγ9Vδ2 subset was also shown to express death ligands such as FasL and TRAIL, as well as innate cytotoxicity markers associated with NK cells.[112] In addition, blocking TRAIL via antibodies *in vitro* inhibited Vγ9Vδ2 killing of colon cancer stem cells,[112] suggesting the importance of death ligands as a mechanism of Vγ9Vδ2 anti-tumor immunity. In a model of neuroblastoma, the Vδ2 subset was also found to kill cancer cells via antibody-dependent cytotoxicity, which requires the expression of CD16 on Vδ2 T cells.[113] While murine γδ T cells do not have a Vγ9Vδ2 subset that recognizes phosphoantigens like the human γδ subset, studies have identified in murine skin cancer models the importance of IFN-γ-producing γδ T cells in enhancing anti-tumor immunity. For example, in a melanoma model where γδ T cells were depleted then reconstituted specifically with the Vγ4 γδ T cell subset, mice achieved better tumor control that was dependent on the potent production of IFN-γ and perforin from this population.[114]

On the other hand, studies suggesting that γδ T cells play a key pro-tumor role largely focus on the pro-tumor effector functions of the γδ T17 subset, which is characterized by its potent secretion of IL-17.[110] Similar to IL-17A production from Th17 cells, IL-17 derived from γδ T17 has been shown to promote angiogenesis in various cancers by inducing VEGF production.[115] In addition, IL-17 from γδ T17 attracted myeloid-derived suppressor cell (MDSC) accumulation in human colorectal cancer, favoring tumor progression.[116] Other studies examining metastasis suggested that γδ T17-derived IL-17 can expand pro-tumor neutrophils, which in turn can suppress CTLs and the anti-tumor Vγ9Vδ2 subset in humans.[117]

Taken together, it appears that the role of γδT cells in cancer may be quite diverse, with different subsets having opposing effector functions in the cancer immune response. Further studies aiming to understand the role of γδ T cells in cancer should focus on elucidating the subsets of γδ T cells present in each tumor model in mice vs. in humans to determine their impact on tumor progression.

1.2 Inducible T-cell costimulator (ICOS)

1.2.1 Structure and expression pattern of ICOS and ICOSL

Inducible T-cell costimulator (ICOS) is a CD28 family costimulatory receptor first discovered in activated human T cells.[118] It is composed of an extracellular immunoglobulin domain, a transmembrane glycoprotein, as well as a cytoplasmic tail (**Figure 1.1**).[1] On the cell surface, ICOS is formed as a sulphide bond-linked homodimer.[1] ICOS is an inducible receptor that is expressed upon TCR ligation, with CD28 costimulation further upregulating its expression, hence the name “inducible” costimulator.[119] The availability of ICOS costimulation is tightly regulated, as ICOS ligation with its only known ligand, ICOS ligand (ICOSL), leads to the internalization of ICOS from the cell surface to prevent T cell hyperactivation.[120] ICOSL can also be shed from the cell surface upon ICOS:ICOSL ligation, a process that is dependent on ADAM family metalloproteinases.[120,121]

ICOS is found to be expressed mainly on T cells, especially on T follicular helper (Tfh) cells, a subset of $CD4^+$ T cells that are involved in humoral immune responses.[1] However, ICOS can also be expressed at varying levels in other activated T cell subsets

such as Th1, Th2, Th17, Treg, and $CD8^+$ CTLs.[1] ICOS can also be found on innate immune cells such as γδ T cells, NK cells, and innate lymphoid cells (ILCs).[122-125]

ICOSL is a B7 family costimulatory ligand whose members also include the CD28 ligands B7.1 and B7.2.[126] ICOSL is predominantly expressed on APCs and B cells to stimulate T cell activation,[127] similar to B7.1 and B7.2. However, studies have also found ICOSL expression in non-hematopoietic cells such as alveolar epithelial cells[128] and podocytes[129], suggesting that ICOS:ICOSL costimulation has unique roles compared to CD28:B7.1/B7.2 costimulation, as B7.1/B7.2 molecules are exclusively presented on APCs. Uniquely in ILCs, ICOS and ICOSL can both be constitutively expressed and are crucial for ILC homeostasis and function,[124,125] which challenges the inducible nature of ICOS.

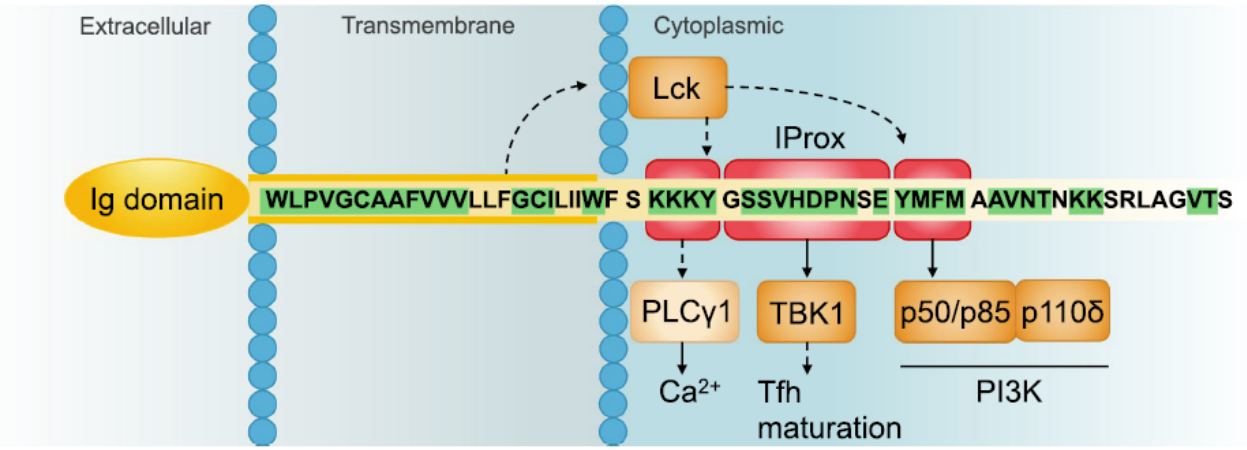


Figure 1.1 Structure of ICOS[1]

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

1.2.2 ICOS signaling mechanisms

ICOS can potentiate 3 distinct signaling pathways through motifs in the cytoplasmic tail: PI3K signaling, PLCγ1-mediated Ca^{2+} flux, and TBK1-mediated signaling (**Figure 1.2**).[1] First, the induction of ICOS-PI3K signaling relies on the key cytoplasmic tail residues Tyr181 in the SH2-binding YMFM motif. Knockin mice with a point mutation that leads to a Tyr181 to Phe substitution at this residue abrogates PI3K activation, without affecting other ICOS-mediated signaling pathways.[130] The ability of ICOS to activate PI3K and downstream signaling is much more potent than CD28, suggesting that ICOS plays a unique costimulatory role in T cell activation in addition to CD28 effects. Second, ICOS has been shown to mobilize intracellular Ca^{2+} to potentiate PLCγ1-mediated Ca^{2+} flux initiated by TCR engagement, a process that is dependent on the KKKY motif on its cytoplasmic tail. Point mutations that lead to the substitution of Tyr170 to Phe within the KKKY motif led to a deficiency in ICOS-mediated Ca^{2+} signaling.[131] Lastly, a signaling motif named IProx was described which was crucial for the recruitment of TBK1 and the activation of TBK1-mediated signaling upon TCR engagement and ICOS:ICOSL interaction.[132] However, this signaling pathway in ICOS has only been identified in Tfh cells, with downstream mechanisms not fully clarified.

1.2.3 ICOS deficiency in Tfh-mediated humoral immunity

The most striking immune defect in both ICOS-deficient mice and humans is the defect in humoral immunity due to a lack of Tfh cells.[119,133-135] Studies in Tfh biology suggest that ICOS is critical for multiple aspects of Tfh cell differentiation and effector functions. Tfh cells are a $CD4^+$ T cell subset that play key roles in sustaining germinal

center (GC) reactions. Upon activation, pre-Tfh cells express the master transcription factor Bcl6 as well as upregulate the expression of CXCR5, allowing pre-Tfh cells to migrate into the T-B border following chemokine gradients.[136,137] ICOS is important for Tfh identity as this stage, as ICOS protects the Bcl6 protein from ubiquitin-dependent degradation,[138] and mice with a deficiency in ICOS-PI3K signaling have reduced Tfh populations.[130] In addition to supporting Tfh lineage, ICOS supports the activity of Tfh cells in the GC. ICOS costimulation is required to maintain Tfh motility as they discriminate cognate B cells from bystander B cells in order to deliver crucial survival signals only to cognate B cells.[139] In mice with an abrogation of ICOS-PI3K, T cells were less mobile in lipid bilayers, with a reduced activation of small GTPases that could remodel actin and promote motility.[131] ICOS-PI3K signaling in Tfh cells can additionally promote IL-4 production, a cytokine important for promoting B cell maturation upon T:B interactions.[140] Therefore, recurrent infections in humans with homozygous null mutations in ICOS can be attributed to the abrogation of humoral immunity in the absence of Tfh cells.[135]

1.2.4 Role of ICOS in CD4$^+$ Tconv cells

While most of the literature examining the role of ICOS in CD4$^+$ T cells focuses on its function in Tfh cells, ICOS has nonetheless important roles in CD4$^+$ Tconv subsets such as Th1, Th2 and Th17 cells that may be involved in tumor immunity. To begin, the role of ICOS in Th1 cells is controversial and appear to depend based on the type of immune challenge used. For example, in *Mycobacterium tuberculosis* lung infection, ICOS deficiency led to a lower chronic bacterial burden along with enhanced Th1 immunity in the form of increased IFN-γ$^+$ Th1 cell frequency, suggesting that ICOS

costimulation suppresses Th1 functions.[141] However, this study utilized germline knockout of ICOS, which may impact cells other than Th1 cells leading to the observed increase in Th1 immunity. On the other hand, other infection models using *Salmonella* and *Listeria* showed that ICOS knockout or blockade instead led to a lack of IFN-γ responses from $CD4^+$ T cells, resulting in poor bacterial control.[142,143] These results suggest that ICOS signaling promotes Th1 responses. While the role of ICOS in Th1 cells in the cancer context have not been fully elucidated, recent studies propose a positive correlation between ICOS expression on tumor-infiltrating Th1 cells, enhanced effector functions, and improved survival in colorectal cancer patients.[105] This seems to suggest that ICOS may play an important costimulatory role to support Th1 cell function in cancer, similar to that observed in *Salmonella* and *Listeria* infection models.[144]

In contrast to Th1 cells, ICOS is well-known to promote Th2 immunity. For one, ICOS promotes the expression of the Th2-polarizing cytokine IL-4 and the IL-4 receptor, leading to enhanced Th2 differentiation.[145] The abrogation of ICOS signaling also led to a decrease in the production of Th2 type cytokines.[133,134,146] Due to its importance in supporting Th2 cells, infection models such as *Nippostrongylus brasiliensis,* where Th2 responses are crucial for parasite eradication, showed impaired parasite control and enhanced parasite egg production in ICOS deficient mice.[147]

ICOS is also thought to support the generation and effector functions of Th17 cells.[148] ICOS is a key regulator of c-Maf expression, a transcription factor that is critical for committed Th17 precursor cells to become stable Th17 cells when exposed to the polarizing cytokine IL-23.[149] ICOS-PI3K signaling was also found to be important for anti-chlamydial Th17 responses, as ICOS deficient $CD4^+$ T cells had impaired production of

IL-17 which resulted in difficulty controlling *Chlamydia* lung infections.[150] In tumor models, ICOS seems to potentiate Th17 signature gene expression and led to better tumor control in one study. It was observed in this study that ICOS-activated Th17 cells expressed higher levels of *Rorc* (encoding RORγt), *MAF* (encoding c-Maf), *Il17a* (encoding IL-17A), and *Il21* (encoding IL-21) compared to CD28-activated Th17 due to potent ICOS-PI3K activation.[151] This suggests that ICOS serves important roles for Th17 cells, but since the role of Th17 cells in cancer is controversial, whether ICOS-mediated Th17 responses is anti- or pro-tumor remains to be fully elucidated.

1.2.5 Role of ICOS in $CD8^+$ CTLs

Past studies investigating the role of ICOS in $CD8^+$ T cells suggested that ICOS is important for supporting CTL effector functions.[152] For example, ICOSL expression on tumor cells has been shown to enhance CTL-mediated tumor cytotoxicity, possibly due to enhanced costimulation because of $ICOS^+$ CTLs ligation with ICOSL expressed on tumors.[153] In addition, injecting $ICOSL^+$ tumor cells led to the efficient destruction of these cells by CTLs compared with the injection of $ICOSL^-$ tumor cells.[153] Other studies in infectious models confirm that ICOS is important for CTL functions, as ICOS blockade in *Listeria monocytogenes* infection led to poor CTL-mediated infection control. This was due to reduced numbers of *Listeria*-specific $CD8^+$ T cells that expressed signature effector cytokines.[143] However, further studies that specifically explore the mechanism of ICOS in supporting $CD8^+$ anti-tumor immunity are required, since there is generally a larger focus on the role of ICOS in $CD4^+$ T cells.

1.2.6 Role of ICOS in Treg cells

Multiple studies have collectively proposed that ICOS is critical for the generation, proliferation, and immunosuppressive capacity of Treg cells. 20% of Foxp3$^+$ Treg cells express ICOS, which suggests that ICOS is likely important for Treg cells.[154] Indeed, ICOS deficiency leads to a decrease in number of Foxp3$^+$ Treg cells.[155] A proposed mechanism of how ICOS supports Treg cells is that ICOS maintains Foxp3 expression, since deficiency led to the downregulation of Foxp3 expression along with increased methylation of the Foxp3 conserved non-coding DNA sequence 2 (CNS2) in Tregs.[156]

In addition, ICOS appears to support Treg proliferation, evident from the observation that ICOS$^+$ Tregs have higher expression of the proliferation marker Ki-67 compared to their ICOS$^-$ counterparts.[88] ICOS is also well-established in promoting the survival of Tregs, as ICOS$^+$ Tregs stimulated *in vitro* with anti-CD3 became hyperproliferative, compared to ICOS$^-$ Tregs which died within hours of stimulation.[157] Furthermore, enhanced ICOS expression in aged mice was shown to attenuate the expression of the pro-apoptotic molecule Bim, sustaining effector Treg survival.[158]

Lastly, ICOS$^+$ Treg cells also seem to have increased immunosuppressive capacities. In a non-obese diabetic (NOD) mouse model, the loss of ICOS expression in pancreatic Tregs was correlated with diabetes progression.[88] ICOS^{hi} Treg cells were also correlated with increased expression of the immunosuppressive cytokine IL-10 in some studies.[159,160] Although precise mechanisms in which ICOS supports Treg suppressive activity remains unclear, it nonetheless highlights ICOS as an important marker with functional roles in Treg cells.

1.3 Role of ICOS in cancer immunity and immunotherapy

1.3.1 Overview

The advent of immune checkpoint blockades like anti-CTLA-4 and anti-PD-1 shows promise in curing cancer by lifting the inhibition on anti-tumor Teff cell activation and function.[161] Nonetheless, many patients do not respond to checkpoint blockade therapies, making urgent the need to elucidate the immune mechanisms underlying patient unresponsiveness to improve therapeutic outcomes.[162]

ICOS first became of interest to the field of cancer immunity when it was discovered that bladder and breast cancer patients receiving CTLA-4 blockade showed elevated levels of ICOS-expressing $CD4^+$ and $CD8^+$ T cells, both in circulation and within the tumor.[163,164] Initial work on the role of ICOS in cancer suggested that increased ICOS expression on $CD4^+$ T cells could be an effective, highly-specific biomarker for monitoring the therapeutic response of cancer patients treated with checkpoint blockade therapy.[165] Subsequent work on ICOS in cancer has since consistently demonstrated that ICOS serves a functional role at the cancer immune interface, beyond its usage as a pharmacodynamic biomarker. However, an important point of contention in the field remains centered around whether ICOS plays a mainly anti- or pro-tumor role. ICOS is expressed on both Teff and Treg cells, two immune cell types that are implicated in opposing sides of the cancer immune response, further confounding the understanding of ICOS and its precise role in cancer immunity.

1.3.2 Evidence supporting the anti-tumor role of ICOS

Early evidence supporting the idea that ICOS serves an anti-tumor function came from studies of cancer patients undergoing CTLA-4 blockade, where it was observed that the elevated population of ICOS-expressing $CD4^+$ and $CD8^+$ Teff cells that arose post-therapy were largely IFN-γ-producing in a tumor Ag-specific manner.[163,164] In addition, the ratio of $CD4^+ICOS^{hi}$ Teff to $Foxp3^+$ Treg cells increased post-treatment, concomitant with an increase in overall Teff:Treg ratio.[163] Given the well-documented role of IFN-γ and Th1 Teff cells in promoting tumor eradication,[166,167] it was proposed that ICOS may play an anti-tumor role by enriching the Teff cell compartment and enhancing their anti-tumor functions. This idea was supported in a study using a model of B16/BL6 murine melanoma, where tumor-bearing mice after CTLA-4 blockade had an increased $ICOS^+$ Teff:Treg ratio that corresponded with elevated levels of IFN-γ production from $ICOS^+$ Teff cells and a subsequent reduction in tumor burden.[168] In contrast, mice deficient in ICOS experienced a drastically dampened CTLA-4 therapeutic response compared to their wild-type counterparts, leading to tumor persistence.[168] Thus, it appears that ICOS:ICOSL engagement may be essential for facilitating the anti-tumor response that is resuscitated during checkpoint blockade therapy, supporting the idea that ICOS plays an anti-tumor function.

Additionally, using a tumor cell vaccine engineered to express ICOSL in murine models of melanoma and prostate cancer, it was shown that ICOS costimulation provided concomitant with CTLA-4 blockade can increase $CD4^+$ Teff cell release of proinflammatory cytokines as well as $CD8^+$ T cell-associated granzyme B cytotoxicity.[169] This enhancement in anti-tumor effector functions again correlated with lower tumor

burden and decreased mortality in mice.[169] Similar augmentations in anti-tumor Teff function were also demonstrated in murine breast cancer models treated with oncolytic viruses engineered to express ICOSL.[170] Furthermore, the observed increase in Teff functional capacity is likely because of ICOS-mediated PI3K signaling effects, which has been shown in the context of CTLA-4 blockade therapy to upregulate T-bet expression and IFN-γ production.[171] Combined together, these studies present an anti-tumor view of ICOS, where ICOS costimulation modulates the ratio of anti-tumor and pro-tumor immune cells in the tumor microenvironment, as well as impacting Teff functional capacity in order to boost anti-tumor immunity.

1.3.3 Evidence supporting the pro-tumor role of ICOS

Other works in the cancer immunity field have challenged the idea that ICOS plays a largely anti-tumor role through studies into the impact of ICOS on Treg cells, an important pro-tumor immune cell type. ICOS is known to be highly expressed on Treg cells, where it is critical for Treg homeostasis, proliferation, and function in autoimmune diabetic mouse models.[88,172,173] Similarly, it has been suggested that ICOS may support the pro-tumor immunosuppressive activity of Tregs in cancer. In human melanoma patients receiving high-dose IL-2 therapy, a high expansion of the ICOS$^+$ Treg compartment was observed, which in turn correlated with poor prognosis.[174] In addition, ICOSL expression has been detected in multiple human cancers. In human metastatic melanomas, around 50% of patients expressed ICOSL to a high degree.[175] ICOSL$^+$ human melanoma cells potently activated Tregs *in vitro* by promoting high CD25 and Foxp3 expression as well as increasing the capacity of Tregs to produce the

immunosuppressive cytokine IL-10.[175] *In vivo* ICOSL-blockade in a mouse melanoma model reduced tumor-infiltrating Foxp3$^+$ICOS$^+$ Tregs.[175] Similarly, acute myeloid leukemia (AML) cells in human patients also expressed ICOSL and can expand ICOS$^+$ IL-10 producing Tregs, a subset of cells that concurrently marked reduced overall patient survival in disease-free conditions.[176] Collectively, these studies propose a tumor-intrinsic model of ICOS costimulation whereby tumors exploited ICOS:ICOSL interactions to actively induce regulatory T cells, then hijacked Treg immunosuppressive functions to aid tumor progression.

In addition to a tumor-intrinsic sources of ICOS costimulation through ICOSL expression, studies have also shown that plasmacytoid dendritic cells (pDCs) may serve as a source of ICOSL in the tumor microenvironment to recruit Treg cells. In human breast and ovarian cancer, Foxp3^{hi} Treg cells were the main T cell subset that expressed ICOS.[177,178] ICOS$^+$ Tregs and ICOSL$^+$ pDCs were co-localized in the tumor site, while ICOS-ICOSL interactions between tumor-infiltrating ICOS$^+$ Tregs and ICOSL$^+$ pDCs amplified IL-10 secretion by Treg cells.[177,178] This interaction also correlated with poor patient prognosis, proposing that ICOS costimulation may overall play a pro-tumor role by supporting ICOS$^+$ Treg cells.[177,178]

1.4 Rationale and objectives

ICOS costimulation seems to play an important role in supporting immune cells such as CD4$^+$ and CD8$^+$ Teff cells, which mediate key anti-tumor immune responses. In bacterial infection models, the abrogation of ICOS signaling decreased IFN-γ production from CD4$^+$ T cells, which could potentially limit macrophage-mediated tumor elimination

reliant on type 1 cytokine activation.[143] Studies have also showed that ICOS blockade can limit $CD8^+$ cytotoxicity, a crucial immune mechanism that facilitates tumor rejection.[153] However, immune cell subsets that oppose anti-tumor immunity such as Treg cells are also supported by ICOS costimulation. ICOS deficiency reduced Treg numbers,[155] and $ICOS^+$ Treg cells have better survival and immunosuppressive capacity via IL-10 production in autoimmune models.[158,160] Further studies in immunotherapy reflect this paradoxical role of ICOS in cancer, with some studies suggesting that ICOS-deficient mice respond less robustly to CTLA-4 blockade,[168] while other studies correlated the expression of ICOSL on tumor cells with increased $ICOS^+$ Treg cells in the tumor that may hasten tumor progression.[176] Since many anti-ICOS antibodies, mainly ICOS agonistic antibodies, have been developed and are actively being tested in phase 1 and 2 clinical trials as a novel cancer therapeutic,[179] there is an urgent need to clarify the precise role of ICOS in cancer immunity to achieve the greatest clinical benefits.

Given these conflicting lines of evidence, we hypothesize that ICOS plays a dual role in promoting anti- and pro-tumor T cell subsets, with each subset having a differential requirement for ICOS. We have two main objectives in this study:

1. Investigate the impact of ICOS costimulation in late-stage metastatic tumor burden and immune cell composition.
2. Elucidate the role of ICOS in modulating various anti- and pro-tumor immune cell types at an early stage of tumor progression, when there are dynamic changes occurring in the immune cell compartment.

Chapter 2: Materials and Methods

2.1 Mice and animal procedures

$CD4^{cre}$ (Jax 017336)[180] and $Foxp3^{YFP\text{-}Cre}$ mice (Jax 016959)[181] were purchased from the Jackson Laboratory. ICOS conditional knockout mice were generated in C57BL/6 background as previously described.[182] Mice used were 6-12 weeks of age unless otherwise specified. For $CD4^{cre}$ lines, female mice were used to allow for co-housing. For $Foxp3^{YFP\text{-}Cre}$ lines, male mice were used. All mice were housed in the Institut de Recherches Cliniques de Montréal animal care facility under specific pathogen-free conditions. Animal experiments were performed in accordance with animal use protocols approved by the Institut de Recherches Cliniques de Montréal Animal Care Committee. For intravenous injections, mice were injected in the tail vein with 6×10^5 B16-OVA cells in 200 µL of PBS.

2.2 Cell culture

The B16-OVA (B16 melanoma cell line transfected with ovalbumin cDNA) was obtained from Dr. John Stagg, Université de Montréal.[183] Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 4.5 g/L L-glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 10% heat-inactivated fetal bovine serum (above all from Wisent), 10 mM HEPES, 100 U/mL penicillin, 100 µg/mL streptomycin, and 55 µM 2-mercaptoethanol (from ThermoFisher) at 37 ˚C with 5% CO_2. Cells were passaged every 2-3 days at 1:10 or 1:12 ratio using 0.05% trypsin-EDTA (ThermoFisher). Cells were harvested for injection using 10 mM EDTA (ThermoFisher) in PBS and were used before the 4^{th} passage to prevent genetic drift.

2.3 Flow cytometry

Tumor-bearing lungs were cut into small pieces using scissors, then digested to obtain single cell suspensions in 300 U/mL collagenase type 2 (Worthington Biochemical) and 10 mg/mL DNase I (MilliporeSigma) for 50 minutes at 37 ˚C, with 300 rpm horizontal shaking. Cells were then filtered using 70 μm and 40 μm cell strainers. To assess cell viability, 1 x 10^6 cells were stained in PBS at 1 x 10^7 cells/mL with 1:200 dilution of fixable viability dye eFluor 450 (ThermoFisher) for 15 minutes at 4 ˚C. Anti-CD16/CD32 (1 μg/sample, BioXCell) was used for 5 minutes at room temperature to block non-specific binding mediated by Fc receptors. Surface stainings were performed for 20 minutes at 4 ˚C in 1% bovine serum albumin (Wisent) in PBS. Intracellular stainings were performed using the Transcription Factor Staining Buffer Set (ThermoFisher) to fix and permeabilize cells (45 minutes at 4 ˚C for each of the steps). The following antibodies were used in stainings. ThermoFisher: anti-CD45 APC-eFluor780 (30-F11), anti-CD4 Alexa Fluor 700 (RM4-5), anti-CD8 PerCP-Cyanine5.5 (53-6.7), anti-Foxp3 PE or APC (FJK-16s), anti-NK1.1 APC (PK136). BioLegend: anti-ICOS FITC (7E.17G9) or Brilliant Violet 650 (C398.4A), anti-CD44 Brilliant Violet 605 (IM7), anti-TCR γ/δ FITC (GL3), anti-NKp46 PE (29A1.4). Antibodies were diluted in 1% bovine serum albumin in PBS according to manufacturer's recommended concentrations. Data was acquired on BD LSRFortessa and analyzed using Flowjo version 10 (BD Biosciences).

2.4 Single cell RNA sequencing

A $CD4^{cre}ICOS^{+/+}$ and a $CD4^{cre}ICOS^{f/f}$ female mouse was intravenously injected with B16-OVA cells and euthanized 10 days post-challenge. Tumor-infiltrating T cells

were isolated from the collagenase digested lungs using the EasySep mouse T cell negative isolation kit (StemCell). 15,000 cells from $CD4^{cre}ICOS^{+/+}$ and $CD4^{cre}ICOS^{f/f}$ mice were sent for library preparation. Libraries were generated using the following components from 10x Genomics: Chromium Next GEM Chip G Single Cell kit, Chromium Next GEM Single Cell 3' GEM, Library & Gel Bead kit v3.1, Chromium i7 Multiplex kit. Sequencing was performed by Genome Québec using a NovaSeq 6000 (Illumina) with a flow cell S1 PE28*91.

For the analysis of the single cell expression matrix, dying or dead cells were filtered out of the dataset by eliminating any cells with more than 10% mitochondrial RNA contamination. Cells expressing less than 200 or more than 7500 unique genes, corresponding to empty droplets and multiplets, were also filtered out. The data was then log normalized. The most differentially expressed genes within the dataset were identified, and the data was subsequently scaled. Linear dimensional reduction was then performed using the Principal Component Analysis (PCA) method based on the 2000 variable features. The first 40 most important principal components generated by the PCA were selected to construct a Shared Nearest Neighbor (SNN) graph, and Modularity Optimizer version 1.3.0 was used to identify 26 clusters.[184] Uniform Manifold Approximation and Projection (UMAP), a non-linear dimensional reduction technique, was used to visualize the cells on a 2D space.[185] Clusters corresponding to T cells were identified using the Seurat FindMarkers function, and were separately clustered. 12 T cell clusters were generated and visualised in a 2D UMAP space. The expression of genes of interest were visualized by generating violin plots using the Seurat VlnPlot function,

and statistical significance was assessed via the Wilcoxon rank sum test provided by the Seurat FindMarkers function.

2.5 Statistical analysis

Two-tailed Student *t* test was used to assess statistical significance in single comparisons. For single cell gene expression comparisons between clusters, the Wilcoxon ranked sum test was used. p values were used to determine statistical significance: ns = $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$. Analysis was performed using Prism 7 (GraphPad software).

Chapter 3: Results

3.1 ICOS deletion in Treg cells, but not in all T cells, decreases tumor burden

To investigate the role of ICOS costimulation in cancer immunity, we first sought to analyze the impact of ICOS deletion in all T cells in a murine model of experimental "metastatic" melanoma, where tumor nodules develop following circulating tumor cell seeding in the lung. This model allows us to quantify tumor burden in the lungs in a synchronous manner compared to other metastatic melanoma models, where mice develop spontaneous pulmonary metastases from primary tumors.[186] Control (*Cd4*^{cre}*Icos*$^{+/+}$) and mice lacking ICOS in all T cells (*Cd4*^{cre}*Icos*$^{f/f}$ here after) were challenged intravenously with B16-OVA melanoma cells, then the lung tumor burden as well as tumor-infiltrating immune cells were assessed after 17 days (**Figure 3.1A**). The metastatic tumor burden was similar between control and *Cd4*^{cre}*Icos*$^{f/f}$ mice (**Figure 3.1B**). We then performed flow cytometry analysis of tumor-infiltrating T cell populations by gating on CD45$^+$CD4$^+$ and CD45$^+$CD8$^+$ T cells (**Supp. Figure 3.1**), since only about 1-3% of CD45$^+$CD4$^+$ T and 0.7-2% of CD45$^+$CD8$^+$ T is TCR-β$^+$ (data not shown). We confirmed that ICOS expression is lost on all T cell subsets (**Supp. Figure 3.2A**). In addition, we found that the percentage and number of total CD4$^+$ and CD8$^+$ cells were similar between genotypes (**Figure 3.1C and D**), while the percentages and number of non-Treg CD4$^+$ cells and Treg cells (**Figure 3.1E and F**) were also comparable between control and *Cd4*^{cre}*Icos*$^{f/f}$ mice. Accordingly, the ratio of CD8$^+$ Teff to Treg cells and non-Treg CD4$^+$ to Treg cells was maintained despite the loss of ICOS in *Cd4*^{cre}*Icos*$^{f/f}$ mice (**Figure 3.1G**). This suggested to us that when ICOS costimulation is missing from all T cell subsets,

there could be a global defect in both anti- and pro-tumor T cell functions without changes to the percentage and number of T cells present in the tumor microenvironment. This could have contributed to the net zero difference in the tumor burden between control and *Cd4*^{cre}*Icos*$^{f/f}$ mice.

Given that ICOS deficiency in all T cells did not affect tumor burden, we next sought to analyze the impact of ICOS deletion selectively in the regulatory T cell compartment. Interestingly, when ICOS is selectively deficient in Foxp3$^+$ Tregs while intact on effector CD4$^+$ and CD8$^+$ T cells (*Foxp3*^{cre}*Icos*$^{f/f}$ here after, **Supp. Figure 3.2B**), the tumor burden was drastically reduced compared to control mice that have ICOS-intact Treg cells (*Foxp3*^{cre}*Icos*$^{+/+}$, **Figure 3.2A**). Unexpectedly, there was a decrease in the percentage and number of tumor-infiltrating CD4$^+$ T as well as the number of CD8$^+$ Teff cells (**Figure 3.2B and C**). Nonetheless, the ratio of CD8$^+$ Teff and non-Treg CD4$^+$ to Treg cells was significantly elevated in *Foxp3*^{cre}*Icos*$^{f/f}$ mice (**Figure 3.2G**), suggesting that the relative anti-tumor T cell compartment was augmented in these mice. There was also a small increase in the percentage of non-Treg CD4$^+$ T cells along with a drastic reduction in the percentage and number of Treg cells in *Foxp3*^{cre}*Icos*$^{f/f}$ mice (**Figure 3.2D and E**). In addition, these mice had slightly reduced CD44$^+$ activated Treg populations compared to controls, while the mean fluorescence intensity (MFI) of Foxp3 remained comparable between genotypes (**Figure 3.2F**). This indicated that ICOS deletion in Treg cells decreased Treg numbers and Treg activation. Taken together, this data suggested that in a cancer context, ICOS may play important roles in both Teff and Treg cell biology so that when ICOS is selectively impaired in Treg cells, pro-tumor immunity is dampened allowing anti-tumor immunity to predominate, ultimately improving tumor control.

3.2 The impact of ICOS deletion in Treg cells is established at an early stage of tumor growth

Since we found that ICOS deletion in all T cells did not seem to alter T cell profiles at a late stage of tumor progression, we next sought to understand the impact of ICOS on T cells at an earlier stage of tumor growth, when there are dynamic changes taking place in the immune cell compartment. We challenged mice with B16-OVA cells, then assessed tumor-infiltrating immune cell profiles 10 days post-challenge when tumor nodules were beginning to become visible in the lung (**Figure 3.3A**). Interestingly, we saw similar trends in the profiles of tumor-infiltrating Teff and Treg cells at this stage compared to later stages of tumor growth. Notably, there were no significant differences in the percentage and number of $CD4^+$ and $CD8^+$ Teff cells (**Figure 3.3B and C**), as well as in the percentage and number of non-Treg $CD4^+$ T and Treg cells between control and *Cd4*^{cre}*Icos*$^{f/f}$ mice (**Figure 3.3D and E**). In addition, the ratio of $CD8^+$ Teff and non-Treg $CD4^+$ to Treg cells remained similar between genotypes (**Figure 3.3F**). This suggests that the impact of ICOS deficiency may impair both Teff and Treg cells even at early stages of tumor progression, and this defect is likely maintained into the later stages of tumor growth.

To confirm that ICOS deficiency impairs T cells at an early stage of tumor progression, we next analyzed the T cell profiles of control and *Foxp3*^{cre}*Icos*$^{f/f}$ mice at an early stage of tumor growth. We observed similar trends as mice in the late stage of tumor progression, including the reduction of tumor-infiltrating $CD4^+$ and $CD8^+$ Teff percentage and numbers (**Figure 3.4A and B**), as well as a small increase in non-Treg $CD4^+$ percentage along with a drastic reduction in Treg cells (**Figure 3.4C and D**). There was

also an augmentation in the ratio of $CD8^+$ and non-Treg $CD4^+$ Teff cells to Treg cells in the *Foxp3^{cre}Icos$^{f/f}$* mice (**Figure 3.4F**). Unlike mice at late stages of tumor progression, there was no significant differences in the activation status of Treg cells in control vs. *Foxp3^{cre}Icos$^{f/f}$* mice at the early stages of tumor growth (**Figure 3.4E**). This data highlights the importance of ICOS in maintaining potent Treg cell activation as tumor cells multiply, likely enhancing the pro-tumor immunosuppressive capacities of Treg cells and favoring tumor progression.

3.3 Single cell transcriptomic analysis reveals augmented expression of proinflammatory chemokines in $CD8^+$ Teff cells

Since the tumor-infiltrating T cell percentages and numbers appear similar at later stages of tumor growth (17 days post-challenge) compared to an earlier stage (10 days post-challenge), we sought to elucidate whether there are alterations in Teff and Treg cell function against cancer cells when ICOS is lost. We performed single cell transcriptomic analysis of tumor-infiltrating T cells in an ICOS-intact control mouse (*Cd4^{cre}Icos$^{+/+}$*) vs. a mouse with ICOS-deficient T cells (*Cd4^{cre}Icos$^{f/f}$*) at an early stage of tumor growth (10 days post-challenge). Tumor-infiltrating T cells were isolated using a pan-T cell isolation kit which achieved about 50% enrichment. UMAP projections revealed that control and *Cd4^{cre}Icos$^{f/f}$* mice have similar clusters, with no new T cell clusters uniquely present in either control or *Cd4^{cre}Icos$^{f/f}$* mice (**Figure 3.5A**). In addition, non-Treg $CD4^+$, $CD8^+$ Teff cells and Treg cells all formed distinctive clusters, with clusters 0, 2, and 5 encompassing non-Treg $CD4^+$ cells, clusters 1, 3, and 6 encapsulating $CD8^+$ Teff cells, while cluster 7 identified Treg cells (**Figure 3.5A and B**). Furthermore, non-Treg $CD4^+$ cells in clusters 2

and 5, $CD8^+$ Teff cells in cluster 6, as well as the Treg cell cluster 7 identified activated T cells due to their high expression of *Cd44*, encoding the protein CD44, and low expression of *Sell*, encoding the protein CD62L (**Figure 3.5C**).

We next analyzed the functional capacities of $CD8^+$ Teff cells in control vs. $Cd4^{cre}Icos^{f/f}$ mice. We saw a trend of increased *Gzmb* and *Prf1* expression, genes encoding for proteins granzyme B and perforin respectively, in the activated $CD8^+$ Teff cluster (cluster 6) in $Cd4^{cre}Icos^{f/f}$ mice compared to other $CD8^+$ clusters. However, there was no statistically significant trend in the expression of key genes associated with $CD8^+$ Teff functions in the activated $CD8^+$ Teff cluster (**Figure 3.5D**). This indicated that $CD8^+$ T effector functions might not be completely reliant on the presence of ICOS costimulation.

We next investigated whether ICOS deletion had an impact on the expression of signature genes associated with Treg cell functions. We observed that Treg cells expressed a trend of lower *Cd44* and *Ctla4* expression in ICOS-deficient mice, while other functional genes such as *Foxp3, Pdcd1, Tgfb1,* and *Il10* were comparable in their expression in control vs. $Cd4^{cre}Icos^{f/f}$ mice (**Figure 3.5E**). This suggested that Treg cells in these ICOS-deficient mice may be slightly less activated and less suppressive compared to their ICOS-intact counterparts. Interestingly, we also observed an increase in the expression of *Ccl5* and *Ccr5* in the activated $CD8^+$ Teff cell cluster, genes that encode the proinflammatory chemokine CCL5 and CCR5 (the receptor for CCL5), respectively (**Figure 3.5F**). CCL5 is an important chemokine that attracts immune cells to proinflammatory sites such as the tumor microenvironment.[187,188] Thus, the elevated expression of *Ccl5* alongside its receptor in $CD8^+$ Teff cells may indicate that $CD8^+$ Teff cells are better able to both secrete chemokines and express receptors that target them

to the tumor microenvironment, allowing them to exert their anti-tumor functions. However, further studies are required to determine if this is a result of the indirect effect of ICOS deficiency in the Treg compartment, or a direct impact of ICOS deficiency in $CD8^+$ Teff cells.

3.4 ICOS deletion decreases the expression level of key genes in Th17 cells

We next aimed to identify additional $CD4^+$ Teff subsets that may be impacted by ICOS deletion. Interestingly, we observed that cluster 5 appeared to have decreased numbers in *Cd4*^{cre}*Icos*$^{f/f}$ mice compared to controls (**Figure 3.5A**). The cluster is identified to be Th17 cells based on the expression of *Rorc*, which encodes the Th17 master transcriptional regulator RORγt, as well as Th17 signature cytokine genes *Il17a* and *Il17f* (dotted lines in **Figure 3.6A**). In addition, we observed that in the *Cd4*^{cre}*Icos*$^{f/f}$ mouse, there was a significant reduction in the expression level of *Rorc*, *Il17f*, and *Maf,* which encodes the transcription factor c-Maf (**Figure 3.6B**). c-Maf is known to play key roles in the development of Th17 cells and is also highly regulated by ICOS costimulation.[148,149] Taken together, this suggests that Th17 cells could be impaired both in their numbers as well as their effector functions in the absence of ICOS. While the role of Th17 cells in tumor immunity has been controversial, its decrease in ICOS-deficient T cells warrants further investigation into its effects on tumor progression.

3.5 Elevated γδ T cells in tumor-challenged ICOS-deficient mice

Since we observed a trend of decreased Treg immunosuppression in tumor-challenged *Cd4*^{cre}*Icos*$^{f/f}$ mice, we sought to identify possible cell subsets that may be altered as a result. We identified a putative IL-17-producing γδ T cell cluster, cluster 4, that appeared to be decreased in numbers in *Cd4*^{cre}*Icos*$^{f/f}$ mice (**Figure 3.5A, 3.6A, and 3.7A)**. However, subsequent flow cytometry analysis showed an increase in the percentage and number of total γδ T cells in mice at early stages of tumor growth (**Figure 3.7B**). This suggests that γδ T cells may be a population that can thrive in the absence of potent Treg immunosuppression. This data also highlights that any potential differences in single cell transcriptomic analysis needs to be validated by flow cytometry using replicates.

We next observed a small cluster of putative NK cells, identified by their co-expression of *Klrb1c* (encoding NK1.1) and *Ncr1* (encoding NKp46), which appeared to have increased in numbers in *Cd4*^{cre}*Icos*$^{f/f}$ mice (**Figure 3.5A, 3.7C**). However, flow cytometry analysis revealed that the percentage and number of NK1.1^{+}NKp46^{+} cells were similar between control and *Cd4*^{cre}*Icos*$^{f/f}$ mice at the early stage of tumor growth (**Figure 3.7D**). Taken together, this data raises the need for further analysis into specific NK or other NK1.1^{+}NKp46^{+} subsets such as NKT cells to identify possible populations that could be impacted by impaired Treg immunosuppression. In addition, the alteration in non-T or non-innate cell compartments should be tested in mice lacking ICOS only in Treg cells.

Chapter 4: Discussion

In our study, we observed that mice with Treg-specific ICOS deficiency had a reduction in tumor burden compared to controls, a phenotype that was not observed in mice with ICOS-deficient T cells. We hypothesized that in *Foxp3*^{cre}*ICOS*$^{f/f}$ mice, Treg cells could be preferentially reduced leading to augmented Teff anti-tumor immunity. Interestingly, we discovered that *Foxp3*^{cre}*ICOS*$^{f/f}$ mice concurrently had reduced Teff cell frequency and number not only at the terminal stages of tumor progression but also at an earlier stage. One plausible explanation for this observation could be that $CD4^+$ and $CD8^+$ Teff cells are more effectively exerting effector functions as they have the support of intact ICOS costimulation, unlike Teff cells in *CD4*^{cre}*ICOS*$^{f/f}$ mice. As a result, the total number of Teff cells may be reduced even at 10 days post-tumor challenge because Teff cells may have already responded in a robust manner to tumor challenge. If this is indeed the case, future studies that profile Teff cell numbers in *Foxp3*^{cre}*ICOS*$^{f/f}$ mice at timepoints earlier than 10 days post-challenge may be able to observe an increase in the Teff compartment. Another explanation for the observed decrease in Teff cells in *Foxp3*^{cre}*ICOS*$^{f/f}$ mice could be due to the transient upregulation of Foxp3 in 10-20% of T cells after activation, leading to a loss of ICOS in these T cells.[189,190] This could contribute to the decrease in Teff cells specifically in *Foxp3*^{cre}*ICOS*$^{f/f}$ mice that is not seen in *CD4*^{cre}*ICOS*$^{f/f}$ mice, where ICOS is deleted in all T cells during thymic development as opposed to post-activation. Future studies where T cells are adoptive transferred into tumor-bearing recipient mice could help us understand whether Teff cells expressing Foxp3 upon activation is indeed relevant in our tumor model.

A caveat in our study of T cells in the tumor is that the entire tumor-bearing lung, as opposed to individual tumor nodules, was digested for immune cell profiling. As a result, we cannot confirm whether the T cells in our flow cytometry data were all inside the tumor nodules. To better refine our analysis, lung immune cell populations in non-tumor-bearing mice need to be examined in comparison to tumor-bearing mice in order to uncover changes unique to the tumor microenvironment. In addition, immunofluorescence microscopy techniques to discern the location of $CD4^+$ and $CD8^+$ Teff cells in relation to Treg and cancer cells should be conducted. Imaging would allow us to identify if Teff cells in *Foxp3*^{cre}*ICOS*$^{f/f}$ mice could be more specific to tumor Ags and/or better localized inside tumor nodules to efficiently eradicate tumor cells compared to *CD4*^{cre}*ICOS*$^{f/f}$ mice.

We also noted that while Treg cells in *Foxp3*^{cre}*ICOS*$^{f/f}$ mice were significantly reduced in number leading to an augmented Teff:Treg cell ratio, there were no significant changes in the Treg numbers or Teff:Treg ratio in *CD4*^{cre}*ICOS*$^{f/f}$ mice which also lost ICOS. Nonetheless, our single cell transcriptomic data revealed trending decreases in the expression of genes associated with Treg activation and effector function in *CD4*^{cre}*ICOS*$^{f/f}$ mice. This could implicate that ICOS may impact Treg biology in other areas such as proliferation, survival, recruitment, or any combination of these aspects. Previous studies have demonstrated that ICOS can have an impact on Treg proliferation, with $ICOS^+$ Tregs demonstrating augmented Ki-67 expression.[88] ICOS expression has also been correlated with reduced expression of the pro-apoptotic molecule Bim in Treg cells, supporting Treg survival.[158] Moreover, autoimmune mouse models demonstrated that ICOS costimulation can increase the expression of CXCR3 on Treg cells, which can bind to ligands such as CXCL9 and CXCL10 to direct Treg migration into inflammatory sites.[173] These studies

provide evidence that ICOS could impact Treg cells in the *CD4^{cre}ICOS$^{f/f}$* mice without overt changes in frequency compared to control mice.

In addition, our single cell transcriptome analysis revealed that CD8^{+} Teff cells in *CD4^{cre}ICOS$^{f/f}$* mice expressed higher levels of the chemokine genes *Ccl5* (encoding CCL5) and *Ccr5* (encoding the CCL5 receptor CCR5). Given that Treg cells are important suppressors of CD8^{+} T cells, the augmentation in chemokine profiles could be an indirect indication of impaired Treg suppression in the absence of ICOS, despite the maintenance of Treg numbers. The CCR5/CCL5 axis has been demonstrated to be important for Teff cell recruitment into tumors[187,188] and CCL5 has been proposed as an adjuvant for cancer immunotherapy.[191] In our *CD4^{cre}ICOS$^{f/f}$* model, this could suggest that tumor-infiltrating ICOS-deficient CD8^{+} Teff cells may be better able to follow proinflammatory chemokine gradients and migrate into tumor sites, while also secreting the same chemokine to recruit more CD8^{+} Teff cells to the TME. However, we cannot definitively confirm from our analysis of *CD4^{cre}ICOS$^{f/f}$* mice whether this augmented chemokine profile was due to a direct impact of ICOS deficiency in CD8^{+} Teff cells or an indirect effect of ICOS deficiency in the Treg department. Thus, a future study to examine the transcriptomes of tumor-challenged control and *Foxp3^{cre}ICOS$^{f/f}$* mice can better reveal the quality of both Teff and Treg cells when ICOS is selective lost in Tregs, a model in which tumor control was improved. This study would help us uncover the increased immune forces that may be present when Tregs lose ICOS.

Interestingly, our single cell transcriptome analysis demonstrated a reduction in the expression level of genes associated with Th17 identity and effector functions. This suggested that Th17 responses are impaired in our model of *CD4^{cre}ICOS$^{f/f}$* tumor-

challenged mice. However, whether the observed decrease in Th17-associated genes was due to the intrinsic lack of ICOS on Th17 cells or the lack of Treg immunosuppression in the *CD4^{cre}ICOS$^{f/f}$* mice remains unclear. On one hand, ICOS is known to support precursor Th17 cell commitment to the Th17 lineage and the production of the effector cytokine IL-17.[149] On the other hand, Treg cells have also been shown to suppress Th17 functions, including the secretion of IL-17 and IL-22 by Th17 cells.[192] Therefore, to parse out these contributing factors to the Th17 defect, experiments should be conducted specifically in *Foxp3^{cre}ICOS$^{f/f}$* mice to investigate Th17 activation and effector functions without impacts from ICOS deficiency in Th17 cells. Moreover, the impact of this Th17 defect on tumor control in our *CD4^{cre}ICOS$^{f/f}$* mice is unclear due to the controversial nature of Th17 cells and IL-17 in cancer immunity. Some studies propose that IL-17A derived from Th17 cells can induce fibroblasts in the TME to produce angiogenic factors, thus favouring tumor growth.[102] Other studies suggest that IL-17 could activate macrophages to produce IL-12, enhancing CTL function and therefore augmenting anti-tumor immunity.[108] Interestingly, we identified in our study a reduction in *Il17f* (encoding the cytokine IL-17F) but not *Il17a* (encoding IL-17A) expression in the Th17 cluster of *CD4^{cre}ICOS$^{f/f}$* mice. Given that a study has proposed an anti-angiogenic effect of IL-17F,[109] we hypothesize that the population of Th17 cells in *CD4^{cre}ICOS$^{f/f}$* mice could have been playing a mostly pro-tumor role. Further studies that can specifically deplete Th17 cells or the IL-17A and IL-17F cytokines separately in *CD4^{cre}ICOS$^{f/f}$* mice would help us better understand the contribution of this T cell subset to overall tumor control.

While screening for potential cells that might be impacted by impaired Treg suppression in *CD4^{cre}ICOS$^{f/f}$* mice, we discovered an increase in the frequency and

number of γδ T cells. This increase is likely not an intrinsic effect of ICOS deficiency in the γδ T subset, because while there are rare subsets of $CD4^+$ γδ T cells, most γδ T cells arise separately from conventional αβ T cells before the double negative 3 (DN3) precursor stage in the thymus, prior to the onset of CD4 and CD8 expression.[193,194] Thus, γδ T cells in *CD4^{cre}ICOS$^{f/f}$* mice would not have been impacted by Cre recombinase activity in *CD4^{cre}ICOS$^{f/f}$* mice and likely still expressed ICOS. As a result, the increase in γδ T numbers could be due to a reduction in function of ICOS-deficient Teff or Treg cells. Studies in the field suggest that Treg suppression is the likely mechanism implicated here, as autoimmune colitis models showed that pathogenic γδ T activation and cytokine production can be repressed by Treg cells.[96] To confirm this implication in our model, *Foxp3^{cre}ICOS$^{f/f}$* mice should be used to observe the impact of ICOS-deficient Treg cells on γδ T cells without possible contributions from ICOS-deficient Teff cells. With regards to the identity of the γδ T cells in our model, we were able to identify a γδ T population in our single cell transcriptomic data with *Il17a* and *Il17f* expression. Thus, we suspected that the increased γδ T population in the *CD4^{cre}ICOS$^{f/f}$* mice could in part be made up of γδ T17 cells that secrete IL-17. The role of γδ T17 cells in cancer, similar to Th17 cells, remains controversial. Studies suggest that different γδ T subsets could confer either anti- or pro-tumor immunity. For example, the human Vγ9Vδ2 T subset has been shown to have cytotoxic activity against cancer cells *in vitro*,[111] while γδ T17 cells tend to be viewed as a pro-tumor immune subset that favor tumor growth via IL-17 release to recruit MDSCs into the TME.[116] Therefore, to understand whether γδ T cells impacts tumor growth in our *CD4^{cre}ICOS$^{f/f}$* model, we will need to profile precisely which murine γδ T cell subset(s) and associated effector functions are involved in the tumor immune response.

Furthermore, we observed in our single cell transcriptome analysis what appeared to be an augmented population of NK cells that co-expressed the genes *Klrb1c* (encoding the protein NK1.1) and *Ncr1* (encoding the protein NKp46), which led us to hypothesize that these NK cells could be enhanced in number when Treg suppression is impaired by ICOS deficiency. However, flow cytometry analysis revealed that the frequency and number of NK1.1^{+}NKp46^{+} NK cells were similar between control and *CD4^{cre}ICOS$^{f/f}$*. Nevertheless, whether the functional capacity of these cells is impaired by intrinsic ICOS deficiency remains unclear. Studies have shown that some activated NK cells express CD4, which would mean that NK cells in our *CD4^{cre}ICOS$^{f/f}$* model could also have lost ICOS.[195] In addition, a recent study has revealed that NK cell activation, effector functions such as IFN-γ and granule-mediated cytotoxicity are dependent on ICOS costimulation.[123] Then, if ICOS is deficient in these putative NK cells in the *CD4^{cre}ICOS$^{f/f}$* mice, it could result in a reduced anti-tumor response from NK cells.

Moreover, the NK1.1^{+}NKp46^{+} subset could include NKT cells that also express these markers.[196] Importantly, NKT cells would be ICOS deficient in our *CD4^{cre}ICOS$^{f/f}$* model, as NKT cells develop independently from Tconv cells in the thymus at the CD4^{+}CD8^{+} double positive (DP) stage after TCR:CD1d engagement.[193] A study using a model of airway hyperreactivity also demonstrated that ICOS:ICOSL interaction is important for CD4^{+} iNKT cell function, and germline ICOS knockout mice lacked IFN-γ, IL-4, and IL-10 production specifically from iNKT cells.[197] Taken together, this could suggest that while the number of NK1.1^{+}NKp46^{+} cells remained similar in control vs. *CD4^{cre}ICOS$^{f/f}$* mice, the effector functions of NK and/or NKT cells could be significantly impaired. NK and NKT cells both play important anti-tumor roles: NK cells utilize cytotoxic

mechanisms such as perforin/granzyme B release and death receptor expression,[59,60] while NKT cells produce large amounts of IFN-γ to support the function of anti-tumor cells such as macrophages and CTLs.[71] Therefore, there could be an overall reduction of NK- and NKT-mediated tumor rejection in *CD4^{cre}ICOS$^{f/f}$* mice. Ultimately, this offers one possible explanation why the tumor burden is similar in control vs. *CD4^{cre}ICOS$^{f/f}$* mice, since both anti-tumor subsets like NK and NKT cells as well as pro-tumor Treg function are dampened, resulting in a net zero effect on tumor burden. Future experiments that utilize the *Foxp3^{cre}ICOS$^{f/f}$* model will help us discern the impact of ICOS^{+} Treg on NK and/or NKT cell responses. Additional studies that can parse out the impacts of ICOS deficiency on different NK and NKT subsets and their associated functions in *CD4^{cre}ICOS$^{f/f}$* mice would better help us solidify our findings.

Nonetheless, it is important to point out a caveat in our single cell transcriptomic analysis of γδ T and NK cells, as these cells should have been removed by the T cell negative selection kit used to isolate T cells for the study. Thus, the γδ T and NK cells identified in our single cell transcriptomic data may not be representative of all relevant γδ T and NK cell populations within the tumor. To obtain pure populations of these immune cells from tumor-bearing mice, future analyses should instead sort out γδ T and NK cells alongside T cells using specific markers of cell lineage.

In sum, our data suggest the idea that ICOS is important for promoting anti-tumor immune subsets such as NK and NKT cells, while augmenting proinflammatory chemokine profiles in CD8^{+} Teff cells. However, ICOS also supports pro-tumor immune subsets like Treg cells, so the impacts of ICOS deficiency seen in anti-tumor immune cells could be due either to intrinsic ICOS defects or indirectly through impaired Treg

responses. In addition, ICOS deficiency can have impacts on subsets of immune cells that have controversial roles in cancer immunity, including Th17 and γδ T cells. As a result, augmented tumor control is only observed in mice with a Treg-specific ICOS deficiency as Treg cells become defective without influencing anti-tumor immune subsets that also rely on ICOS.

Chapter 5: Concluding Remarks

The aim of the study presented in this thesis was to elucidate the role of ICOS in cancer immunity. Current studies have largely focused on the impact of ICOS in anti-tumor immune cells vs. in pro-tumor immune cells, with strong evidence suggesting that ICOS could play a dual role in these opposing cell types. Using a murine model of metastatic melanoma, we showed that when ICOS is lost on all T cells, the tumor burden is similar to that of control mice. However, when ICOS is specifically deficient in Treg cells, we observed improved tumor control. Our single cell transcriptome analysis in mice with ICOS-deficient T cells indeed revealed that ICOS deficiency could have impacts on anti-tumor immune cells such as $CD8^+$ T, NK, and/or NKT cells, while leading to a defective pro-tumor Treg population. We also uncovered that ICOS could have effects in cell types that play a controversial role in cancer, such as Th17 and $\gamma\delta$ T cells. Further studies are required to determine whether these impacts of ICOS deficiency could be due to intrinsic defects caused by loss of ICOS in the T cell subsets, or indirect effects because of reduced Treg immunosuppression. Nonetheless, this study could have important implications in the use of anti-ICOS antibodies in clinical trials for cancer. In particular, the dual role of ICOS could mean cancers with high Teff and NK cell infiltration will benefit much more from agonistic therapies, while cancers with high Treg content given the same treatment may not confer similar benefits. Ultimately, this underlies the importance of fully elucidating the role of ICOS in cancer immunity to design novel therapeutics most effectively.

Figures and Figure Legends

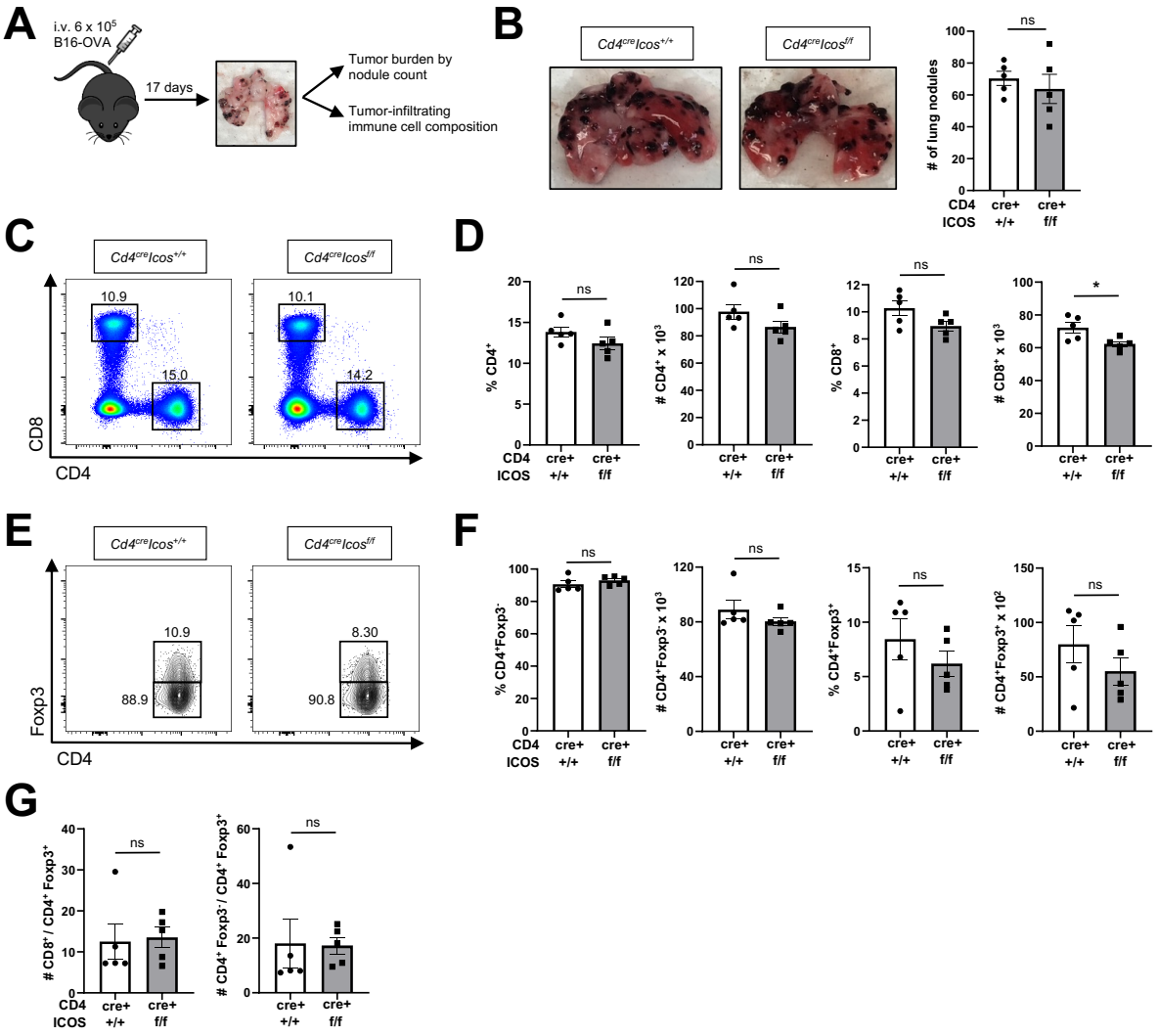


Figure 3.1 No significant differences in tumor burden and tumor-infiltrating T cell populations in T cell-specific ICOS deficient mice. (A) Experimental setup for murine model of metastatic melanoma. (B-G) 9- to 12-weeks old female *Cd4*^{cre}*Icos*$^{+/+}$ and *Cd4*^{cre}*Icos*$^{f/f}$ mice (n = 5 each) that have been co-housed for at least 2 weeks were challenged with tumor cells as in (A). (B) Representative images of lung tumor burden in *Cd4*^{cre}*Icos*$^{+/+}$ versus *Cd4*^{cre}*Icos*$^{f/f}$ mice, and statistical analysis of lung nodule count. (C-G) Flow cytometry analysis of tumor-infiltrating immune cells as obtained from mouse lungs in (B). (C) Representative flow cytometry plots of $CD4^+$ and $CD8^+$ cells pre-gated on $CD45^+$ cells. (D) Statistical analysis of $CD4^+$ and $CD8^+$ cell percentage and numbers. (E)

Representative flow cytometry plots of non-Treg ($CD4^+Foxp3^-$) and Treg ($CD4^+Foxp3^+$) cells pre-gated on $CD4^+$ cells. (F) non-Treg $CD4^+$ and Treg cells percentage and numbers. (G) Ratio of $CD8^+$ effector T cells to Treg cells and non-Treg $CD4^+$ cells to Treg cells. Data shown as mean with error bars denoting SEM, ns = not statistically significant. *p<0.05. Data is representative of three independent experiments.

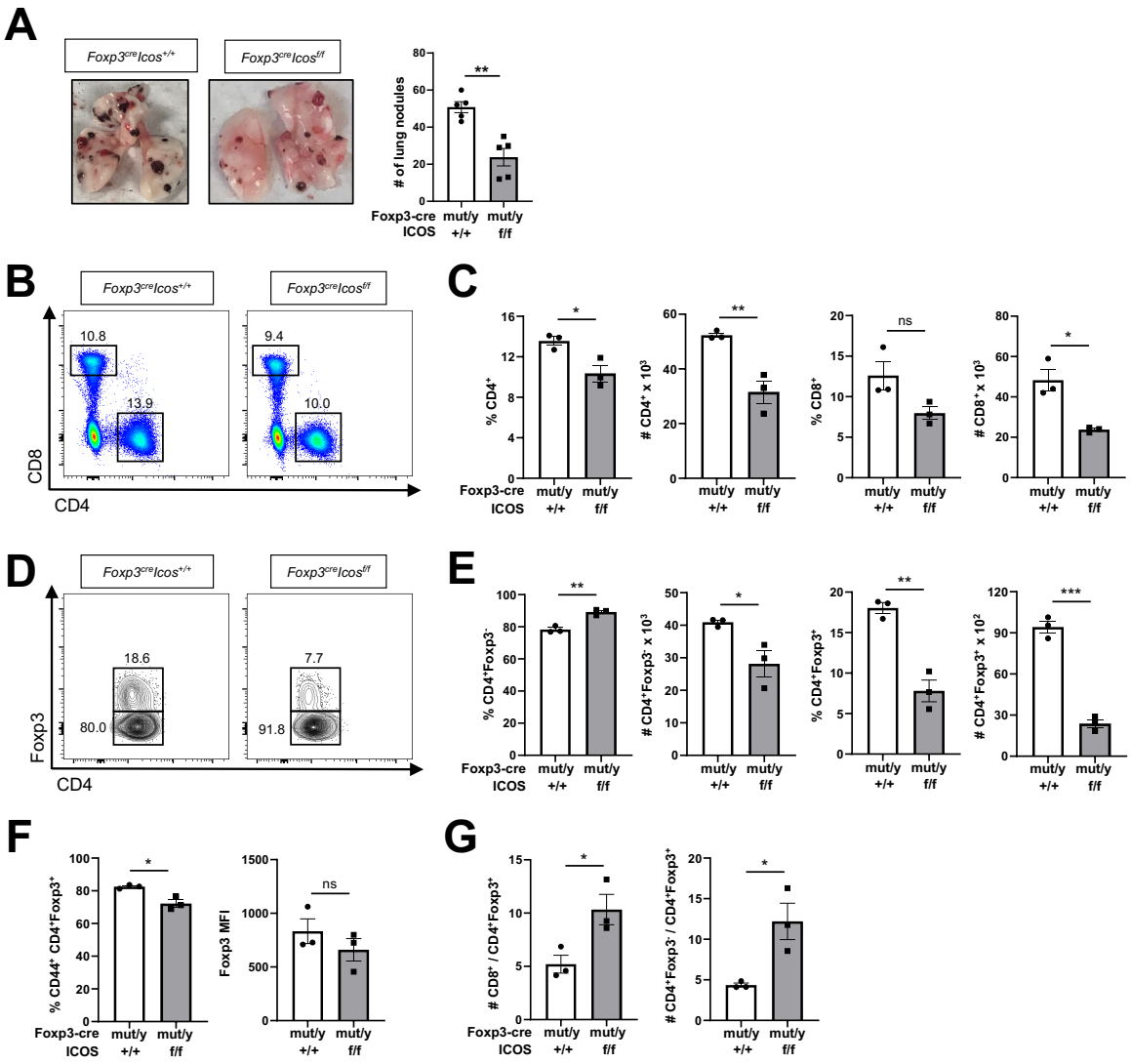


Figure 3.2 Reduced tumor burden and augmented Teff:Treg ratio in Treg-specific ICOS deficient mice. 8- to 11-weeks old *Foxp3*$^{YFP\text{-}cre}$*Icos*$^{+/+}$ (abbreviated as *Foxp3*^{cre}*Icos*$^{+/+}$) and *Foxp3*$^{YFP\text{-}cre}$*Icos*$^{f/f}$ (abbreviated as *Foxp3*^{cre}*Icos*$^{f/f}$) male mice were challenged with tumor cells as in Figure 3.1A (n = 5 each). (A) Representative images of lung tumor burden in *Foxp3*^{cre}*Icos*$^{+/+}$ vs. *Foxp3*^{cre}*Icos*$^{f/f}$ mice, and statistical analysis of lung nodule count. (B-G) Flow cytometry analysis of tumor-infiltrating immune cells as obtained from mouse lungs in (A). (B) Representative flow cytometry plots of CD4$^+$ and CD8$^+$ cells pre-gated on CD45$^+$ cells. (C) Statistical analysis of CD4$^+$ and CD8$^+$ cell percentage and numbers. (D) Representative flow cytometry plots of non-Treg (CD4$^+$Foxp3$^-$) and Treg (CD4$^+$Foxp3$^+$) cells pre-gated on CD4$^+$ cells. (E) non-Treg CD4$^+$

and Treg cells percentage and numbers. (F) Percentage of activated Treg ($CD44^+CD4^+Foxp3^+$) cells pre-gated on $CD4^+$ cells and Foxp3 mean fluorescence intensity (MFI) in Treg cells. (G) Ratio of $CD8^+$ effector T cells to Treg cells and non-Treg $CD4^+$ cells to Treg cells. Data shown as mean with error bars denoting SEM, *p<0.05, **p<0.01, ***p<0.001. Data is representative of at least two independent experiments.

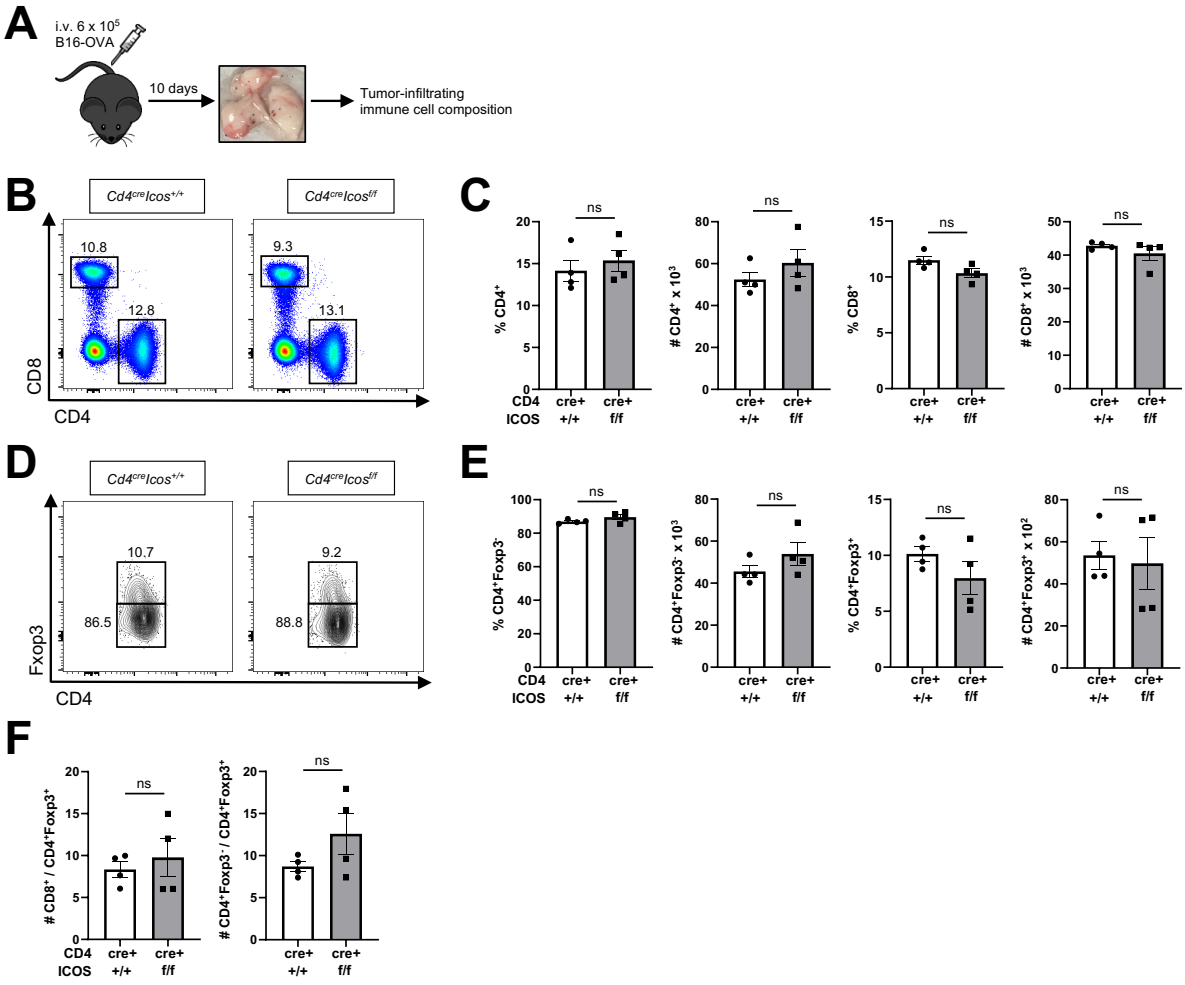


Figure 3.3 No significant differences in tumor-infiltrating T cell populations in T cell-specific ICOS deficient mice at early stage of tumor progression. (A) Experimental setup for murine model of metastatic melanoma. (B-G) 9- to 12-weeks old female *Cd4*^{cre}*Icos*$^{+/+}$ and *Cd4*^{cre}*Icos*$^{f/f}$ mice (n = 4 each) that have been co-housed for at least 2 weeks were challenged with tumor cells as in (A). (B-F) Flow cytometry analysis of tumor-infiltrating immune cells obtained from tumor-challenged mouse lungs. (B) Representative flow cytometry plots of CD4$^+$ and CD8$^+$ cells pre-gated on CD45$^+$ cells. (C) Statistical analysis of CD4$^+$ and CD8$^+$ cell percentage and numbers. (D) Representative flow cytometry plots of non-Treg (CD4$^+$Foxp3$^-$) and Treg (CD4$^+$Foxp3$^+$) cells pre-gated on CD4$^+$ cells. (E) non-Treg CD4$^+$ and Treg cells percentage and numbers. (F) Ratio of CD8$^+$ effector T cells to Treg cells and non-Treg CD4$^+$ cells to Treg cells. Data shown as mean

with error bars denoting SEM, ns = not statistically significant. Data is representative of at least three independent experiments.

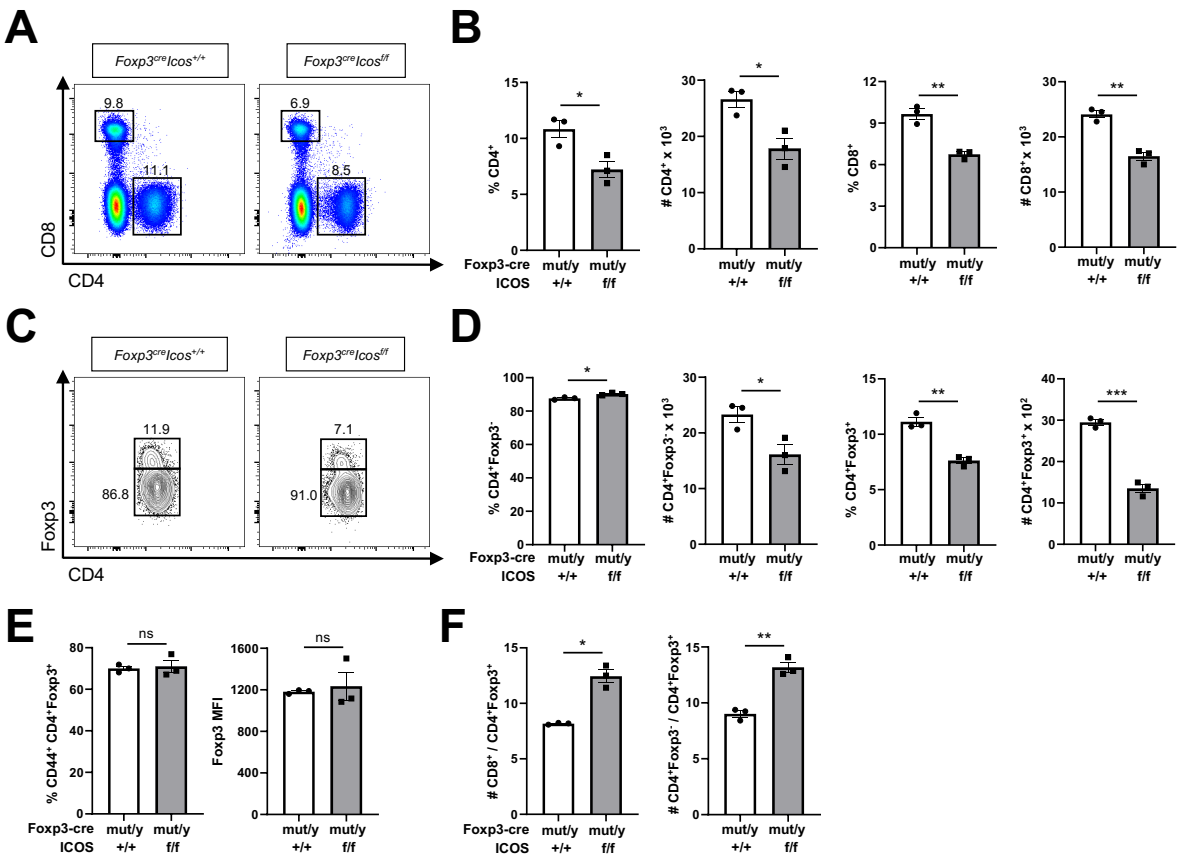


Figure 3.4 Augmented Teff:Treg ratio in Treg-specific ICOS deficient mice at early stage of tumor progression. 10- to 13-weeks old *Foxp3*$^{YFP\text{-}cre}$*Icos*$^{+/+}$ (abbreviated as *Foxp3*^{cre}*Icos*$^{+/+}$) and *Foxp3*$^{YFP\text{-}cre}$*Icos*$^{f/f}$ (abbreviated as *Foxp3*^{cre}*Icos*$^{f/f}$) male mice were challenged with tumor cells as in Figure 3.3A (n = 3 each). (A-F) Flow cytometry analysis of tumor-infiltrating immune cells obtained from tumor-challenged mouse lungs. (A) Representative flow cytometry plots of $CD4^+$ and $CD8^+$ cells pre-gated on $CD45^+$ cells. (B) Statistical analysis of $CD4^+$ and $CD8^+$ cell percentage and numbers. (C) Representative flow cytometry plots of non-Treg ($CD4^+Foxp3^-$) and Treg ($CD4^+Foxp3^+$) cells pre-gated on $CD4^+$ cells. (D) non-Treg $CD4^+$ and Treg cells percentage and numbers. (E) Percentage of activated Treg ($CD44^+CD4^+Foxp3^+$) cells pre-gated on $CD4^+$ cells and Foxp3 MFI in Treg cells. (F) Ratio of $CD8^+$ effector T cells to Treg cells and non-Treg $CD4^+$ cells to Treg cells. Data shown as mean with error bars denoting SEM, ns = not statistically significant, *p<0.05, **p<0.01, ***p<0.001. Data is representative of at two independent experiments.

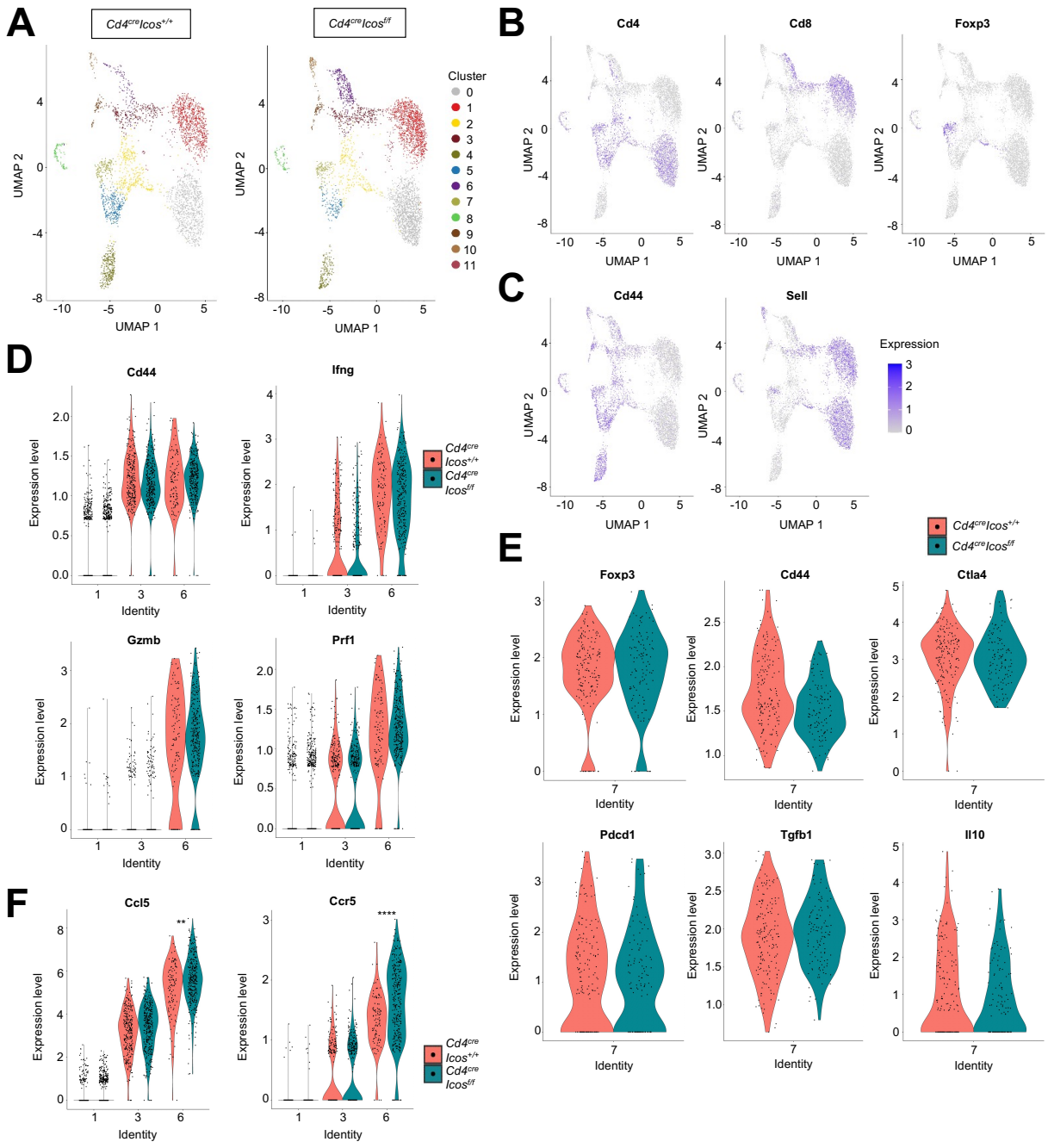


Figure 3.5 ICOS-deficient mice show enhanced expression of proinflammatory chemokine and receptor on CD8⁺ Teff cells, with no significant changes in expression of CD8⁺ T and Treg effector molecules. Single cell transcriptomes of magnetically sorted CD3ε⁺CD3γ⁺ tumor-infiltrating T cells from a *Cd4*^{cre}*Icos*$^{+/+}$ and a *Cd4*^{cre}*Icos*$^{f/f}$ mouse 10 days post-intravenous challenge with B16-OVA cells. (A) UMAP

projection of T cell clusters in *Cd4*^{cre}*Icos*$^{+/+}$ and *Cd4*^{cre}*Icos*$^{f/f}$ mice. (B) Feature plots of *Cd4, Cd8, Foxp3,* (C) *Cd44* and *Sell* expression. (D) Violin plots of *Cd44, Ifng, Gzmb,* and *Prf1* showing their gene expression levels in CD8^{+} Teff cells, subdivided by cluster identities defined in (A) and (B). (E) Violin plots of *Foxp3, Cd44, Ctla4, Pdcd1, Tgfb1,* and *Il10* showing their gene expression levels in Treg cells, with cluster identity defined in (A) and (B). (F) Violin plots of *Ccl5* and *Ccr5* showing their gene expression levels in CD8^{+} Teff cells, subdivided by cluster identities defined in (A) and (B). Each dot represents one cell. ***p*<0.01, *****p*<0.0001.

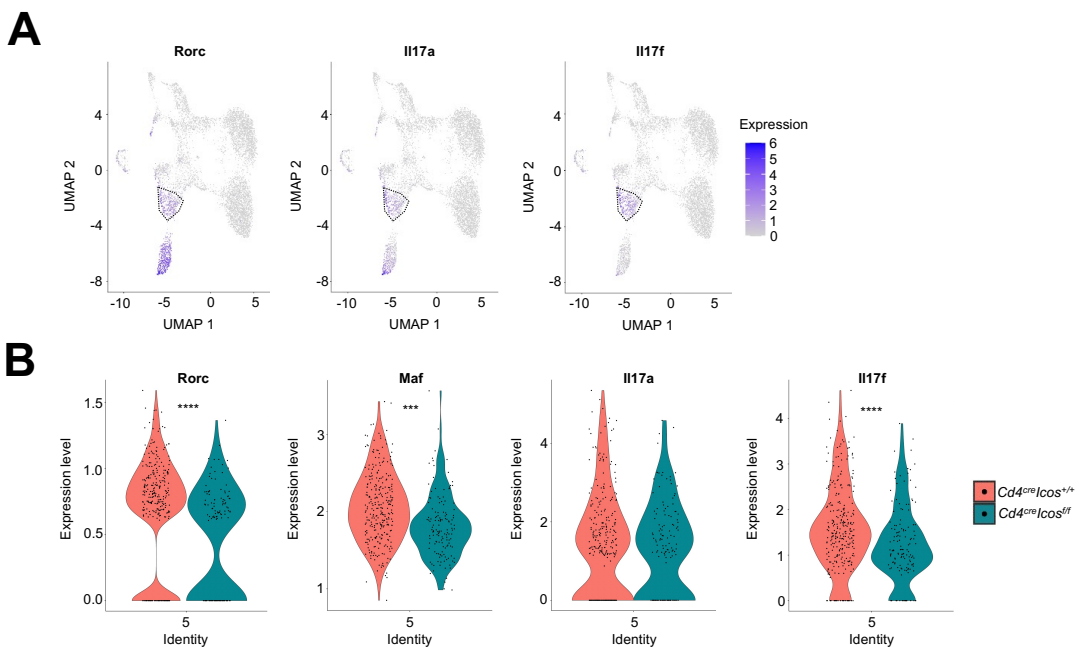


Figure 3.6 Reduction of Th17 cells in ICOS-deficient mice. Single cell transcriptomes of magnetically sorted CD3ε⁺CD3γ⁺ tumor-infiltrating T cells from a *Cd4*^{cre}*Icos*$^{+/+}$ and a *Cd4*^{cre}*Icos*$^{f/f}$ mouse 10 days post-intravenous challenge with B16-OVA cells. (A) Feature plots of *Rorc, Il17a, Il17f* expression. Dotted line outlines the Th17 cell cluster. (B) Violin plots of *Rorc, Maf, Il17a, Il17f* showing their gene expression levels in Th17 cells, with Th17 cluster identity defined in (A). Each dot represents one cell. ***p<0.001, ****p<0.0001.

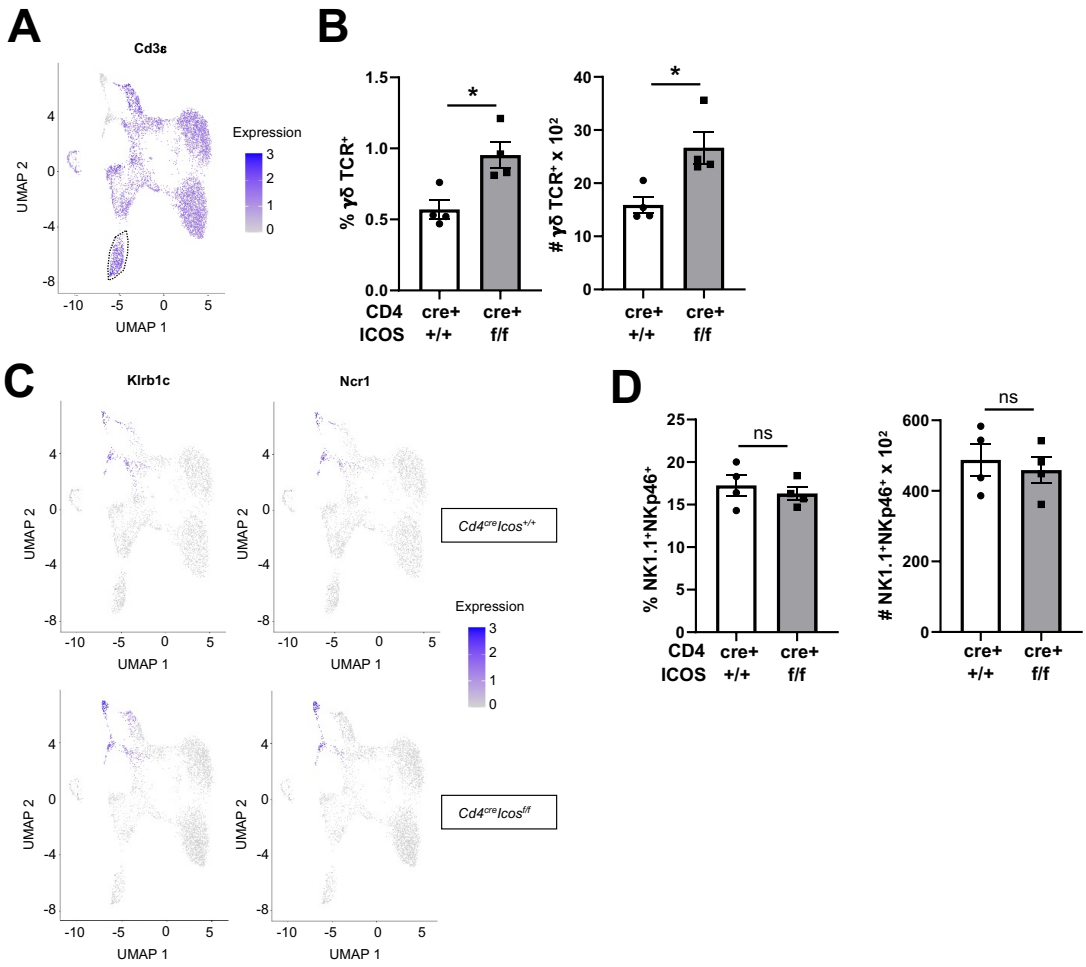
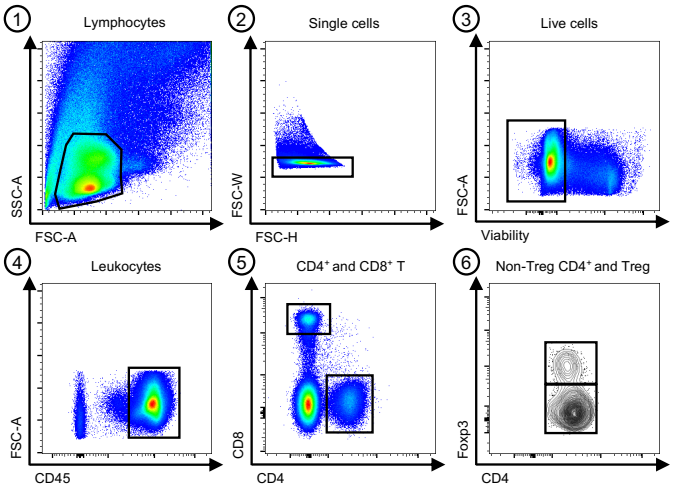
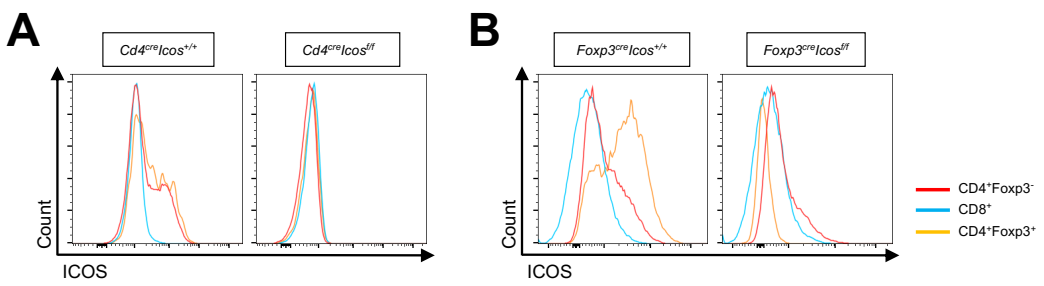


Figure 3.7 Augmented γδ T cell compartments with no changes in NK cell compartments in ICOS-deficient mice. (A, C) Single cell transcriptomes of magnetically sorted tumor-infiltrating immune cells from a *Cd4*^{cre}*Icos*$^{+/+}$ and a *Cd4*^{cre}*Icos*$^{f/f}$ mouse 10 days post-intravenous challenge with B16-OVA cells. (B, D) Flow cytometry analysis of tumor-infiltrating immune cells in *Cd4*^{cre}*Icos*$^{+/+}$ and *Cd4*^{cre}*Icos*$^{f/f}$ mice 10 days post-intravenous challenge with B16-OVA cells. (A) Feature plot of *Cd3ε* expression. Dotted line outlines the putative IL-17^{+} γδ T cell cluster. (B) γδ T cells as a percentage of CD45^{+} cells and numbers. Data shown as mean with error bars denoting SEM, *p<0.05. Data is representative of two independent experiments. (C) Feature plots of *Klrb1c* and *Ncr1* expression. (D) Putative NK cells (NK1.1^{+}NKp46^{+}) as a percentage of CD45^{+} cells. Data shown as mean with error bars denoting SEM, ns = not statistically significant.

Supplementary Figures



Supplemental Figure 3.1 Gating strategy to identify lung tumor-infiltrating T cell populations. Lymphocytes were gated from total lung cells, with singlets and live cells further selected. Then, $CD45^+$ leukocytes were gated to define tumor-infiltrating $CD4^+$ and $CD8^+$ Teff cells. Lastly, within $CD4^+$ cells, non-Treg $CD4^+$ Teff and Treg cells were gated based on the level of Foxp3 expression.



Supplemental Figure 3.2 ICOS expression levels in T cell subsets from tumor-challenged control and ICOS deficient mice. Mice with indicated genotypes were challenged as in Figure 3.1A. Tumor-infiltrating T cell populations were analyzed by flow cytometry. (A) ICOS expression levels in tumor-infiltrating non-Treg $CD4^+$ T cells ($CD4^+Foxp3^-$, red), $CD8^+$ Teff cells ($CD8^+$, blue), and Treg cells ($CD4^+Foxp3^+$, orange) in *Cd4*^{cre}*Icos*$^{+/+}$ and *Cd4*^{cre}*Icos*$^{f/f}$ mice. (B) ICOS expression levels in tumor-infiltrating non-Treg $CD4^+$ T cells ($CD4^+Foxp3^-$, red), $CD8^+$ Teff cells ($CD8^+$, blue), and Treg cells ($CD4^+Foxp3^+$, orange) in *Foxp3*$^{YFP\text{-}cre}$*Icos*$^{+/+}$ (abbreviated as *Foxp3*^{cre}*Icos*$^{+/+}$) and *Foxp3*$^{YFP\text{-}cre}$*Icos*$^{f/f}$ (abbreviated as *Foxp3*^{cre}*Icos*$^{f/f}$) mice.

References

1. Panneton, V., Chang, J., Witalis, M., Li, J. & Suh, W.K. Inducible T-cell co-stimulator: Signaling mechanisms in T follicular helper cells and beyond. *Immunol Rev* **291**, 91-103 (2019).
2. Decker, W.K. & Safdar, A. Bioimmunoadjuvants for the treatment of neoplastic and infectious disease: Coley's legacy revisited. *Cytokine Growth Factor Rev* **20**, 271-281 (2009).
3. Decker, W.K.*, et al.* Cancer Immunotherapy: Historical Perspective of a Clinical Revolution and Emerging Preclinical Animal Models. *Front Immunol* **8**, 829 (2017).
4. Dunn, G.P., Bruce, A.T., Ikeda, H., Old, L.J. & Schreiber, R.D. Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol* **3**, 991-998 (2002).
5. Spits, H. Development of alphabeta T cells in the human thymus. *Nat Rev Immunol* **2**, 760-772 (2002).
6. Kim, H.J. & Cantor, H. CD4 T-cell subsets and tumor immunity: the helpful and the not-so-helpful. *Cancer Immunol Res* **2**, 91-98 (2014).
7. Gerloni, M. & Zanetti, M. CD4 T cells in tumor immunity. *Springer Semin Immunopathol* **27**, 37-48 (2005).
8. Linnemann, C.*, et al.* High-throughput epitope discovery reveals frequent recognition of neo-antigens by CD4+ T cells in human melanoma. *Nat Med* **21**, 81-85 (2015).
9. Antony, P.A.*, et al.* CD8+ T cell immunity against a tumor/self-antigen is augmented by CD4+ T helper cells and hindered by naturally occurring T regulatory cells. *J Immunol* **174**, 2591-2601 (2005).
10. Bos, R. & Sherman, L.A. CD4+ T-cell help in the tumor milieu is required for recruitment and cytolytic function of CD8+ T lymphocytes. *Cancer Res* **70**, 8368-8377 (2010).
11. Greenberg, P.D., Cheever, M.A. & Fefer, A. Eradication of disseminated murine leukemia by chemoimmunotherapy with cyclophosphamide and adoptively transferred immune syngeneic Lyt-1+2- lymphocytes. *J Exp Med* **154**, 952-963 (1981).
12. Cabrera, T., Ruiz-Cabello, F. & Garrido, F. Biological implications of HLA-DR expression in tumours. *Scand J Immunol* **41**, 398-406 (1995).
13. Ossendorp, F., Mengede, E., Camps, M., Filius, R. & Melief, C.J. Specific T helper cell requirement for optimal induction of cytotoxic T lymphocytes against major histocompatibility complex class II negative tumors. *J Exp Med* **187**, 693-702 (1998).
14. Bennett, S.R.*, et al.* Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature* **393**, 478-480 (1998).
15. Thaiss, C.A., Semmling, V., Franken, L., Wagner, H. & Kurts, C. Chemokines: a new dendritic cell signal for T cell activation. *Front Immunol* **2**, 31 (2011).
16. Mitchison, N.A. & O'Malley, C. Three-cell-type clusters of T cells with antigen-presenting cells best explain the epitope linkage and noncognate requirements of the in vivo cytolytic response. *Eur J Immunol* **17**, 1579-1583 (1987).
17. Ridge, J.P., Di Rosa, F. & Matzinger, P. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. *Nature* **393**, 474-478 (1998).
18. Feau, S.*, et al.* The CD4(+) T-cell help signal is transmitted from APC to CD8(+) T-cells via CD27-CD70 interactions. *Nat Commun* **3**, 948 (2012).

19. Castellino, F.*, et al.* Chemokines enhance immunity by guiding naive CD8+ T cells to sites of CD4+ T cell-dendritic cell interaction. *Nature* **440**, 890-895 (2006).
20. Ahrends, T.*, et al.* CD4(+) T Cell Help Confers a Cytotoxic T Cell Effector Program Including Coinhibitory Receptor Downregulation and Increased Tissue Invasiveness. *Immunity* **47**, 848-861 e845 (2017).
21. Tay, R.E., Richardson, E.K. & Toh, H.C. Revisiting the role of CD4(+) T cells in cancer immunotherapy-new insights into old paradigms. *Cancer Gene Ther* **28**, 5-17 (2021).
22. Wang, J.C. & Livingstone, A.M. Cutting edge: CD4+ T cell help can be essential for primary CD8+ T cell responses in vivo. *J Immunol* **171**, 6339-6343 (2003).
23. Nishimura, T.*, et al.* Distinct role of antigen-specific T helper type 1 (Th1) and Th2 cells in tumor eradication in vivo. *J Exp Med* **190**, 617-627 (1999).
24. Braumuller, H.*, et al.* T-helper-1-cell cytokines drive cancer into senescence. *Nature* **494**, 361-365 (2013).
25. Naganuma, H.*, et al.* Increased susceptibility of IFN-gamma-treated neuroblastoma cells to lysis by lymphokine-activated killer cells: participation of ICAM-1 induction on target cells. *Int J Cancer* **47**, 527-532 (1991).
26. Shklovskaya, E.*, et al.* Tumour-specific CD4 T cells eradicate melanoma via indirect recognition of tumour-derived antigen. *Immunol Cell Biol* **94**, 593-603 (2016).
27. Quezada, S.A.*, et al.* Tumor-reactive CD4(+) T cells develop cytotoxic activity and eradicate large established melanoma after transfer into lymphopenic hosts. *J Exp Med* **207**, 637-650 (2010).
28. Xie, Y.*, et al.* Naive tumor-specific CD4(+) T cells differentiated in vivo eradicate established melanoma. *J Exp Med* **207**, 651-667 (2010).
29. Pages, F.*, et al.* Effector memory T cells, early metastasis, and survival in colorectal cancer. *N Engl J Med* **353**, 2654-2666 (2005).
30. Durgeau, A., Virk, Y., Corgnac, S. & Mami-Chouaib, F. Recent Advances in Targeting CD8 T-Cell Immunity for More Effective Cancer Immunotherapy. *Front Immunol* **9**, 14 (2018).
31. Williford, J.M.*, et al.* Recruitment of CD103(+) dendritic cells via tumor-targeted chemokine delivery enhances efficacy of checkpoint inhibitor immunotherapy. *Sci Adv* **5**, eaay1357 (2019).
32. Merad, M., Sathe, P., Helft, J., Miller, J. & Mortha, A. The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. *Annu Rev Immunol* **31**, 563-604 (2013).
33. de Saint Basile, G., Menasche, G. & Fischer, A. Molecular mechanisms of biogenesis and exocytosis of cytotoxic granules. *Nat Rev Immunol* **10**, 568-579 (2010).
34. Anikeeva, N.*, et al.* Distinct role of lymphocyte function-associated antigen-1 in mediating effective cytolytic activity by cytotoxic T lymphocytes. *Proc Natl Acad Sci U S A* **102**, 6437-6442 (2005).
35. Le Floc'h, A.*, et al.* Alpha E beta 7 integrin interaction with E-cadherin promotes antitumor CTL activity by triggering lytic granule polarization and exocytosis. *J Exp Med* **204**, 559-570 (2007).
36. Lopez, J.A.*, et al.* Perforin forms transient pores on the target cell plasma membrane to facilitate rapid access of granzymes during killer cell attack. *Blood* **121**, 2659-2668 (2013).

37. Voskoboinik, I., Whisstock, J.C. & Trapani, J.A. Perforin and granzymes: function, dysfunction and human pathology. *Nat Rev Immunol* **15**, 388-400 (2015).
38. van den Broek, M.E.*, et al.* Decreased tumor surveillance in perforin-deficient mice. *J Exp Med* **184**, 1781-1790 (1996).
39. Kagi, D.*, et al.* Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature* **369**, 31-37 (1994).
40. Lowin, B., Hahne, M., Mattmann, C. & Tschopp, J. Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways. *Nature* **370**, 650-652 (1994).
41. Finnberg, N., Klein-Szanto, A.J. & El-Deiry, W.S. TRAIL-R deficiency in mice promotes susceptibility to chronic inflammation and tumorigenesis. *J Clin Invest* **118**, 111-123 (2008).
42. Bhat, P., Leggatt, G., Waterhouse, N. & Frazer, I.H. Interferon-gamma derived from cytotoxic lymphocytes directly enhances their motility and cytotoxicity. *Cell Death Dis* **8**, e2836 (2017).
43. Qin, Z.*, et al.* A critical requirement of interferon gamma-mediated angiostasis for tumor rejection by CD8+ T cells. *Cancer Res* **63**, 4095-4100 (2003).
44. Herberman, R.B., Holden, H.T., Ting, C.C., Lavrin, D.L. & Kirchner, H. Cell-mediated immunity to leukemia virus- and tumor-associated antigens in mice. *Cancer Res* **36**, 615-621 (1976).
45. Chiossone, L., Dumas, P.Y., Vienne, M. & Vivier, E. Natural killer cells and other innate lymphoid cells in cancer. *Nat Rev Immunol* **18**, 671-688 (2018).
46. Huntington, N.D.*, et al.* Interleukin 15-mediated survival of natural killer cells is determined by interactions among Bim, Noxa and Mcl-1. *Nat Immunol* **8**, 856-863 (2007).
47. Bottcher, J.P.*, et al.* NK Cells Stimulate Recruitment of cDC1 into the Tumor Microenvironment Promoting Cancer Immune Control. *Cell* **172**, 1022-1037 e1014 (2018).
48. Bauer, S.*, et al.* Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science* **285**, 727-729 (1999).
49. Amin, P.J. & Shankar, B.S. Sulforaphane induces ROS mediated induction of NKG2D ligands in human cancer cell lines and enhances susceptibility to NK cell mediated lysis. *Life Sci* **126**, 19-27 (2015).
50. Narni-Mancinelli, E.*, et al.* Complement factor P is a ligand for the natural killer cell-activating receptor NKp46. *Sci Immunol* **2**(2017).
51. Siewiera, J.*, et al.* Natural cytotoxicity receptor splice variants orchestrate the distinct functions of human natural killer cell subtypes. *Nat Commun* **6**, 10183 (2015).
52. Textor, S.*, et al.* The proto-oncogene Myc drives expression of the NK cell-activating NKp30 ligand B7-H6 in tumor cells. *Oncoimmunology* **5**, e1116674 (2016).
53. Kumar, S. Natural killer cell cytotoxicity and its regulation by inhibitory receptors. *Immunology* **154**, 383-393 (2018).
54. Kim, S.*, et al.* Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature* **436**, 709-713 (2005).
55. Dhatchinamoorthy, K., Colbert, J.D. & Rock, K.L. Cancer Immune Evasion Through Loss of MHC Class I Antigen Presentation. *Front Immunol* **12**, 636568 (2021).

56. Diefenbach, A., Jamieson, A.M., Liu, S.D., Shastri, N. & Raulet, D.H. Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages. *Nat Immunol* **1**, 119-126 (2000).
57. Guerra, N.*, et al.* NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy. *Immunity* **28**, 571-580 (2008).
58. Brandt, C.S.*, et al.* The B7 family member B7-H6 is a tumor cell ligand for the activating natural killer cell receptor NKp30 in humans. *J Exp Med* **206**, 1495-1503 (2009).
59. Wu, S.Y., Fu, T., Jiang, Y.Z. & Shao, Z.M. Natural killer cells in cancer biology and therapy. *Mol Cancer* **19**, 120 (2020).
60. Backes, C.S.*, et al.* Natural killer cells induce distinct modes of cancer cell death: Discrimination, quantification, and modulation of apoptosis, necrosis, and mixed forms. *J Biol Chem* **293**, 16348-16363 (2018).
61. Jorgovanovic, D., Song, M., Wang, L. & Zhang, Y. Roles of IFN-gamma in tumor progression and regression: a review. *Biomark Res* **8**, 49 (2020).
62. Song, M.*, et al.* Low-Dose IFNgamma Induces Tumor Cell Stemness in Tumor Microenvironment of Non-Small Cell Lung Cancer. *Cancer Res* **79**, 3737-3748 (2019).
63. Briesemeister, D.*, et al.* Tumor rejection by local interferon gamma induction in established tumors is associated with blood vessel destruction and necrosis. *Int J Cancer* **128**, 371-378 (2011).
64. Lu, Y.*, et al.* Responsiveness of stromal fibroblasts to IFN-gamma blocks tumor growth via angiostasis. *J Immunol* **183**, 6413-6421 (2009).
65. Baer, C.*, et al.* Suppression of microRNA activity amplifies IFN-gamma-induced macrophage activation and promotes anti-tumour immunity. *Nat Cell Biol* **18**, 790-802 (2016).
66. Metelitsa, L.S.*, et al.* Natural killer T cells infiltrate neuroblastomas expressing the chemokine CCL2. *J Exp Med* **199**, 1213-1221 (2004).
67. Tachibana, T.*, et al.* Increased intratumor Valpha24-positive natural killer T cells: a prognostic factor for primary colorectal carcinomas. *Clin Cancer Res* **11**, 7322-7327 (2005).
68. Kawano, T.*, et al.* CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science* **278**, 1626-1629 (1997).
69. Bassiri, H.*, et al.* iNKT cell cytotoxic responses control T-lymphoma growth in vitro and in vivo. *Cancer Immunol Res* **2**, 59-69 (2014).
70. Wingender, G., Krebs, P., Beutler, B. & Kronenberg, M. Antigen-specific cytotoxicity by invariant NKT cells in vivo is CD95/CD178-dependent and is correlated with antigenic potency. *J Immunol* **185**, 2721-2729 (2010).
71. Nakagawa, R.*, et al.* Antitumor activity of alpha-galactosylceramide, KRN7000, in mice with the melanoma B16 hepatic metastasis and immunohistological study of tumor infiltrating cells. *Oncol Res* **12**, 51-58 (2000).
72. Kitamura, H.*, et al.* The natural killer T (NKT) cell ligand alpha-galactosylceramide demonstrates its immunopotentiating effect by inducing interleukin (IL)-12 production by dendritic cells and IL-12 receptor expression on NKT cells. *J Exp Med* **189**, 1121-1128 (1999).
73. Tomura, M.*, et al.* A novel function of Valpha14+CD4+NKT cells: stimulation of IL-12 production by antigen-presenting cells in the innate immune system. *J Immunol* **163**, 93-101 (1999).

74. Takahashi, T.*, et al.* Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *Int Immunol* **10**, 1969-1980 (1998).
75. Bennett, C.L.*, et al.* The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet* **27**, 20-21 (2001).
76. Fontenot, J.D., Gavin, M.A. & Rudensky, A.Y. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* **4**, 330-336 (2003).
77. Brunkow, M.E.*, et al.* Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet* **27**, 68-73 (2001).
78. Hsieh, C.S., Lee, H.M. & Lio, C.W. Selection of regulatory T cells in the thymus. *Nat Rev Immunol* **12**, 157-167 (2012).
79. Kretschmer, K.*, et al.* Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol* **6**, 1219-1227 (2005).
80. Onizuka, S.*, et al.* Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor alpha) monoclonal antibody. *Cancer Res* **59**, 3128-3133 (1999).
81. Shimizu, J., Yamazaki, S. & Sakaguchi, S. Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity. *J Immunol* **163**, 5211-5218 (1999).
82. Sakaguchi, S., Miyara, M., Costantino, C.M. & Hafler, D.A. FOXP3+ regulatory T cells in the human immune system. *Nat Rev Immunol* **10**, 490-500 (2010).
83. Saito, T.*, et al.* Two FOXP3(+)CD4(+) T cell subpopulations distinctly control the prognosis of colorectal cancers. *Nat Med* **22**, 679-684 (2016).
84. Seed, R.I.*, et al.* A tumor-specific mechanism of Treg enrichment mediated by the integrin alphavbeta8. *Sci Immunol* **6**(2021).
85. Walker, L.S. & Sansom, D.M. The emerging role of CTLA4 as a cell-extrinsic regulator of T cell responses. *Nat Rev Immunol* **11**, 852-863 (2011).
86. Qureshi, O.S.*, et al.* Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. *Science* **332**, 600-603 (2011).
87. Wing, K.*, et al.* CTLA-4 control over Foxp3+ regulatory T cell function. *Science* **322**, 271-275 (2008).
88. Kornete, M., Sgouroudis, E. & Piccirillo, C.A. ICOS-dependent homeostasis and function of Foxp3+ regulatory T cells in islets of nonobese diabetic mice. *J Immunol* **188**, 1064-1074 (2012).
89. Asseman, C., Mauze, S., Leach, M.W., Coffman, R.L. & Powrie, F. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J Exp Med* **190**, 995-1004 (1999).
90. Chen, M.L.*, et al.* Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF-beta signals in vivo. *Proc Natl Acad Sci U S A* **102**, 419-424 (2005).
91. Smyth, M.J.*, et al.* CD4+CD25+ T regulatory cells suppress NK cell-mediated immunotherapy of cancer. *J Immunol* **176**, 1582-1587 (2006).
92. Lundqvist, A., Yokoyama, H., Smith, A., Berg, M. & Childs, R. Bortezomib treatment and regulatory T-cell depletion enhance the antitumor effects of adoptively infused NK cells. *Blood* **113**, 6120-6127 (2009).

93. Gondek, D.C., Lu, L.F., Quezada, S.A., Sakaguchi, S. & Noelle, R.J. Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. *J Immunol* **174**, 1783-1786 (2005).
94. Cao, X.*, et al.* Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance. *Immunity* **27**, 635-646 (2007).
95. Thornton, A.M. & Shevach, E.M. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med* **188**, 287-296 (1998).
96. Yurchenko, E., Levings, M.K. & Piccirillo, C.A. CD4+ Foxp3+ regulatory T cells suppress gammadelta T-cell effector functions in a model of T-cell-induced mucosal inflammation. *Eur J Immunol* **41**, 3455-3466 (2011).
97. Li, L. & Wu, C.Y. CD4+ CD25+ Treg cells inhibit human memory gammadelta T cells to produce IFN-gamma in response to M tuberculosis antigen ESAT-6. *Blood* **111**, 5629-5636 (2008).
98. Chung, Y.*, et al.* Critical regulation of early Th17 cell differentiation by interleukin-1 signaling. *Immunity* **30**, 576-587 (2009).
99. Wei, L., Laurence, A., Elias, K.M. & O'Shea, J.J. IL-21 is produced by Th17 cells and drives IL-17 production in a STAT3-dependent manner. *J Biol Chem* **282**, 34605-34610 (2007).
100. Steinman, L. A brief history of T(H)17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage. *Nat Med* **13**, 139-145 (2007).
101. Asadzadeh, Z.*, et al.* The paradox of Th17 cell functions in tumor immunity. *Cell Immunol* **322**, 15-25 (2017).
102. Takahashi, H., Numasaki, M., Lotze, M.T. & Sasaki, H. Interleukin-17 enhances bFGF-, HGF- and VEGF-induced growth of vascular endothelial cells. *Immunol Lett* **98**, 189-193 (2005).
103. Huang, Q.*, et al.* IL-17 Promotes Angiogenic Factors IL-6, IL-8, and Vegf Production via Stat1 in Lung Adenocarcinoma. *Sci Rep* **6**, 36551 (2016).
104. Benevides, L.*, et al.* IL17 Promotes Mammary Tumor Progression by Changing the Behavior of Tumor Cells and Eliciting Tumorigenic Neutrophils Recruitment. *Cancer Res* **75**, 3788-3799 (2015).
105. Tosolini, M.*, et al.* Clinical impact of different classes of infiltrating T cytotoxic and helper cells (Th1, th2, treg, th17) in patients with colorectal cancer. *Cancer Res* **71**, 1263-1271 (2011).
106. Punt, S.*, et al.* A beneficial tumor microenvironment in oropharyngeal squamous cell carcinoma is characterized by a high T cell and low IL-17(+) cell frequency. *Cancer Immunol Immunother* **65**, 393-403 (2016).
107. Kryczek, I.*, et al.* Phenotype, distribution, generation, and functional and clinical relevance of Th17 cells in the human tumor environments. *Blood* **114**, 1141-1149 (2009).
108. Jovanovic, D.V.*, et al.* IL-17 stimulates the production and expression of proinflammatory cytokines, IL-beta and TNF-alpha, by human macrophages. *J Immunol* **160**, 3513-3521 (1998).
109. Xie, Y., Sheng, W., Xiang, J., Ye, Z. & Yang, J. Interleukin-17F suppresses hepatocarcinoma cell growth via inhibition of tumor angiogenesis. *Cancer Invest* **28**, 598-607 (2010).

110. Zhao, Y., Niu, C. & Cui, J. Gamma-delta (gammadelta) T cells: friend or foe in cancer development? *J Transl Med* **16**, 3 (2018).
111. Niu, C.*, et al.* In vitro analysis of the proliferative capacity and cytotoxic effects of ex vivo induced natural killer cells, cytokine-induced killer cells, and gamma-delta T cells. *BMC Immunol* **16**, 61 (2015).
112. Todaro, M.*, et al.* Efficient killing of human colon cancer stem cells by gammadelta T lymphocytes. *J Immunol* **182**, 7287-7296 (2009).
113. Fisher, J.P.*, et al.* Neuroblastoma killing properties of Vdelta2 and Vdelta2-negative gammadeltaT cells following expansion by artificial antigen-presenting cells. *Clin Cancer Res* **20**, 5720-5732 (2014).
114. He, W.*, et al.* Naturally activated V gamma 4 gamma delta T cells play a protective role in tumor immunity through expression of eomesodermin. *J Immunol* **185**, 126-133 (2010).
115. Patil, R.S.*, et al.* IL17 producing gammadeltaT cells induce angiogenesis and are associated with poor survival in gallbladder cancer patients. *Int J Cancer* **139**, 869-881 (2016).
116. Wu, P.*, et al.* gammadeltaT17 cells promote the accumulation and expansion of myeloid-derived suppressor cells in human colorectal cancer. *Immunity* **40**, 785-800 (2014).
117. Coffelt, S.B.*, et al.* IL-17-producing gammadelta T cells and neutrophils conspire to promote breast cancer metastasis. *Nature* **522**, 345-348 (2015).
118. Hutloff, A.*, et al.* ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. *Nature* **397**, 263-266 (1999).
119. McAdam, A.J.*, et al.* Mouse inducible costimulatory molecule (ICOS) expression is enhanced by CD28 costimulation and regulates differentiation of CD4+ T cells. *J Immunol* **165**, 5035-5040 (2000).
120. Lownik, J.C.*, et al.* ADAM10-Mediated ICOS Ligand Shedding on B Cells Is Necessary for Proper T Cell ICOS Regulation and T Follicular Helper Responses. *J Immunol* **199**, 2305-2315 (2017).
121. Marczynska, J.*, et al.* The role of metalloproteinase ADAM17 in regulating ICOS ligand-mediated humoral immune responses. *J Immunol* **193**, 2753-2763 (2014).
122. Buus, T.B., Schmidt, J.D., Bonefeld, C.M., Geisler, C. & Lauritsen, J.P. Development of interleukin-17-producing Vgamma2+ gammadelta T cells is reduced by ICOS signaling in the thymus. *Oncotarget* **7**, 19341-19354 (2016).
123. Montes-Casado, M.*, et al.* ICOS deficiency hampers the homeostasis, development and function of NK cells. *PLoS One* **14**, e0219449 (2019).
124. Maazi, H.*, et al.* ICOS:ICOS-ligand interaction is required for type 2 innate lymphoid cell function, homeostasis, and induction of airway hyperreactivity. *Immunity* **42**, 538-551 (2015).
125. Paclik, D., Stehle, C., Lahmann, A., Hutloff, A. & Romagnani, C. ICOS regulates the pool of group 2 innate lymphoid cells under homeostatic and inflammatory conditions in mice. *Eur J Immunol* **45**, 2766-2772 (2015).
126. Wang, S.*, et al.* Costimulation of T cells by B7-H2, a B7-like molecule that binds ICOS. *Blood* **96**, 2808-2813 (2000).
127. Aicher, A.*, et al.* Characterization of human inducible costimulator ligand expression and function. *J Immunol* **164**, 4689-4696 (2000).

128. Qian, X., *et al.* The ICOS-ligand B7-H2, expressed on human type II alveolar epithelial cells, plays a role in the pulmonary host defense system. *Eur J Immunol* **36**, 906-918 (2006).
129. Wiendl, H., *et al.* Muscle fibres and cultured muscle cells express the B7.1/2-related inducible co-stimulatory molecule, ICOSL: implications for the pathogenesis of inflammatory myopathies. *Brain* **126**, 1026-1035 (2003).
130. Gigoux, M., *et al.* Inducible costimulator promotes helper T-cell differentiation through phosphoinositide 3-kinase. *Proc Natl Acad Sci U S A* **106**, 20371-20376 (2009).
131. Leconte, J., Bagherzadeh Yazdchi, S., Panneton, V. & Suh, W.K. Inducible costimulator (ICOS) potentiates TCR-induced calcium flux by augmenting PLCgamma1 activation and actin remodeling. *Mol Immunol* **79**, 38-46 (2016).
132. Pedros, C., *et al.* A TRAF-like motif of the inducible costimulator ICOS controls development of germinal center TFH cells via the kinase TBK1. *Nat Immunol* **17**, 825-833 (2016).
133. Tafuri, A., *et al.* ICOS is essential for effective T-helper-cell responses. *Nature* **409**, 105-109 (2001).
134. Dong, C., *et al.* ICOS co-stimulatory receptor is essential for T-cell activation and function. *Nature* **409**, 97-101 (2001).
135. Grimbacher, B., *et al.* Homozygous loss of ICOS is associated with adult-onset common variable immunodeficiency. *Nat Immunol* **4**, 261-268 (2003).
136. Vinuesa, C.G., Linterman, M.A., Yu, D. & MacLennan, I.C. Follicular Helper T Cells. *Annu Rev Immunol* **34**, 335-368 (2016).
137. Baumjohann, D., Okada, T. & Ansel, K.M. Cutting Edge: Distinct waves of BCL6 expression during T follicular helper cell development. *J Immunol* **187**, 2089-2092 (2011).
138. Leavenworth, J.W., Verbinnen, B., Yin, J., Huang, H. & Cantor, H. A p85alpha-osteopontin axis couples the receptor ICOS to sustained Bcl-6 expression by follicular helper and regulatory T cells. *Nat Immunol* **16**, 96-106 (2015).
139. Xu, H., *et al.* Follicular T-helper cell recruitment governed by bystander B cells and ICOS-driven motility. *Nature* **496**, 523-527 (2013).
140. Gigoux, M., *et al.* Inducible costimulator facilitates T-dependent B cell activation by augmenting IL-4 translation. *Mol Immunol* **59**, 46-54 (2014).
141. Nouailles, G., *et al.* Impact of inducible co-stimulatory molecule (ICOS) on T-cell responses and protection against Mycobacterium tuberculosis infection. *Eur J Immunol* **41**, 981-991 (2011).
142. Vidric, M., Bladt, A.T., Dianzani, U. & Watts, T.H. Role for inducible costimulator in control of Salmonella enterica serovar Typhimurium infection in mice. *Infect Immun* **74**, 1050-1061 (2006).
143. Mittrucker, H.W., *et al.* Inducible costimulator protein controls the protective T cell response against Listeria monocytogenes. *J Immunol* **169**, 5813-5817 (2002).
144. Zhang, Y., *et al.* The clinical impact of ICOS signal in colorectal cancer patients. *Oncoimmunology* **5**, e1141857 (2016).
145. Watanabe, M., *et al.* ICOS-mediated costimulation on Th2 differentiation is achieved by the enhancement of IL-4 receptor-mediated signaling. *J Immunol* **174**, 1989-1996 (2005).

146. Wilson, E.H., *et al.* B7RP-1-ICOS interactions are required for optimal infection-induced expansion of CD4+ Th1 and Th2 responses. *J Immunol* **177**, 2365-2372 (2006).
147. Miyahira, Y., *et al.* Involvement of ICOS-B7RP-1 costimulatory pathway in the regulation of immune responses to Leishmania major and Nippostrongylus brasiliensis infections. *Immunol Lett* **89**, 193-199 (2003).
148. Paulos, C.M., *et al.* The inducible costimulator (ICOS) is critical for the development of human T(H)17 cells. *Sci Transl Med* **2**, 55ra78 (2010).
149. Bauquet, A.T., *et al.* The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells. *Nat Immunol* **10**, 167-175 (2009).
150. Gao, X., *et al.* Anti-chlamydial Th17 responses are controlled by the inducible costimulator partially through phosphoinositide 3-kinase signaling. *PLoS One* **7**, e52657 (2012).
151. Majchrzak, K., *et al.* beta-catenin and PI3Kdelta inhibition expands precursor Th17 cells with heightened stemness and antitumor activity. *JCI Insight* **2**(2017).
152. Wallin, J.J., Liang, L., Bakardjiev, A. & Sha, W.C. Enhancement of CD8+ T cell responses by ICOS/B7h costimulation. *J Immunol* **167**, 132-139 (2001).
153. Liu, X., *et al.* B7H costimulates clonal expansion of, and cognate destruction of tumor cells by, CD8(+) T lymphocytes in vivo. *J Exp Med* **194**, 1339-1348 (2001).
154. Duhen, T., Duhen, R., Lanzavecchia, A., Sallusto, F. & Campbell, D.J. Functionally distinct subsets of human FOXP3+ Treg cells that phenotypically mirror effector Th cells. *Blood* **119**, 4430-4440 (2012).
155. Burmeister, Y., *et al.* ICOS controls the pool size of effector-memory and regulatory T cells. *J Immunol* **180**, 774-782 (2008).
156. Landuyt, A.E., Klocke, B.J., Colvin, T.B., Schoeb, T.R. & Maynard, C.L. Cutting Edge: ICOS-Deficient Regulatory T Cells Display Normal Induction of Il10 but Readily Downregulate Expression of Foxp3. *J Immunol* **202**, 1039-1044 (2019).
157. Chen, Y., Shen, S., Gorentla, B.K., Gao, J. & Zhong, X.P. Murine regulatory T cells contain hyperproliferative and death-prone subsets with differential ICOS expression. *J Immunol* **188**, 1698-1707 (2012).
158. Raynor, J., *et al.* IL-6 and ICOS Antagonize Bim and Promote Regulatory T Cell Accrual with Age. *J Immunol* **195**, 944-952 (2015).
159. Lohning, M., *et al.* Expression of ICOS in vivo defines CD4+ effector T cells with high inflammatory potential and a strong bias for secretion of interleukin 10. *J Exp Med* **197**, 181-193 (2003).
160. Kohyama, M., *et al.* Inducible costimulator-dependent IL-10 production by regulatory T cells specific for self-antigen. *Proc Natl Acad Sci U S A* **101**, 4192-4197 (2004).
161. Wei, S.C., Duffy, C.R. & Allison, J.P. Fundamental Mechanisms of Immune Checkpoint Blockade Therapy. *Cancer Discov* **8**, 1069-1086 (2018).
162. Sharma, P. & Allison, J.P. The future of immune checkpoint therapy. *Science* **348**, 56-61 (2015).
163. Liakou, C.I., *et al.* CTLA-4 blockade increases IFNgamma-producing CD4+ICOShi cells to shift the ratio of effector to regulatory T cells in cancer patients. *Proc Natl Acad Sci U S A* **105**, 14987-14992 (2008).

164. Vonderheide, R.H.*, et al.* Tremelimumab in combination with exemestane in patients with advanced breast cancer and treatment-associated modulation of inducible costimulator expression on patient T cells. *Clin Cancer Res* **16**, 3485-3494 (2010).
165. Ng Tang, D.*, et al.* Increased frequency of ICOS+ CD4 T cells as a pharmacodynamic biomarker for anti-CTLA-4 therapy. *Cancer Immunol Res* **1**, 229-234 (2013).
166. Castro, F., Cardoso, A.P., Goncalves, R.M., Serre, K. & Oliveira, M.J. Interferon-Gamma at the Crossroads of Tumor Immune Surveillance or Evasion. *Front Immunol* **9**, 847 (2018).
167. Medler, T.R., Cotechini, T. & Coussens, L.M. Immune response to cancer therapy: mounting an effective antitumor response and mechanisms of resistance. *Trends Cancer* **1**, 66-75 (2015).
168. Fu, T., He, Q. & Sharma, P. The ICOS/ICOSL pathway is required for optimal antitumor responses mediated by anti-CTLA-4 therapy. *Cancer Res* **71**, 5445-5454 (2011).
169. Fan, X., Quezada, S.A., Sepulveda, M.A., Sharma, P. & Allison, J.P. Engagement of the ICOS pathway markedly enhances efficacy of CTLA-4 blockade in cancer immunotherapy. *J Exp Med* **211**, 715-725 (2014).
170. Zamarin, D.*, et al.* Intratumoral modulation of the inducible co-stimulator ICOS by recombinant oncolytic virus promotes systemic anti-tumour immunity. *Nat Commun* **8**, 14340 (2017).
171. Chen, H.*, et al.* CD4 T cells require ICOS-mediated PI3K signaling to increase T-Bet expression in the setting of anti-CTLA-4 therapy. *Cancer Immunol Res* **2**, 167-176 (2014).
172. Herman, A.E., Freeman, G.J., Mathis, D. & Benoist, C. CD4+CD25+ T regulatory cells dependent on ICOS promote regulation of effector cells in the prediabetic lesion. *J Exp Med* **199**, 1479-1489 (2004).
173. Kornete, M.*, et al.* Th1-Like ICOS+ Foxp3+ Treg Cells Preferentially Express CXCR3 and Home to beta-Islets during Pre-Diabetes in BDC2.5 NOD Mice. *PLoS One* **10**, e0126311 (2015).
174. Sim, G.C.*, et al.* IL-2 therapy promotes suppressive ICOS+ Treg expansion in melanoma patients. *J Clin Invest* **124**, 99-110 (2014).
175. Martin-Orozco, N.*, et al.* Melanoma cells express ICOS ligand to promote the activation and expansion of T-regulatory cells. *Cancer Res* **70**, 9581-9590 (2010).
176. Han, Y.*, et al.* Acute Myeloid Leukemia Cells Express ICOS Ligand to Promote the Expansion of Regulatory T Cells. *Front Immunol* **9**, 2227 (2018).
177. Faget, J.*, et al.* ICOS-ligand expression on plasmacytoid dendritic cells supports breast cancer progression by promoting the accumulation of immunosuppressive CD4+ T cells. *Cancer Res* **72**, 6130-6141 (2012).
178. Conrad, C.*, et al.* Plasmacytoid dendritic cells promote immunosuppression in ovarian cancer via ICOS costimulation of Foxp3(+) T-regulatory cells. *Cancer Res* **72**, 5240-5249 (2012).
179. Solinas, C., Gu-Trantien, C. & Willard-Gallo, K. The rationale behind targeting the ICOS-ICOS ligand costimulatory pathway in cancer immunotherapy. *ESMO Open* **5**(2020).
180. Lee, P.P.*, et al.* A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. *Immunity* **15**, 763-774 (2001).
181. Rubtsov, Y.P.*, et al.* Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity* **28**, 546-558 (2008).

182. Panneton, V., Bagherzadeh Yazdchi, S., Witalis, M., Chang, J. & Suh, W.K. ICOS Signaling Controls Induction and Maintenance of Collagen-Induced Arthritis. *J Immunol* **200**, 3067-3076 (2018).
183. Kedl, R.M.*, et al.* CD40 stimulation accelerates deletion of tumor-specific CD8(+) T cells in the absence of tumor-antigen vaccination. *Proc Natl Acad Sci U S A* **98**, 10811-10816 (2001).
184. Waltman, L. & van Eck, N.J. A smart local moving algorithm for large-scale modularity-based community detection. *The European Physical Journal B* **86**, 471 (2013).
185. McInnes, L., Healy, J. & Melville, J. UMAP: Uniform Manifold Approximation and Projection for Dimension Reduction. arXiv:1802.03426 (2018).
186. Timmons, J.J., Cohessy, S. & Wong, E.T. Injection of Syngeneic Murine Melanoma Cells to Determine Their Metastatic Potential in the Lungs. *J Vis Exp*, 54039 (2016).
187. Gonzalez-Martin, A., Gomez, L., Lustgarten, J., Mira, E. & Manes, S. Maximal T cell-mediated antitumor responses rely upon CCR5 expression in both CD4(+) and CD8(+) T cells. *Cancer Res* **71**, 5455-5466 (2011).
188. Gonzalez-Martin, A., Mira, E. & Manes, S. CCR5 in cancer immunotherapy: More than an "attractive" receptor for T cells. *Oncoimmunology* **1**, 106-108 (2012).
189. Wang, J., Ioan-Facsinay, A., van der Voort, E.I., Huizinga, T.W. & Toes, R.E. Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. *Eur J Immunol* **37**, 129-138 (2007).
190. Miyao, T.*, et al.* Plasticity of Foxp3(+) T cells reflects promiscuous Foxp3 expression in conventional T cells but not reprogramming of regulatory T cells. *Immunity* **36**, 262-275 (2012).
191. Lapteva, N. & Huang, X.F. CCL5 as an adjuvant for cancer immunotherapy. *Expert Opin Biol Ther* **10**, 725-733 (2010).
192. Crome, S.Q.*, et al.* Inflammatory effects of ex vivo human Th17 cells are suppressed by regulatory T cells. *J Immunol* **185**, 3199-3208 (2010).
193. Pellicci, D.G., Koay, H.F. & Berzins, S.P. Thymic development of unconventional T cells: how NKT cells, MAIT cells and gammadelta T cells emerge. *Nat Rev Immunol* **20**, 756-770 (2020).
194. Petrie, H.T., Scollay, R. & Shortman, K. Commitment to the T cell receptor-alpha beta or -gamma delta lineages can occur just prior to the onset of CD4 and CD8 expression among immature thymocytes. *Eur J Immunol* **22**, 2185-2188 (1992).
195. Bernstein, H.B.*, et al.* CD4 expression on activated NK cells: ligation of CD4 induces cytokine expression and cell migration. *J Immunol* **177**, 3669-3676 (2006).
196. Yu, J.*, et al.* NKp46 identifies an NKT cell subset susceptible to leukemic transformation in mouse and human. *J Clin Invest* **121**, 1456-1470 (2011).
197. Akbari, O.*, et al.* ICOS/ICOSL interaction is required for CD4+ invariant NKT cell function and homeostatic survival. *J Immunol* **180**, 5448-5456 (2008).