Modulation of T Cell Antitumor Immunity Through Acetylcholine Signaling

By

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B.Comm., McMaster University, 2017

B.Sc., University of Victoria, 2021

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Abstract

Immunotherapies such as chimeric antigen receptor T cells (CAR-T cells) have shown promising results in many cancer patients but are still limited in solid cancers. Solid cancers contain immunosuppressive factors in the tumor microenvironment (TME), such as hypoxia and glucose deprivation. Antigen heterogeneity reduces therapy effectiveness in many cancers as CAR-T receptors need to recognize specific antigen that may be absent. This thesis investigates the function of a prominent TME metabolite: the neurotransmitter acetylcholine (ACh). Primary data from our lab shows that T cells infiltrating the TME have elevated ACh. Recent publications show ACh signaling influences mouse T cells to express a transcription factor FoxP3, a marker for regulatory T cells (Tregs) that contribute to the suppressive TME. However, there is little progress in testing the impact of these ACh-stimulated T cells' anticancer functions. Data from my thesis indicates that folate receptor alpha (FRα) CAR-T cell antitumor effector function is enhanced, rather than suppressed by ACh. ACh promotes transient expression of human FOXP3 in activated proinflammatory effector T cells (Teffs) expressing interferon gamma (IFN-γ). My results identify the alpha 7 nicotinic acetylcholine receptor (α7 nAChR) is involved in increasing FOXP3, but not IFN-γ expression in human T cells and the enzyme choline acetyltransferase (ChAT) which catalyzes the rate-limiting step in the synthesis of ACh, is required for self-regulation of FOXP3 and the activation marker CD25 following activation. Helios, a transcription factor and Treg stability marker is likewise transiently expressed by active Teffs and only FOXP3+/Helios-, IFN-γ+ Teffs proliferate throughout expansion. Taken together, my results indicate that ACh signaling *in vitro* enhances T cell activation and differentiation into antitumor Teffs, and this could be used for novel methods to increase the efficiency of current solid cancer therapies through manipulation of FOXP3 and/or IFN-γ.

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Acknowledgements

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Next, I would like to thank my supervisor Dr. Boulanger, as he was the person who initially connected me with Dr. Walter and Dr. Lum, which allowed me to get my foot in the door and introduce myself as a potential graduate student. Without Dr. Boulanger's continual connection and guidance, I would not have built the connections to be in the position I see myself in now.

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Chapter 1 – Introduction

1.1 - Prologue

The purpose of this thesis is to understand the impact of the neurotransmitter acetylcholine (ACh) on the complex signaling pathways involved in T cell activation to enhance solid cancer immunotherapies. The introductory chapter provides a background on ACh and the current knowledge as an immune-modularly molecule. I will first describe ACh signaling within the nervous system and other bodily systems. Second, I will describe the current knowledge on T cells and cholinergic signaling, highlighting outstanding questions for cancer treatments. Finally, I will discuss use for ACh signaling in T cells to tackle challenges in T cell-based cancer therapies.

In this introductory chapter, I extracted preliminary data from published work by a colleague and personal mentor in the Lum Laboratory, Dr. Marisa Kilgour. During my undergraduate studies as a directed studies student, I took Dr. Kilgour's published data and synthesized it further to form the foundation for my thesis. These results will be discussed near the end of the introduction.

1.2 - Acetylcholine

1.2.1 - What is acetylcholine?

Acetylcholine was the first neurotransmitter discovered[1,2] and is one of the primary excitatory signaling molecules in the central and peripheral nervous systems[3,4]. Acetylcholine is formed by transferring the acetyl group from acetyl coenzyme A (acetyl CoA, synthesized from glucose) onto the essential nutrient choline, found in foods such as meats, nuts, eggs, and some vegetables[2,5,6]. The enzyme choline acetyltransferase (ChAT) catalyzes the rate-limiting step in

ACh synthesis, and neurons expressing ChAT are considered "cholinergic"[7–9]. ACh signaling is primarily terminated by acetylcholine esterase (AChE) a transmembrane protein that hydrolyses ACh into choline and acetic acid[10]. After hydrolysis, choline is reabsorbed by cells to recycle back into ACh in preparation for the next signal transduction.

1.2.2 - Acetylcholine's primary function in the nervous system

ACh is involved in a diverse amount of signal pathways. It is the primary transmitter in the parasympathetic nervous system and preganglionic sympathetic nerves (postganglionic sympathetic nerves typically release norepinephrine), and is released at neuromuscular junctions where it transmits signals that cause muscle contraction[10,11]. ACh is rapidly released at cholinergic nerve synapses at high concentrations. Neurons release upwards of 3000 ACh molecules per vesicle into the synaptic cleft, and there can be up to a million ACh vesicles per axon terminal[5,12]. ACh subsequently travels the synaptic gap and binds to one of many acetylcholine receptors (AChRs) on the cell surface[5,12]. This binding initiates a cascade of signal transduction within the cell. ACh signals are rapidly terminated in the synaptic cleft by the enzyme AChE. AChE is a transmembrane protein commonly found near AChRs and is one of the fastest-acting enzymes in the human body, breaking down an ACh molecule every 60μs on average[13]. ACh signals are both transduced and terminated rapidly, as expected of nerve signals.

1.3 - Tumor innervation

A relatively new field of study is tumor innervation. Some recent studies identify high nerve infiltration in tumors with highly aggressive and proliferative cancer[14–17]. However, cholinergic nerve infiltration in tumors may have opposing results depending on the context.

For example, Hayakawa *et al*. identified that cholinergic nerve fibers promoted tumorigenesis and tumor cell proliferation in a feedback loop. Cholinergic stimulation of gastric epithelial cells by ACh promoted nerve growth factor (NGF) expression, which expanded cholinergic nerve growth and promoted cancer cell progression[18]. Treating gastric tumor cells with ACh directly promoted NGF expression and tumor growth[18].

On the other hand, parasympathetic nerve stimulation on human breast cancer xenograft mice suppressed tumor progression[15]. The xenograft mice had increased tumor burden when sympathetic neurons were instead stimulated. Furthermore, the researchers performed a retrospective analysis of 29 human breast cancer patients. They identified that increased sympathetic and decreased parasympathetic nerve infiltration in tumors was associated with high immune checkpoint molecules such as programmed death ligand 1 (PD-L1) and forkhead box P3 (FOXP3) and poor clinical outcomes[15]. PD-L1 binds to the programmed death protein 1 (PD-1) on adaptive immune cells and promotes programmed cell death, or apoptosis, in effector immune cells and activation of this pathway is a leading cause of cancer immunosuppression[15].

Likewise, tumor infiltrating nerves may control tumor infiltrating lymphocytes (TILs). The same researchers above noted that expression of checkpoint inhibitor molecules PD-1 and FOXP3 in TILs was reduced by either sympathetic denervation or parasympathetic stimulation[15]. Decreased expression of PD-1 and FOXP3 in TILs is a positive prognostic factor in patients[19–21].

This indicates that the type of nerve fiber infiltrating the tumor and the type of cancer are important in evaluating the clinical outcomes of nerve infiltration. These results require further investigation to identify the cause of the good or poor clinical outcomes. Elucidating mechanisms of tumor innervation on the immune system to manipulate existing immunotherapies may be key in improving clinical outcomes.

1.4 - Acetylcholine and the immune system

An emerging field is the cholinergic modulation of the immune system[7,22,23]. ACh has been identified in many cell classes outside of the nervous system, like immune cells[2,22–24]. Knowledge of ACh's function in immune cells is still not well understood. Many immune cell classes, including innate (macrophages, dendritic cells, natural killer cells) and adaptive (B cells, T cells) express AChRs, ChAT, and AChE following an immune response[2,22,25–27]. Furthermore, evolutionary conserved ChAT, AChE, and AChRs have been identified in unicellular eukaryotes, suggesting that ACh has a long evolutionary history[28].

Crosstalk between the nervous system and the immune system forms the study of neuroimmunology. Interestingly, cholinergic nerve cells can be modulated by immune cell receptors expressed on their own cell membrane. Cholinergic nerve cells can express major histocompatibility complex (MHC) and T cell receptors (TCR), and TCR engagement may reduce the activity and expression of specific AChRs[29,30]. There is a deep interconnectivity between the immune and nervous systems that open doors for a multitude of therapies ranging from neurodegenerative diseases such as Alzheimer's disease and Parkinson disease to autoimmune diseases such as multiple sclerosis, lupus, and potentially cancer[26,31,32]. This thesis focuses on the impact of cholinergic signaling on T cell function and its potential impact on cancer immunity.

1.4.1 – Adaptive T cells and nerve signals

The immune system has two main components - the innate immune system and the adaptive immune system. The innate immune system responds rapidly to foreign bodies but has less specificity, and the adaptive immune system has a slower response time but is highly

specific and confers long-term memory. T cells are a unique type of adaptive immune cell which develop in the bone marrow and mature in the thymus; they are key mediators of the adaptive cell-mediated immune response[33–35]. T cells carry out many functions in adaptive responses and are characterized by the expression of surface coreceptors known as cluster of differentiation (CD) molecules[36]. T cells are identified by CD3 expression, a cofactor in the TCR complex[36]. Typically, innate antigen presenting cells (APCs) like dendritic cells and macrophages activate T cells by phagocytosing foreign antigen and then processing the foreign proteins into small peptide fragments to present their MHC receptors, providing a link between the innate and adaptive immune systems. This is known as antigen processing and presentation.

T cells can be split into "helper" and "cytotoxic" classes that carry out distinct functions in the immune response. Cytotoxic T cells are identified by CD8 expression on their cell surface and are key contributors to cell-mediated adaptive killing of foreign cells; helper T cells are identified by CD4 expression and mediate signals by secreting cytokines to control and direct the immune response, activating other cells such as macrophages or suppressing an overreactive immune response[36,37]. Much of the adaptive immune response involves either direct or indirect action of T cells and are key targets in a multitude of immunotherapies.

In recent years, T cells have been identified as key mediators of the crosstalk between the nervous and immune systems[38–40]. T cells can respond and adapt external neurotransmitter signals released from proximal nerve or epithelial cells by producing cytokines and self-produced neurotransmitters[7,41]. T cells express both adrenergic and cholinergic receptors which control immune responses[7,32,42,43]. For example, norepinephrine (NE) stimulation of adrenergic receptors on cytotoxic T cells inhibits inflammatory cytokine release and infiltration into infected tissues; this has been linked to tumor progression due to an impaired immune response[44]. The

cholinergic signaling pathway has had limited study in T cells and will be discussed in the sections below.

1.5 - Current knowledge of acetylcholine's influence on immune system function

As mentioned, T cells both respond to and produce their own ACh following activation[45–47]. For example, activation of T cells with lipopolysaccharide (LPS) causes T cells to upregulate ChAT and AChRs, indicating that cholinergic signaling in T cells is "turned on" following activation[48]. AChE rapidly hydrolyses ACh so cholinergic signals usually are transmitted in short distances[5]. Communication systems in the body require a network of cells to propagate signals, of which T cells likely play a key intermediary or termination role[24]. Due to ACh's rapid hydrolysis by AChE, T cell-derived ACh is primarily utilized for autocrine or paracrine signaling of nearby cells exclusively[24,26,43]. T cells likely use self-derived ACh to modulate themselves and others such as blood vessel endothelial cells in a very short spatial distance[45,49]. Some cholinergic pathways mediated by T cells have been identified, but there is little known about this process. The following sections will describe some of the characterized functions of T cell-based ACh signaling.

1.5.1 - Cholinergic anti-inflammatory pathway

The most well-studied physiological process mediated by T cell cholinergic signaling is known as the cholinergic anti-inflammatory pathway (CAP)[32,32,43,50,51]. The CAP was originally discovered by Borovikova *et al*. in 2000[50]. They discovered that macrophages treated with ACh significantly reduced the production of inflammatory cytokines such as tumor necrosis factor

alpha (TNF-α), and direct stimulation of the vagus nerve in rats *in vivo* in response to endotoxins also resulted in lower TNF-α synthesis and prevention of lethal inflammatory shock[50]. The pathway has since garnered a significant amount of interest, and researchers have now identified that ChAT+ T cells are key sources of ACh in the CAP and T cell-produced ACh acts upon alpha 7 nicotinic acetylcholine receptor (α7 nAChR) subunits on macrophages to reduce inflammation[31,32].

It is now understood that vagus nerve stimulation during systemic inflammation in turn stimulates the splenic nerve, which causes the release of NE in the spleen that acts upon adrenergic receptors on T cells. T cells respond to NE stimulation by upregulating ChAT and releasing ACh, which subsequently acts upon the α7 nAChRs on macrophages to reduce systemic inflammation[42,44,48,51]. Through this pathway, T cells not only respond to neurotransmitter signals such as NE, but they respond by synthesizing their own ACh to control macrophages. T cells may control the immune response as an intermediary between nerve and immune cells. ACh-producing T cells act as an inhibitor of inflammation in this context. A summary of their findings is shown in **Figure 1** below.

Of note, a publication released in April 2023 has identified that ACh-producing T cells may be redundant in the CAP and NE can stimulate macrophages directly to achieve the same effect[52]. Therefore, the involvement of ACh-producing T cells in the CAP may be controversial. More research needs to be completed to replicate these findings.

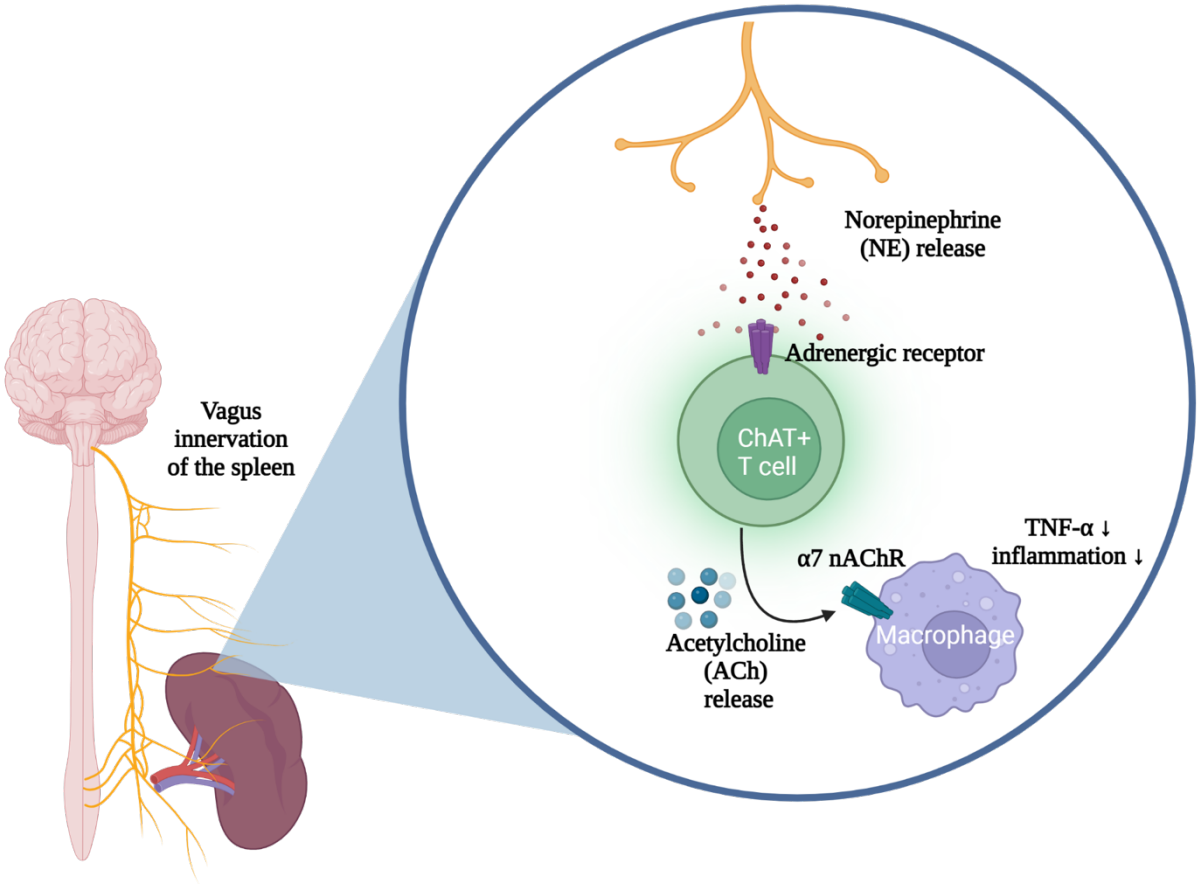

Figure 1. Representative diagram of the cholinergic anti-inflammatory pathway. Schematic of the release of norepinephrine (NE) from splenic nerves, which acts on T cells and causes upregulation of ChAT and release of acetylcholine (ACh). ACh then acts upon alpha 7 nicotinic acetylcholine receptors (α7 nAChR) on macrophages. Macrophages respond by lowering the production of tumor necrosis factor alpha (TNF-α) and causing ablated inflammation. Information in figure consolidated from multiple sources[32,43,51,53]

1.5.2 - ChAT+ T cells - vasodilation and viral infections

Vasodilation mediated by ChAT+ T cells was reported by Cox *et al*. in 2016 and they further elucidated this process in 2019[49,54]. The researchers tested the genetic knockout of ChAT in mice (ChAT-KO) and identified that ACh produced by T cells caused dilation of local blood vessels and reduced blood pressure[2,45,49,54]. The reduced blood pressure and blood flow from dilated vessels promoted the infiltration of T cells into viral-infected tissue to control infection. ChAT-KO T cells had difficulty infiltrating infected tissues and controlling viral infection. The researchers also determined that ACh synthesis by T cells was induced in an interleukin 21 (IL-21) dependent manner and that treatment with the vasodilator drug minoxidil restored viral control in ChAT-KO mice[49,54]. Here, ACh-producing cells act in an inflammatory-promoting function as they directly cause vasodilation to infiltrate infected tissues. A summary of their findings is shown in **Figure 2** below.

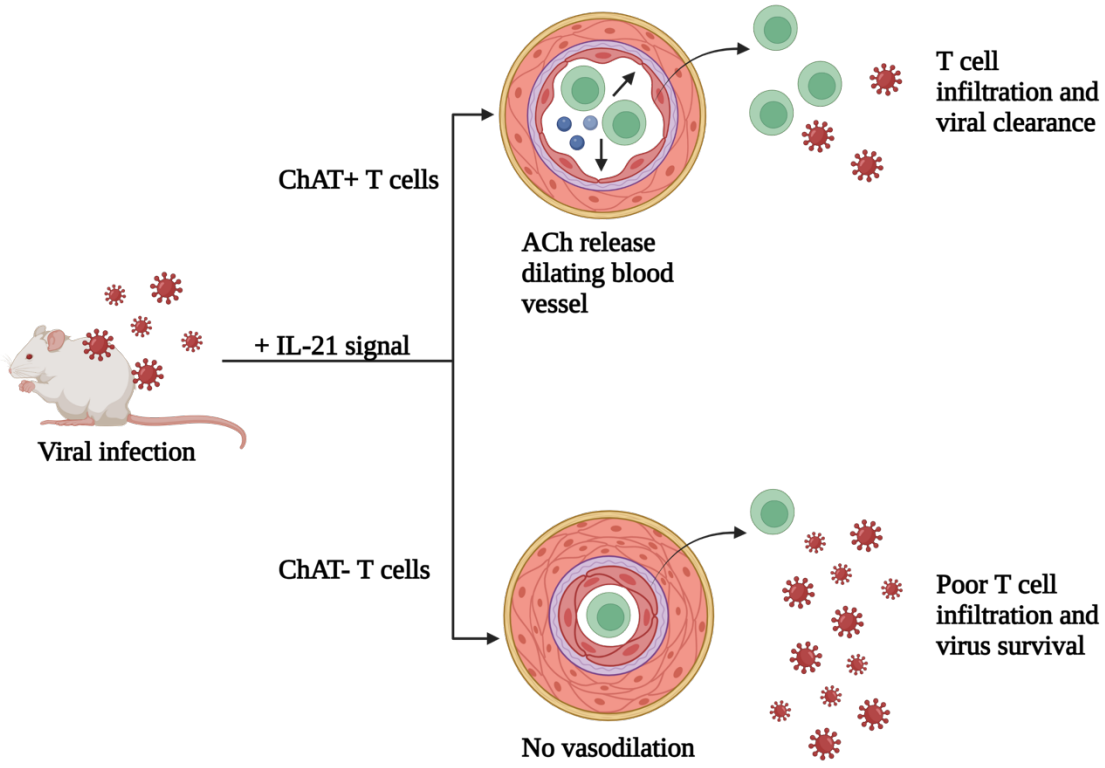

Figure 2. Representative diagram ChAT+ T cells causing dilation of blood vessels to infiltrate viral infected tissues. Schematic of the release of acetylcholine (ACh) from ChAT+ T cells, which causes dilation of blood vessels near infected tissues in mice that facilitates infiltration into infected tissues and viral clearance. ChAT- T cells had insufficient vasodilation and subsequently poor infiltration into infected tissues and the virus was allowed to persist. Information in figure adapted from the publication from Cox *et al*[49].

1.5.3 - Cholinergic signaling and mouse T cell differentiation

A few studies have laid the groundwork for the effects of cholinergic signaling on T cell activation and differentiation. Mashimo and Fujii *et al*. have published a few papers on cholinergic signaling and mouse T cell differentiation[7,27,41,55]. Their first experiments identified that stimulation of α7 nAChRs on mouse T cells resulted in an increase in expression of the transcription factor FoxP3 (similar, but not identical to human FOXP3) in CD4+ T cells, classified as induced Tregs (iTregs)[7,56]. The researchers also noted that α7 nAChR stimulation alone did not significantly change IFN-γ production. Therefore, they initially indicated that α7 nAChR signaling on mouse T cells may result in iTreg formation but not change IFN-γ production[7]. They then suggested that since α7 nAChRs in mouse T cells control the formation of immunosuppressive iTregs, they could be an option for therapies that wish to manipulate Tregs, such as autoimmune diseases or cancer.

However, follow-up studies indicate that α7 nAChR cholinergic signaling in murine T cells is more complicated and T cell phenotypes are dependent on activation conditions[41]. Mashimo and Fujii *et al*. later tested α7 nAChR signaling with different activation conditions to identify if mouse T cell differentiation is dependent on the method of activation. They investigated if a selective α7 nAChR agonist drug, GTS-21 (otherwise known as DMXBA), impacted mouse T cell differentiation under different activation conditions. They used ovalbumin (OVA, a protein found in egg whites and used to induce immune responses in culture), and antigen-presenting cells (APCs) such as dendritic cells and macrophages. They tested: 1) OVA antigen processing and APC-dependent activation of mouse T cells, 2) OVA peptide (OVAp) antigen processing-independent, APC-dependent activation of mouse T cells, and 3) APC-independent activation of mouse T cells using anti-CD3/CD28 antibodies.

The results of their study were quite interesting. They found that T cell differentiation was suppressed following OVA activation with GTS-21 but found the opposite effect from OVAp and anti-CD3/CD28 activation[41]. When OVA is added to culture containing mouse APCs and T cells, they found that GTS-21 suppressed antigen processing mechanisms in APCs and subsequently suppressed the activation and differentiation of mouse T cells. However, when pre-processed OVAp was added instead (and therefore antigen processing was not required), they found that differentiation into all T cell subsets were increased. Further, when APCs were removed and mouse T cells were instead activated with anti-CD3/CD28 antibody, they also saw that GTS-21 increased differentiation into all subsets – including FoxP3-expressing iTregs and IFN-γ-expressing effector T cells (Teffs)[41]. A summary of their findings is shown in **Figure 3** below. These disparate results indicate further context is needed when testing cholinergic signaling and T cells.

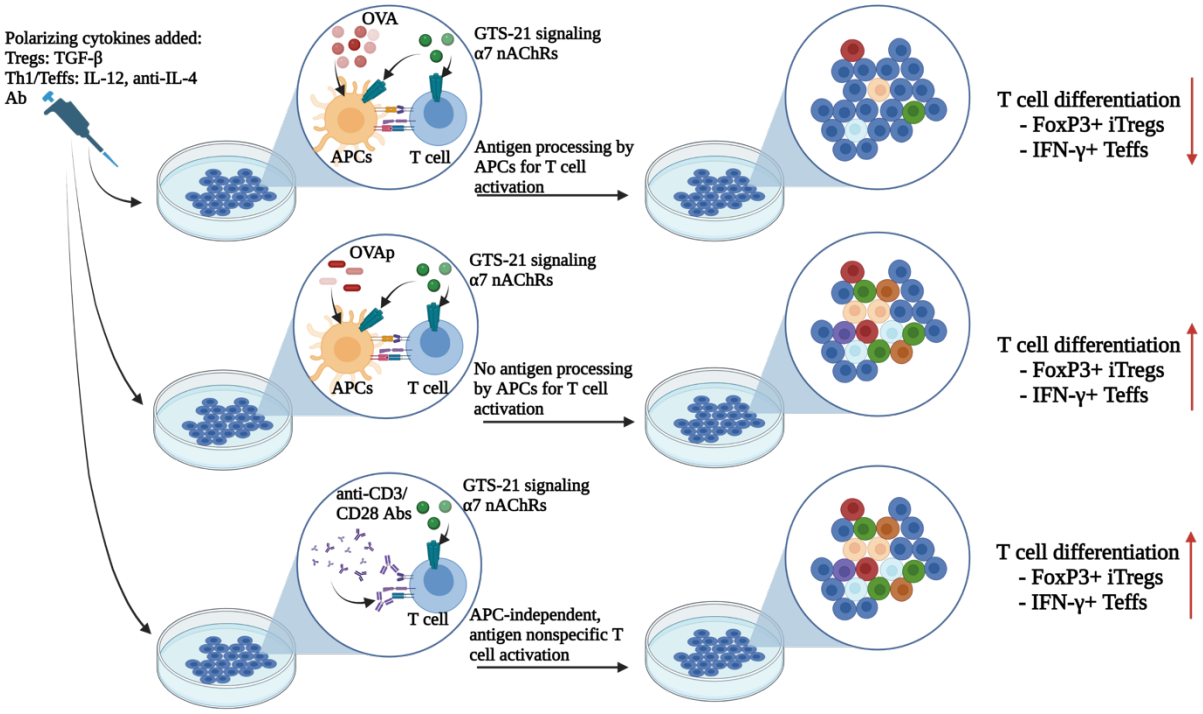

Figure 3. Representative diagram of α7 nAChR signaling on mouse T cell differentiation. Mouse T cells were activated under three possible conditions: Antigen presentation with full-length OVA requiring antigen processing (top), antigen presentation with pre-processed OVA peptides requiring no processing (middle), and antigen-nonspecific activation using anti-CD3/CD28 antibodies (bottom). All conditions were supplemented with GTS-21 and additional cytokines to polarize T cells into specific subclasses. Schematic detailing decreased T cell differentiation under the first condition and increased differentiation under the other two conditions. Abbreviations: TGF-β – transforming growth factor beta. Th1 – T cell helper class 1. Teffs – effector T cells. IL – interleukin cytokine. APCs – antigen presenting cells. α7 nAChRs – alpha 7 nicotinic acetylcholine receptors. CD – cluster of differentiation. Tregs – regulatory T cells. FoxP3 – forkhead box P3. IFN-γ – interferon gamma. Diagram represents a summary of the publication by Mashimo *et al*[41].

1.5.4 – Contextual Effects of cholinergic signaling on T cells

The effects of T cell stimulation and production of ACh, respectively, are highly dependent on outside factors and appear to have opposing effects depending on context. ACh production by T cells has anti-inflammatory effects by stimulating α7 nAChRs on macrophages but has a pro-inflammatory effect by promoting virus-infected tissue infiltration when acting on blood vessels[32,43,44,49,54]. Further, α7 nAChRs may decrease antigen processing by mouse APCs, which suppresses T cell differentiation, but mouse T cell differentiation was increased when antigen processing is not required[41]. As such, every study with cholinergic signaling and T cells need to carefully account for methodological context used before extrapolating results.

The multitude of effects on T cells are potentially novel in the clinical context. Control of Treg development has many implications in autoimmune diseases and cancer[33,57–59]. T cell-mediated control of tissue infiltration and inflammation, T cell activation state, and suppressive iTregs are all important in developing cancer treatments and will be the focus of my thesis. If ACh can control T cell-mediated immune response to cancer, this is a potential ground-breaking and relatively unexplored mechanism.

Finally, many studies on ACh signaling in T cells have been on mice and rat samples and few have extended into human T cells[27,45,54]. Many of these effects may be seen only in mouse and rat T cells and is not relevant in a human context. Therefore, validation in human T cells is vital before they can be extrapolated to cancer therapies.

Understanding the role T cells have in cancer responses essential to identify how ACh may be used to manipulate these processes. The following sections will discuss T cell anticancer functions and difficulties in detail to outline potential areas that ACh may be used to manipulate existing therapies.

1.6 - T cells and cancer

1.6.1 - T cell anti-cancer functions

As mentioned above, T cells play a key role in immune responses to cancer. T cells directly kill cancer cells through CD8+ cytotoxic T cell activity, where CD8+ cytotoxic T cells recognize damaged or altered MHC class I peptides on tumor cells and release granzyme and perforin to induce apoptosis[44,60,61]. T cells also act as key communicators that control and direct other immune cells primarily through the action of CD4+ helper T cell classes[36,62–64]. CD4+ helper T cells can be further differentiated by produced cytokines and expressed transcription factors. CD4+ T cell class differentiation is a complicated and detailed process. The key classes of T cells I will discuss in this thesis are suppressive regulatory T cells (Tregs) and an overarching term for T cells which express IFN-γ and promote immune clearance of tumors, deemed effector T cells (Teffs)[64–66].

Completely subdividing CD8+ and CD4+ T cell classes as if they had mutually exclusive functions in cancer responses is problematic. Some CD8+ T cells have been shown to contain suppressive capacity (CD8+ Tregs) whereas some preclinical and clinical studies suggest that CD4+ T cells have tumor cytotoxic properties[66–70]. Therefore, while my data segregate T cell populations into CD8+ and CD4+ groups to identify if they respond to ACh differently, I will be examining the regulatory and effector functions of both groups.

T cells control cancer responses through cytokine release as well as cell-cell interactions. IFN-γ released by Teffs promote the activation of macrophages to kill tumor cells and also increases MHC class I expression in cancer cells. This in turn promotes recognition by the CD8+ cytotoxic Teffs[71–74]. As such, IFN-γ-producing Teffs promote anti-tumor function. High

infiltration of active IFN-γ producing Teffs, including cytotoxic CD8+ T cells, is a good prognostic factor for cancer patients[61].

On the other hand, Tregs promote tumor growth by suppressing the function of immune cells through cell-cell surface antigen interactions and secretion of suppressive cytokines such as transforming growth factor beta (TGF-β) and interleukin 10 (IL-10), which reduce the effectiveness of Teffs[59,75,76]. Also, Tregs can out-compete Teffs for resources such as IL-2 (which is essential for the continuous activation and growth of T cells) due to elevated expression of the IL-2 receptor alpha chain (IL2RA, or CD25)[77,78]. By suppressing the action of other T cells and immune cells, Tregs promote the growth of tumors. High Treg infiltration into tumors is a negative prognostic factor[19,61,79–81].

1.6.2 - Suppression of T cell function within tumors

One of the hallmarks of cancer is metabolic reprogramming or changing cellular metabolism to suit their proliferative needs[82–85]. Cancer cells can change their metabolic processes to divert resources from other regions of the body and create an environment that is suppressive for immune cells[86–88]. For example, cancer cells commonly upregulate glycolysis, increasing uptake of glucose and secretion of lactate which suppresses immune cells function, such as inducing apoptosis in natural killer (NK) cells[88,89]. This process is otherwise known as the Warburg Effect[90]. This metabolic environment in solid tumors influences the infiltration and function of T cells, preventing them from entering the tumor microenvironment (TME)[73,81,86]. Furthermore, cancer cells often downregulate the expression of MHC I on their cell surface to escape detection by cytotoxic T cells[91,92]. Tumor cells can also reduce the effectiveness TILs by secreting immune-suppressing cytokines such as TGF-β or expressing inhibitory receptor ligands

on their membrane PD-L1[93–95]. This creates a TME that reduces the effectiveness of immune-based therapies. The precise mechanisms for hindered TIL infiltration, upregulation of immunosuppressive signals, and depletion of nutrients have seen breakthroughs, but much is still unclear. Essentially, cancer cells in solid tumors can reprogram their metabolism to out-compete other "healthy" cells in the body by monopolizing resources and subsequently creating an environment where they thrive and immune cells suffer, which causes a feedback loop that allows solid cancers to evade destruction by the immune system.

Figure 4 highlights a summary of the last two sections, detailing the functions of Tregs and Teffs and how the TME can change the delicate balance between these T cell subtypes. A question arises from these details, is tumor innervation and ACh contributing to immune modulation in tumors? What are current immunotherapies developed and how do they address the difficulties in treating tumors?

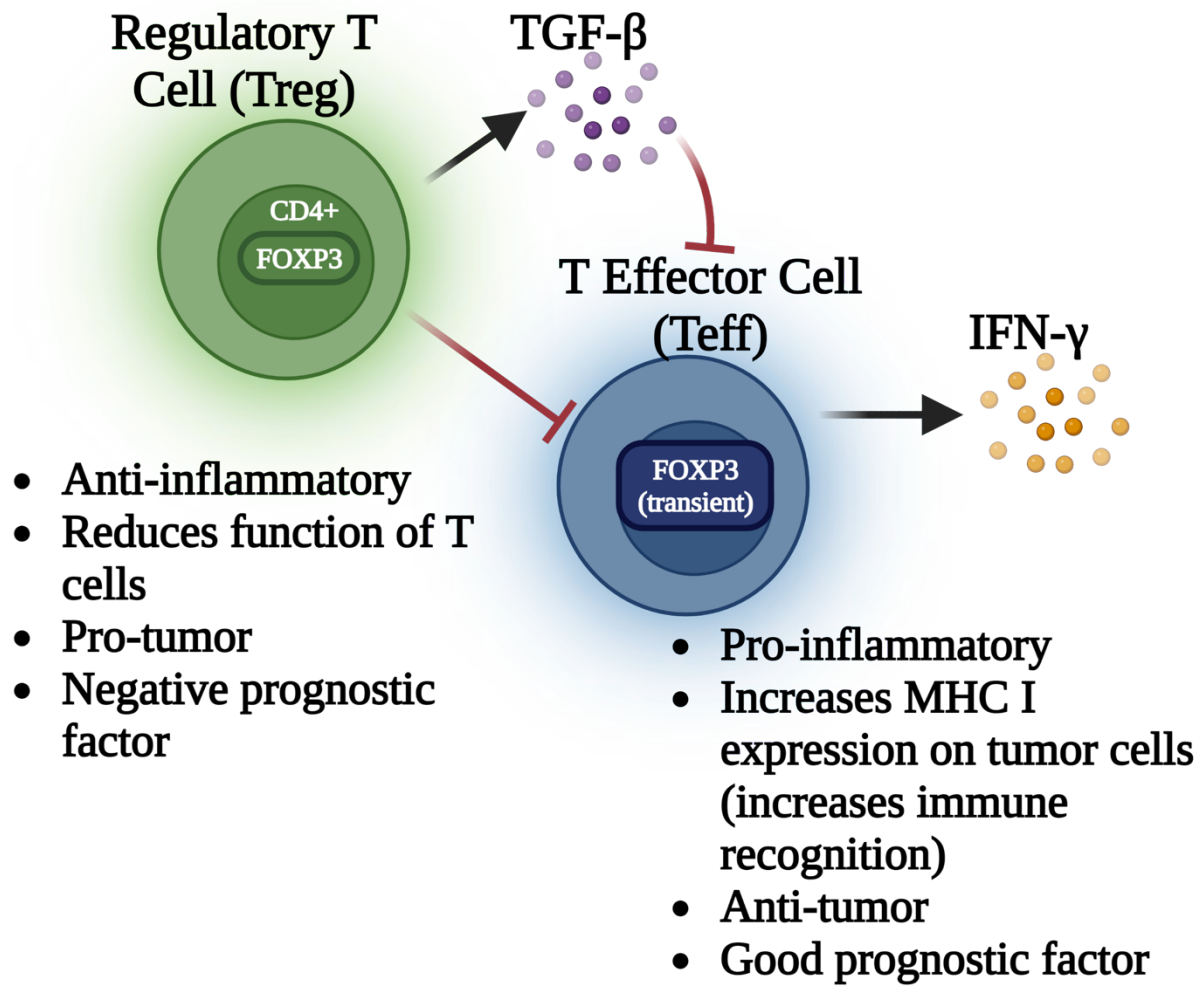

Figure 4. Representative diagram Tregs and Teffs and the balance affecting cancer prognosis. (A) Representation of Treg functions and suppression of Teffs through cell-cell contact or secretion of TGF-β. Teffs secrete IFN-γ and exhibit multiple pro-inflammatory, anti-tumor functions. (B) Representation of the change in the balance between Tregs and Teffs in the TME affecting the prognosis of cancer. Acronyms: TGF-β – transforming growth factor beta. TME – tumor microenvironment.

1.6.3 - T cell-based therapies to treat cancer – CAR-T cells

One of the greatest difficulties the immune system faces is the recognition of cancer cells. As discussed, tumor cells can escape immune recognition by downregulating MHC class I on their surface, often becoming "invisible" to cytotoxic T cells[92]. To combat this, T cells can be genetically engineered to express chimeric antigen receptor domains that allow them to recognize and kill tumor cells that they otherwise could not detect[96,97].

CAR-T cells are created by extracting a patient's T cells and genetically inserting a set of genes that cause the T cell to express what is known as a chimeric antigen receptor, or CAR. CARs contain a subset of complex functional domains: an extracellular antigen receptor domain that recognizes and binds to a known cancer antigen (for example, CD19), a transmembrane anchor, and costimulatory domains (often CD28 and CD3 costimulatory domains)[98,99]. CAR-T cells can bypass many limitations that T cells experience as they avoid antigen recognition by MHC class I. CAR-T cells can bypass the need for antigen recognition from APCs and directly activate by antigen recognition directly on tumor cells to kill them[96,97]. This allows CAR-T cells to recognize tumor cells more than naïve T cells which improve tumor clearance. Once CAR-T cells have been genetically modified to express the CAR, they are activated and cultured in a lab until they have grown enough to be re-infused back into the patient, at which time they should recognize and kill tumor cells expressing the chosen target antigen[100].

The typical methods for CAR-T cell generation either involve transfection *in vitro* with the genetic material containing the CAR using a viral vector such as lentivirus, or using the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and Cas9 (CRSIPR/Cas9) system to insert genes of interest[100]. Standard protocols for activation of CAR-T cells during cell culture *in vitro* use anti-CD3/CD28 antibodies[100].

Masimo and Fujii *et al*. showed that ACh stimulation (by the cholinergic drug GTS-21) with anti-CD3/CD28 antibody activation increased mouse T cell differentiation[41]. It is possible CAR-Ts may respond in the same way, however, there is very little research completed on this, which will be a key factor in my thesis.

Currently, only a handful of CAR-T cell antigens are approved by the Food and Drug Administration (FDA) to be used for the treatment of blood-based cancers such as leukemias and lymphomas[101,102]. As there are currently hundreds of CAR-T cell clinical trials ongoing, this number is expected to continue to grow. Furthermore, CAR-T cell therapies for solid cancers are currently being investigated, although the many difficulties discussed limit their effectiveness. Can ACh be used to manipulate CAR-T cell therapy?

1.6.4 - Current limitations of CAR-T cell therapies

CAR-T cells are highly effective in eliminating blood-based cancers such as leukemias but remain inefficient at treating solid tumors[96,103–105]. CAR-T cells still are subject to many of the difficulties solid cancers impair on the immune system, which inhibit their ability to attack solid cancers reliably. CAR-T cells are highly efficient at eliminating cells that express their target antigen, but have reduced effectiveness on cancer cells that downregulate them[104]. Furthermore, CAR-T cells may recognize target antigens on unintended healthy cells, causing an overreactive response and death of healthy cells instead of cancerous cells[106,107]. Therefore, the design of CARs by selecting an antigen that is expressed on tumor cells and not off-target cells is essential. This takes extensive time and resources before being able to be put in clinical trials[107].

Next, CAR-T cells also have difficulty in infiltrating the TME much like naïve T cells. Even after CAR-T cells have infiltrated the TME, the suppressive cytokines/metabolites and

immune cell classes (such as Tregs and tumor-promoting M2 macrophages), acidic conditions, and hypoxia reduces their effectiveness once inside the tumor[97,103].

While CAR-T cell therapy is an exciting and promising treatment option, it is not commercially affordable and widely available as an effective treatment for solid cancers. Understanding the mechanisms involved in the TME that suppress CAR-T cell function to design new approaches to create more robust, efficient, and effective responses is essential before CAR-T cell therapy can be a truly feasible option for solid cancers.

1.6.5 – Unknown links between CAR-T cell therapies and the nervous system – especially ACh

The link between CAR-T cell function and nerve signals is poorly understood. CAR-T cells can infiltrate off-target organ systems and release dangerous amounts of inflammatory signals, known as cytokine release syndrome (CRS)[107]. CRS occurs when CAR-T cells overreact and release cytokines to promote dangerous systemic inflammation which can damage healthy organs and lead to death. CRS is often followed by another CAR-T related side effect: immune effector cell-associated neurotoxicity syndrome (ICANS)[108]. ICANS often occurs when severe systemic inflammation disrupts the blood brain barrier, allowing immune cells to infiltrate the central nervous system where they disrupt nerve function and kill nerve cells[108].

Despite these known complications involving CAR-T cells and the nervous system, there is little understood about neurotransmitters and CAR-T cell function/toxicity. Treatments to manage CAR-T systemic toxicity, cancer efficiency, and tissue infiltration typically involve manipulating cytokines or checkpoint molecules, not neurotransmitter signals. It is unknown if ACh released by either nerve or ChAT+ CAR-T cells mediate any of these effects. The link

between ACh and T cell-mediated control of systemic inflammation, tissue infiltration, and T cell differentiation has yet to expand to CAR-T cells. These present opportunities for therapy development. Harnessing the mechanism ACh acts to manipulate CAR-T cells may be a novel approach to combat limitations in immunotherapies and improve clinical outcomes.

1.7 – Preliminary data

1.7.1 - Metabolically profiling the ovarian cancer TME

To identify novel metabolites contributing to the suppressive TME, our lab developed novel methods to measure intracellular metabolite abundance in TILs utilizing samples from ovarian cancer patients[109]. Late-stage ovarian cancer patients often present a fluid-filled abdominal region called "ascites" that contains tumorigenic factors such as growth factors, cancer cells, and immune cells[110,111]. This presents a unique opportunity to investigate differences between liquid cancerous environments (ascites) and solid cancers (ovarian tumors) while controlling for inter-patient diversity. The differences in both phenotype metabolism between immune cells in ascites and tumors in the same patient can be attributed to the differences between the environments instead of differences between patients. Kilgour *et al*. used magnetic bead isolation on extracted ovarian cancer patient samples to isolate CD4+ helper T cells, CD8+ cytotoxic T cells, and tumor cells (CD45-) within ascites and tumor environments[109]. They subsequently used a combination of bead isolation and liquid chromatography-tandem mass spectrometry (LC-MS/MS) to measure intracellular abundances of 99 target metabolites and compared them to matched patient-controlled pairs to assess differences in specific cell compartments.

Using these techniques, my colleagues were able to identify novel metabolites that potentially suppress the immune system within ovarian cancer. 1-methylnicotinamide (1-MNA) was identified as a significantly enriched metabolite in CD4+ tumor-infiltrating T cells (TIL-Ts) and was chosen as the metabolite of focus for Kilgour *et al*.'s publication and doctoral thesis. At the time, 1-MNA was relatively unstudied and Kilgour *et al*.'s work elucidated its immunosuppressive functions[109]. Due to the focus on 1-MNA by my colleagues, there are many other possible candidate metabolites that have yet to be identified.

1.7.2 – Acetylcholine's presence within the TME

During my undergraduate directed studies and before beginning my graduate thesis, I worked with colleagues in the Lum Lab to take Kilgour *et al*.'s published dataset and analyze it further to identify if more novel metabolites are enriched in the TME. I took the dataset and analyzed it similarly to their publication. I compared the relative change in metabolite abundance of metabolites in each cell subset between ascites and tumors. By comparing the degree of change in the abundance of metabolites between matched-patient pairs in specific cell compartments, I could identify metabolites and their pathways that are enriched in ovarian cancer which can be promising candidates for research, akin to finding the "next" 1-MNA for our laboratory.

I identified ACh as a metabolite that was enriched in T cells infiltrating the solid tumors of ovarian cancer patients **(Figure 5B)**. ACh was significantly intracellularly enriched in helper CD4+ TILs. Interestingly, ACh had the 2nd-highest fold change in abundance in CD4+ TILs, second only to 1-MNA[109]. Furthermore, ACh was comparably enriched to the same degree that other well-studied inhibitory metabolites such as kynurenine and adenosine were in Kilgour *et*

al.'s dataset[112–118], which further reinforces that ACh may be a significant, yet understudied metabolite in cancer immunotherapy. Also, choline (the precursor metabolite to ACh) was not significantly enriched in any cell tumor cell compartment **(Figure 5C)**, indicating that choline levels are not changed but ACh either is formed or taken up by T cells externally.

Given that ACh is currently understudied as an immune-modulating metabolite, tumor innervation has controversial impacts on tumor prognosis and progression, and we have measured significantly enriched amounts of ACh within TIL-Ts of ovarian cancer patients, ACh was selected as the target molecule for my thesis.

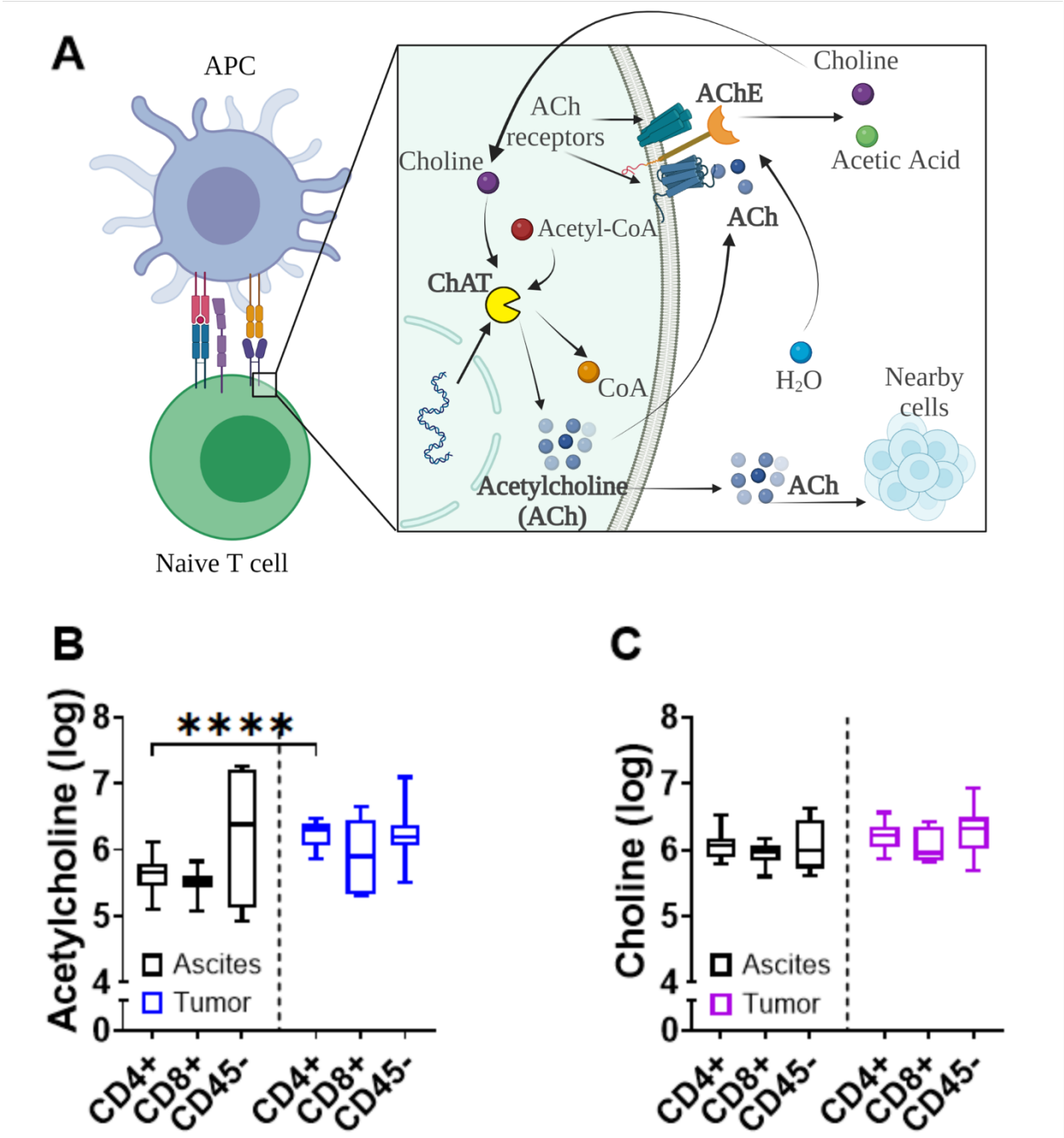

Figure 5. ACh production by T cells and intracellular acetylcholine abundance in cell compartments in ovarian cancer patient samples. (A) Schematic diagram of ACh production in T cells following activation signals. Graphs represent logarithmic abundance of ACh (B) and ACh precursor choline (C) within CD4+, CD8+, and CD45- cells within ovarian cancer patient ascites (black) and infiltrating tumor (blue and purple, respectively) compartments. Graphs represent medians (lines), interquartile range (hinges), and minimum to maximum (whiskers). (n=6, Student's paired t-test. ****p<0.0001).

1.8 – Summary and Rationale

Acetylcholine is a widely studied neurotransmitter, however, its function is not limited to the nervous system. ACh signaling has been found to modulate various cell types, especially immune cells. One of the key lymphocytes modulated by ACh are T cells. Some functions of T cells controlled by ACh includes the CAP, tissue infiltration, and T cell differentiation. ACh signaling on T cells appears to be immune-suppressive or promoting based on the context involved. Preliminary data published from our lab has metabolically analyzed TME and ascites environments of ovarian cancer patients, and I identified ACh as a significantly enriched metabolite within TILs. Solid cancers like ovarian cancer are highly immunosuppressive and impair current immunotherapies, there is a pressing need to improve current techniques and possibly design new ones. ACh has been shown to modulate immune function, potentially inducing differentiation of T cells into suppressive iTregs. Since our lab has found increased levels within the cancer environment, ACh may contribute to immune cell difficulties and elucidating the effects and mechanisms involved is crucial.

CAR-T cells are a ground-breaking immunotherapy development, but there are many unanswered difficulties to address before they are feasible for a wide range of solid cancer types. ACh's function in CAR-T cells is unknown, but given that ACh has been shown to manipulate immune function and ACh is significantly elevated in the immunosuppressive TME, is it possible that ACh is suppressing T cell function? By understanding the effect of ACh on T cell regulatory function, I seek to provide the groundwork for interventions to improve cancer therapy and hopefully save patients that have unresponsive, difficult to treat cancers.

1.9- Hypothesis

Acetylcholine signaling promotes human T cell differentiation into T regulatory cells and this impairs effector T cell function in cancer.

1.10 - Objectives

1. Determine the immune-modulating function of ACh on human T cell function through:
    a. ACh regulating the induction of transcription factors FOXP3 and Helios (Chapters 2 and 3, respectively).
    b. ACh regulating the production of cytokines IFN-γ and TGF-β. (Chapters 2 and 3, respectively)
2. Determine how ACh impacts the cytotoxic function of CAR-T cells (Chapter 2).
3. Evaluate the mechanism of ACh supplementation on the differentiation of T cells (Chapter 3).

Chapter 2: Acetylcholine enhances, rather than suppresses human T cell effector function, and improves tumor cell cytotoxicity

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R.G., J.J.L., and M.K. initially conceptualized the project. R.G. conducted experiments after initial mentorship and training provided by laboratory members. M.K. and S.M. mentored R.G. in T cell culture and flow cytometry. T.Z. mentored R.G. in western blot. G.K. mentored R.G. in CAR-T co-culture luciferase assay. Lentivirus for transduction of folate receptor alpha (FRα) CAR-T cells was provided by S.M. and V.C.

J.J.L, R.G., M.K., G.K., and T.Z. contributed 40%, 40%, 10%, 5%, and 5% respectively in experimental design and scientific suggestions.

2.1 - Abstract

Acetylcholine is a widely ubiquitous neurotransmitter, involved in a vast amount of signaling pathways in and out of the nervous system. Primary data from our lab shows ovarian cancer TILs contain elevated ACh. ACh production and signaling have been identified in lymphocytes, although phenotypic effects are still poorly understood. In this chapter, my data will show ACh signaling influences T cells to express the transcription factor FOXP3, which is often linked to a regulatory phenotype. However, my data show ACh signaling also increases IFN-γ production, which has multiple antitumor, immune-promoting properties. These data indicate multiple effects that ACh exhibits on T cells which may be used to manipulate immune responses. Furthermore, the generation of tumor-killing folate receptor alpha (FRα) CAR-T cells in the presence of ACh enhances their ability to recognize and kill SKOV3 ovarian cancer cells *in vitro,* paving the way for novel methods to increase the efficiency of current solid cancer therapies.

2.2 - Introduction

2.2.2 - T cell functions in detail

As discussed in Chapter 1, T cells have many functions in the immune response. Upon stimulation with antigen, T cells begin a complex signaling cascade that will cause differentiation into a multitude of subclasses with different functions. T cell differentiation can be influenced by the strength of the activation signal (i.e. the strength of antigen binding) and additional costimulatory signals such as CD4, CD8, or CD28, plus different cytokines[119–121]. Effector T cells actively fight against foreign pathogens and cancer cells and can be further subdivided into T helper subtypes such as type 1 (Th1), helper type 2 (Th2), or Th17[122,123]. Also,

T cells can differentiate into Tregs that control the degree of the immune response and aid in turning down immune cells after the pathogen is cleared out[76]. The delicate balance of T cell differentiation needs to be finely tuned to choose the appropriate response to specific pathogens or cancer. Disruption of this balance can have severe consequences as an immune response may not be strong enough to clear a pathogen, or the response will be too strong and have negative consequences as overreacting T cells can damage healthy tissues[124,125]. Dysregulation of this balance is especially apparent in cancer, and therefore understanding how to improve this is key to developing holistic cancer treatments.

Effector T cells and IFN-γ

As discussed, CD4+ T cells can be subdivided into distinct helper subclasses and they have different functions depending on the degree of response required and the type of pathogen. For cancer, Teffs that secrete IFN-γ are key[71,72,74]. IFN-γ coordinates immune responses in multiple ways. My focus is on its ability to promote antitumor functions of T cells and macrophages[126,127]. IFN-γ promotes differentiation into Th1 T cells that secrete IFN-γ (providing a positive feedback loop) and promotes differentiation into type M1 macrophages that aid in inflammatory responses that have anti-tumor properties[128]. Furthermore, IFN-γ promotes the expression of MHC class I molecules on cancer cells, increasing recognition and therefore destruction of tumors by cytotoxic T cells. IFN-γ may also prevent tumor-associated angiogenesis[129]. IFN-γ is a key cytokine used to identify Teffs that have antitumor properties and therapies that increase the production of IFN-γ often improve cancer responses[72,127,129].

Other than CD4+ Teffs, activated CD8+ cytotoxic effector T cells also secrete high levels of IFN-γ[130]. Therefore, measurement of IFN-γ production by activated T cells is indicative of CD4+ and CD8+ effector T cell activity.

Regulatory T cells

Aside from Teffs, another key T cell subclass is Tregs. Opposing Teffs, Tregs' main function is to control and suppress the immune response[76]. Tregs control the immune response by suppressing the activity of Teffs, macrophages, dendritic cells, and many other immune cells. Typically, Tregs control immune responses by direct cell-cell contact or secretion of inhibitory cytokines such as TGF-β[75].

Circulating human Tregs are typically indicated by CD4+ T cells expressing the transcription factor forkhead box P3 (FOXP3)[20,131]. Activated suppressive Tregs usually express high levels of the interleukin 2 receptor alpha chain (IL-2Rα, or CD25), although it is usually used in combination with FOXP3 to identify Tregs as activated Teffs express CD25 as well[132].

Tregs dampen immune responses and are key in controlling overreactive immune responses and preventing autoimmune diseases such as lupus. They are an essential component of a healthy functioning immune system[31,133]. However, a substantial skew in the Teff/Treg balance towards Tregs can cause an immune system to be ineffective at clearing a pathogen. Monitoring the balance between Teffs and Tregs in cancer is a key indicator of good or poor outcomes[125,134]. Cancer cells often secrete cytokines like transforming growth factor beta (TGF-β) that promote Treg differentiation to control immune responses and promote tumor growth[135]. Understanding mechanisms that contribute to Treg differentiation by cancer cells and overcoming this is important in building cancer therapies.

Mouse FoxP3 and human FOXP3 in T cells - differences and similarities

It is common for researchers to mislabel the mouse FoxP3 and human FOXP3[131,136]. Typically, FoxP3 refers to the gene and protein expressed in mice, while FOXP3 is expressed in humans[136]. Mouse FoxP3 and human FOXP3 share about a ~86% similarity[137] and both are used to identify Treg cells[131]. FOXP3 is a master transcription factor regulating the development of natural Tregs (nTregs) in the body. Disruption of expression of FOXP3 through mutations or other methods leads to dysregulation of Treg subtypes and development of various autoimmune diseases such as Immune Polyendocrinopathy, X-linked (IPEX) syndrome[138].

FOXP3 can act as either a transcription activator or suppressor depending on acetylation or histone modification state[139]. FOXP3 binds to multiple genetic loci to inhibit or promote transcription, eventually leading to Treg lineage commitment during development in the thymus[136,137]. While FOXP3 is labelled as the "master transcription factor" determining nTregs circulating in the body, FOXP3 is also transiently expressed by activated conventional T cells (Tconvs) and Teffs under cell culture conditions *in vitro*[136,137,140]. Solely using FOXP3 as a marker for induced Tregs (iTregs) derived from naïve human T cells in cell culture is not optimal. Transient expression of FOXP3 *in vitro* has also been shown to reduce T cell proliferation and influence cytokine production in Teffs and Tconvs without indicating Treg phenotypes[141]. Activation-induced FOXP3 may slow T cell proliferation to modulate immune responses and control the phenotype of Teffs[136].

Human FOXP3 has been found to exist in alternatively spliced isoforms, and despite the sequence similarity between mouse FoxP3 and human FOXP3, alternatively spliced mouse FoxP3 has not been found[136]. FOXP3 in humans is predominantly found in two isoforms - the full-length FOXP3 isoform containing all exons (FOXP3fl) and the isoform of FOXP3 lacking

exon 2 (FOXP3Δ2), together making up almost 95% of total expressed FOXP3[136]. There also exists a third isoform lacking exon 2 and exon 7 (FOXP3Δ2Δ7), however, it is expressed in very small amounts, sometimes undetectable and its function is not widely understood[136]. The exon 2 locus in FOXP3fl protein encodes for a nuclear transport protein and may facilitate the export of FOXP3 into the cytoplasm[142]. Since FOXP3 is a transcription factor, it is expected that nuclear localization is essential for its function, and therefore transport out of the nucleus would reduce Treg formation. However, studies have been split on the functional use of FOXP3fl and FOXP3Δ2 isoforms. Sato *et al*. identified that co-expression of both isoforms was required for stable, suppressive Tregs and found that loss of either isoform conferred less suppressive Tregs and ablated latency associated peptide expression (LAP, a protein complexed with TGF-β)[143]. Further, other researchers have proposed that Th17 cells primarily express the FOXP3Δ2 isoform since exon 2 has been shown to associate with and block expression of Th17 transcription factors retinoid acid receptor-related orphan receptors (ROR)-α and ROR-γt. However, *ex vivo* analysis indicates that Th17 cells are more associated with FOXP3fl[136]. it appears that FOXP3fl and FOXP3Δ2 isoforms may have overlapping functions and their overexpression due to *in vitro* activation conditions may lead to unintended associations[136].

With all these caveats considered, FOXP3 expression *in vitro* from a top-down, high-level perspective is not sufficient to fully identify its function in Tregs and other functional markers or mechanisms need to be considered before *in vitro* cultured T cells can be considered iTregs. Nonetheless, FOXP3 remains the most robust, yet flawed, cellular marker currently understood to delineate Treg populations. More importantly, the differences between mouse FoxP3 and human FOXP3 highlight the need to validate results from mouse studies in humans before any conclusions can be extrapolated. Just because an experiment shows induction of

mouse FoxP3 *in vitro*, this cannot be immediately transferred to the human context to conclude that stable human iTregs can be generated through the same methods. This difficulty in defining stable iTreg from an activated Teff in culture is a major factor in the beginning stages of my thesis and will be highlighted in my results.

2.3 - Methods

2.3.1 - T cell isolation and Cell Culture

A visual summary of cell culture methods is shown in **Figure 6** below. Healthy naïve T cells were isolated from healthy donor peripheral blood mononuclear cells (PBMCs), supplied by StemCell Technologies. T cells were isolated from healthy donor PBMCs using CD3-conjugated MACS microbead column separation kits (Mylteni Biotec) according to the manufacturer's protocols. T cells were cultured in StemCell Technologies' ImmunoCult-XF T cell Expansion medium (1% Penicillin/Streptomycin (Hyclone) added), supplemented with 300U/ml IL-2 (eBioscience), as per the manufacturer's instructions. T cells were activated with 25µl ImmunoCult Human CD3/CD28 T Cell Activator antibodies. 1e6 T cells per sample were cultured at 1ml/sample in 48-well tissue culture plates at 37°C and 5% CO_2 for 3 days. After 3 days, samples were expanded 8-fold by transferring to 6-well tissue culture plates or T25 cell culture flasks and supplemented with fresh ImmunoCult media and IL-2. Samples were then cultured for an additional 3 days prior to analysis on day 6. For experiments extending beyond 6 days, cell samples were split 4-fold every two days (i.e., days 6, 8, and 10) with additional IL-2 and fresh ImmunoCult medium each day of splitting.

ACh administration

To test the effects of ACh on T cell function during activation, ACh-treated T cell samples were initially supplemented with a dose response of 0-10mM acetylcholine (chloride) (Cayman Chemical Co.) after isolation and activation as described above. Cells were assessed for proliferation, viability, FOXP3 expression, and IFN-γ expression to determine optimal dosage. Results shown in **supplemental Figure 25 in the Appendix.** 10mM ACh was determined to be the optimal ACh dose tested and was used in all subsequent experiments. ACh was administered into sample media after activation antibodies and IL-2 was added. Control samples received activation antibodies and IL-2 but no additional ACh. Unless otherwise stated, ACh-treated samples were re-supplemented with fresh ACh on every day that cells were split or media was added (i.e., days 0, 3, 6, 8, and 10) to maintain a constant concentration of 10mM.

TGF-β administration

For TGF-β supplementation experiments, 2.5ng/ml mature active human TGF-β1 (premium grade, Mylteni Biotec) was also supplemented into T cell culture media after CD3/CD28 activation antibodies and IL-2 was added. "No TGF-β" samples received no additional supplementation of TGF-β, "TGF-β" samples received TGF-β but no additional ACh. "ACh+TGF-β" samples received both TGF-β and 10mM ACh.

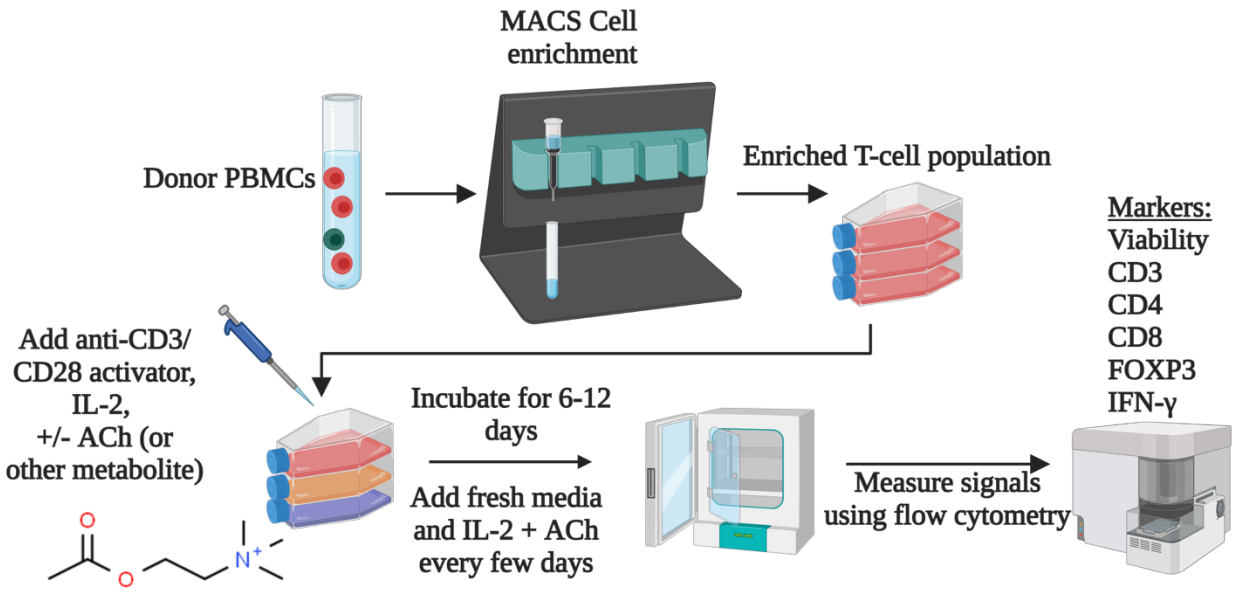

Figure 6. Representative diagram of T cell acetylcholine culture methods. Schematic diagram outlining MACS microbead enrichment of naïve T cells from healthy donor peripheral blood mononuclear cells (PBMCs). After enrichment, T cell populations are activated with anti-CD3/CD28 activator and supplemented IL-2 with or without additional ACh (or other metabolites as stated in other sections of this thesis). Cell media is refreshed every 2-3 days, adding fresh new IL-2 and ACh in ACh-supplemented samples. After 6 or 12 days of culturing, T cells are stained and analyzed through flow cytometry.

2.3.2 - Flow cytometry

To activate the production of cytokines for intracellular staining through flow cytometry, samples were incubated with 20ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich), 1µl/ml ionomycin (Sigma-Aldrich), and 1x GolgiStop™ (BD Biosciences) for 4 hours at 37°C and 5% CO_2. Next, samples were washed with phosphate-buffered saline (PBS). Next, samples were incubated with 1:5000 eBioscience™ efluor™ 450 fixable viability dye (ThermoFisher) for 15 minutes at 4°C and then fixed and permeabilized with eBioscience FOXP3/transcription factor staining buffer set (ThermoFisher) according to the manufacturer's protocols. Samples were then stained with fluorescent-labelled antibodies for cell markers, transcription factors, and cytokines according to **Table 1 in the Appendix.** *Please note*: unless otherwise stated, the FOXP3 antibody used is from clone 259D/C7, which recognizes all FOXP3 isoforms. Therefore, FOXP3 flow cytometry staining measures the expression of both the FOXP3fl and FOXP3Δ2 isoforms. All data were collected on a Cytek Aurora flow cytometer and analyzed using FlowJo version 10.8 software (Tree Star Inc.).

2.3.3 - Gating strategy

To measure samples through flow cytometry, the following gating strategy was used. Single cells were separated based on viability, CD3 positivity, and CD4 vs CD8 positivity. Then, cells expressing IFN-γ and FOXP3 could be measured. **Figure 7** below visually represents the gating strategy flowthrough used to identify viable CD4+ or CD8+ samples and IFN-γ and FOXP3-expressing T cells for Chapter 2.

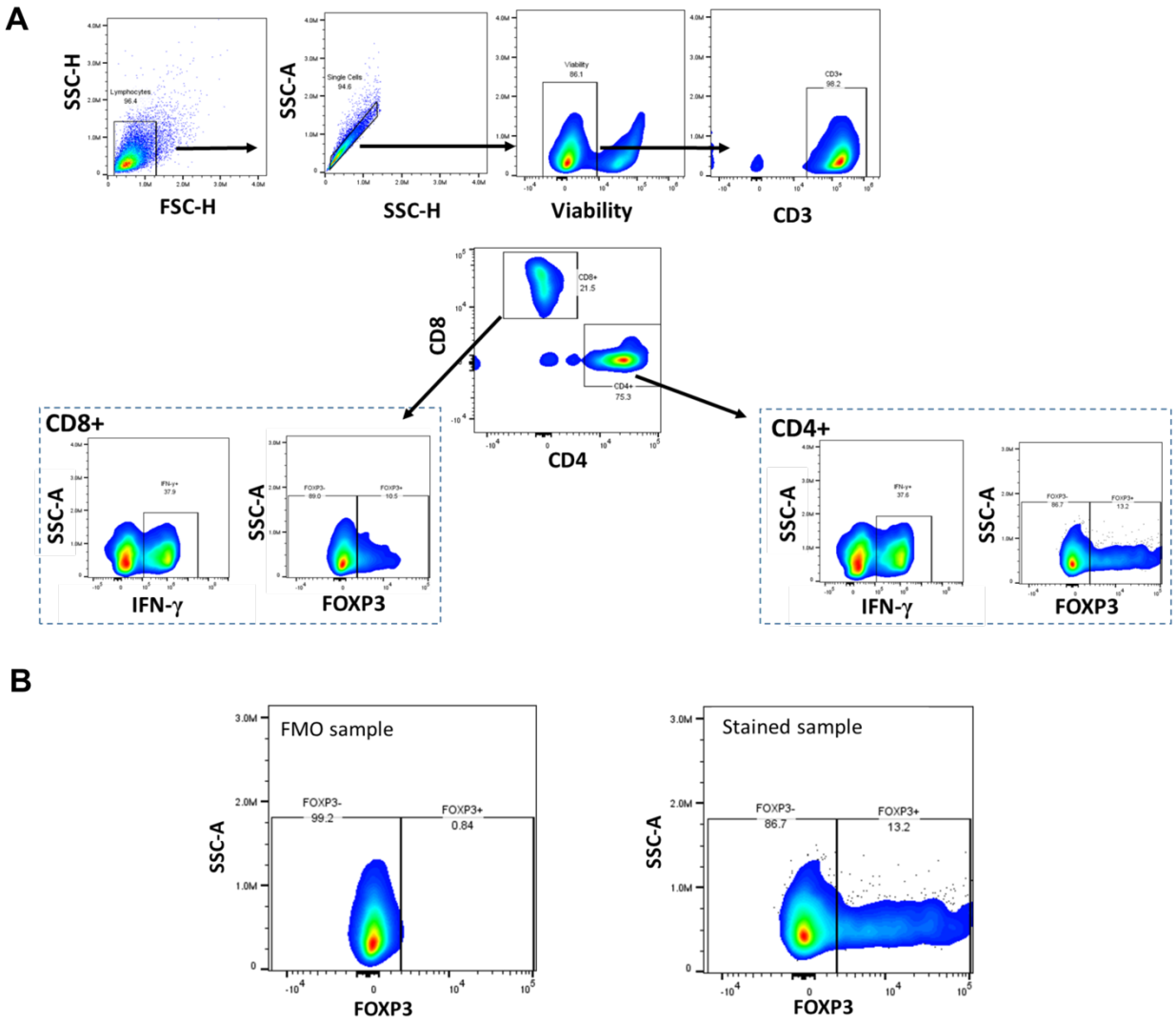

Figure 7. Gating strategy used for flow cytometry analysis. T cell samples were stained with antibodies according to **Table 1** in the Appendix and all data were collected on a Cytek Aurora flow cytometer and analyzed using FlowJo version 10.8 software (Tree Star Inc.). (A) Representative gating strategy showing methods to identify CD4+ and CD8+ T cells, and expression of IFN-γ and FOXP3. (B) Representative plots representing samples stained with all antibodies aside from FOXP3 (Fluorescence minus one, FMO) (left) and stained with all antibodies (right) to determine gates for positive and negative expression of FOXP3.

2.3.4 - Western blotting

Cells were pelleted using a microcentrifuge and aspirated. Cell samples were then lysed with RIPA buffer containing Pierce™ Protease and Phosphatase Inhibitor (ThermoFisher). Cell lysates were mixed with 10X NuPAGE™ Sample Reducing Agent and 4X LDS Buffer (Invitrogen) and heated for 10 minutes at 70°C. Samples were run on a pre-cast NuPAGE™ 4-12% Bis-tris gels (Invitrogen) in 1X MES Running Buffer prepared in-house. Samples were run for 60-75 minutes at 125V through the gels. Samples were transferred onto nitrocellulose membrane (Life Sciences) and in-house semi-dry transfer buffer. Samples blocked in Intercept Blocking Buffer optimized for *Odyssey* infrared imaging systems. Blots were incubated with the following rabbit primary antibodies for analysis at a 1:1000 dilution: β-actin, FOXP3, mTOR, and pmTOR (Ser2447) (Cell Signaling Technology®). Blots were incubated overnight on a rocking plate at 4°C and then washed 4 times in PBST buffer at room temperature and then incubated with 1:10,000 secondary antibody, washed, and then imaged using a LiCOR Odyssey imaging system.

2.3.5 - FRα-CAR-T Cell Generation

This protocol is adapted from a standard operating procedure (SOP) developed by Sarah MacPherson and Gabrielle Kowalchuk, my colleagues in the Lum Lab. T cells were isolated and expanded on day 0 in the presence or absence of 10mM ACh utilizing the same methodology outlined in *T Cell Isolation and Cell Culture* and *ACh Administration (2.3.1)*. On day 1 of T cell expansion, 1µl of lentivirus containing the FRα CAR domain and GFP reporter domain was added to the CAR-T samples for transduction. To measure CAR transduction efficiency on day 5, 1e6 of each CAR-T and untransduced control sample were taken from cell culture and stained

with viability and CD3 antibodies to be analyzed through flow cytometry using the protocol outlined in the *Flow Cytometry* section above. CAR-positivity rate for samples was measured through the expression of the GFP reporter gene. The CAR-positivity rate was then used for calculations for the co-culturing SKOV3 luciferase assay. CAR-T cells were cultured until day 6 before harvesting for co-killing assay. FRα lentivirus was developed and validated by colleagues in our laboratory Sarah MacPherson and Vanessa Chan and provided to me for this experiment.

2.3.6 - FRα-CAR-T Cell, SKOV-3 Luciferase Co-Killing Assay

This protocol is adapted from a standard operating procedure (SOP) developed by Sarah MacPherson and Gabrielle Kowalchuk, my colleagues in the Lum Lab. SKOV3 tumor cells expressing FRα surface antigen and luciferase were placed in a 96-well flat bottom opaque plate at 2e4 SKOV3 cells/well for 48 hours before co-culturing to allow the tumor cells to adhere to the plate. At day 6 of CAR-T cell expansion, the FRα T cells were taken out of culture and centrifuged at 1500rpm for 5 minutes and washed with PBS to remove all traces of T cell media. When the CAR-T cells were transferred into the co-culturing conditions, all trace of original T cell culture media was removed and no additional ACh was then added. Therefore, ACh was only present during the activation and differentiation stage of CAR-T cell development and did not interfere with the co-killing assay. This is to specifically test the influence of ACh on T cell expansion while controlling for unwanted/unexpected effects during tumor co-killing that may skew data.

After media removal, FRα CAR-T cells were transferred to the 96-well plate containing SKOV3 cells at effector to target ratios (E:T ratios) of 1:1, 1:2, 1:4, 1:8, 1:16, and 1:32. The E:T ratios were calculated using the CAR+ rate determined on day 5 of CAR-T cell expansion. For

example, if the CAR+ rate was 50% in the culture, then 4e4 T cells from the CAR-T cell culture (2e4 CAR+ T cells) were transferred to the 1:1 E:T samples, and so on.

The FRα CAR-T/SKOV3 co-culture was incubated at 37°C and 5% CO_2 for 48 hours before analysis. After 48 hours of co-culturing, 1X XenoLight™ D-luciferin (Cedarlane) was added to the samples and incubated for 5 minutes in the dark at room temperature. Luminescence then was immediately read using *Varioskan Lux* Plate Reader (ThermoFisher) with ScanIT RE 6.1 software. The viable (i.e. still living) SKOV3 cells would cleave the luciferin and fluoresce which would be readable by the plate detector. Therefore, the percent of CAR-specific killing of tumor cells could be measured by the reduction in fluorescence in co-cultured samples compared to SKOV3 cultures that contain no CAR-T cells. A visual representation is provided in **Figure 8** below.

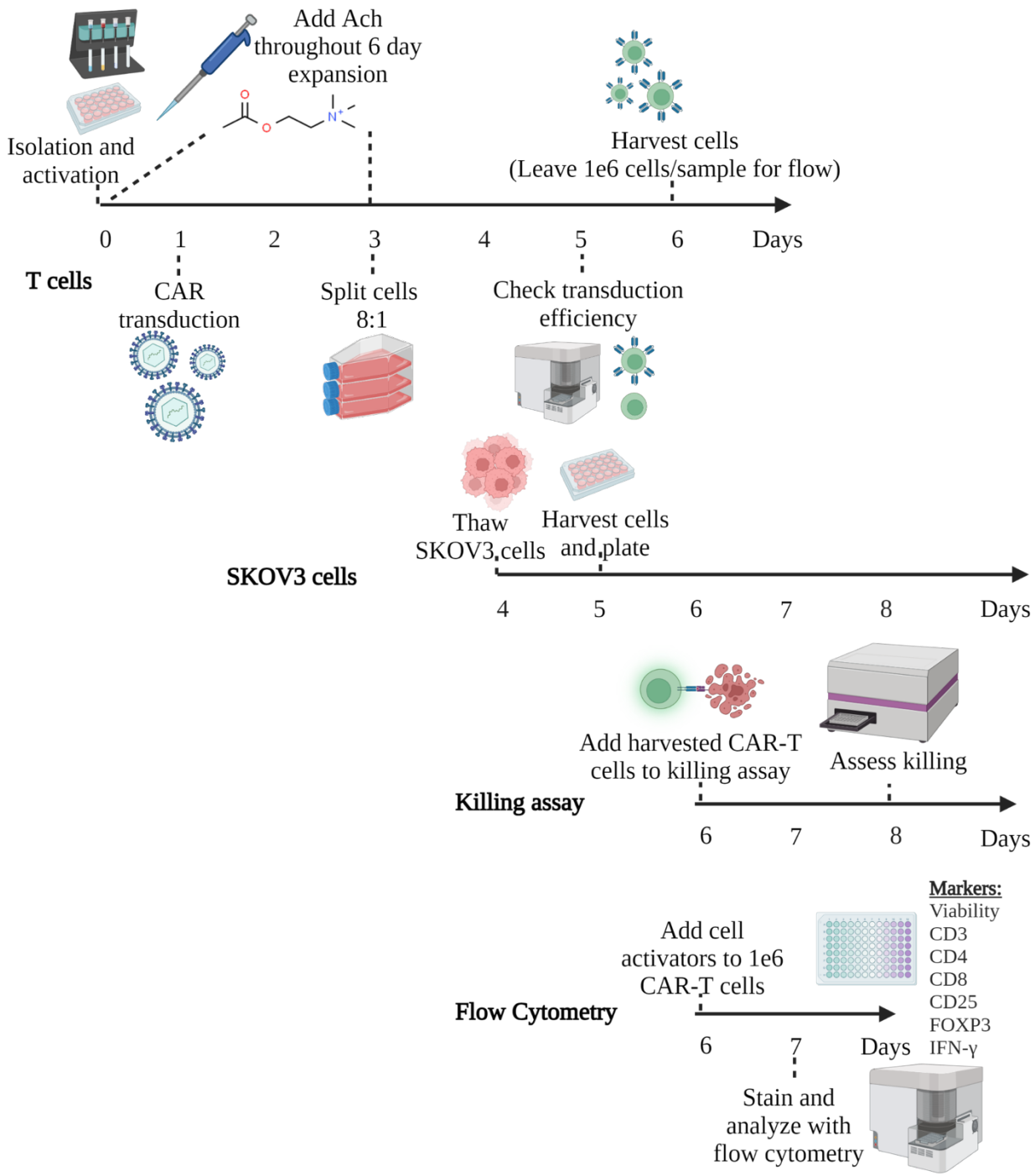

Figure 8. Representative diagram for FRα CAR-T cell generation and co-killing assay with SKOV3 tumor cells. The protocol is adapted from a standard operating procedure (SOP) developed by my colleagues in the Lum Laboratory Sarah MacPherson and Gabrielle Kowalchuk.

2.3.7 – Statistical analysis

Statistical analyses were completed using a student's paired *t*-test, a one-way paired ANOVA test, or mixed-effects model plus a Dunnett's post-test and Geisser-greenhouse correction for paired variances, where appropriate. Statistical calculations were completed using GraphPad Prism version 9.5 software. P values < 0.05 were considered statistically significant.

2.4 - Results

2.4.1 - ACh stimulation reduces T cell proliferation and slightly increases the viability

Healthy naive T cells were isolated and activated using the methods described in *T Cell Isolation and Cell Culture* and *ACh Administration (2.3.1.).* Day 6 cell counts in 10mM ACh-treated samples (ACh-T cells) reduced by 18.75% compared to 0mM ACh control samples (Control-T cells). Cell counts were 1.43e7 cells (SE = 6.2e6 cells) for ACh-Ts and 1.76e7 cells (SE = 7.2e6 cells) for Control-T cells, respectively **(Figure 9A)**. Cell viability was slightly increased in ACh-T cells compared to Control-T cells (86.64% (SE = 1.28%), and 84.78% (SE = 1.56%), respectively) **(Figure 9B).** These results are interesting as some previous publications investigating murine cells have shown conflicting results, suggesting ACh signaling may increase or decrease murine T cell proliferation depending on activation context[7,41].

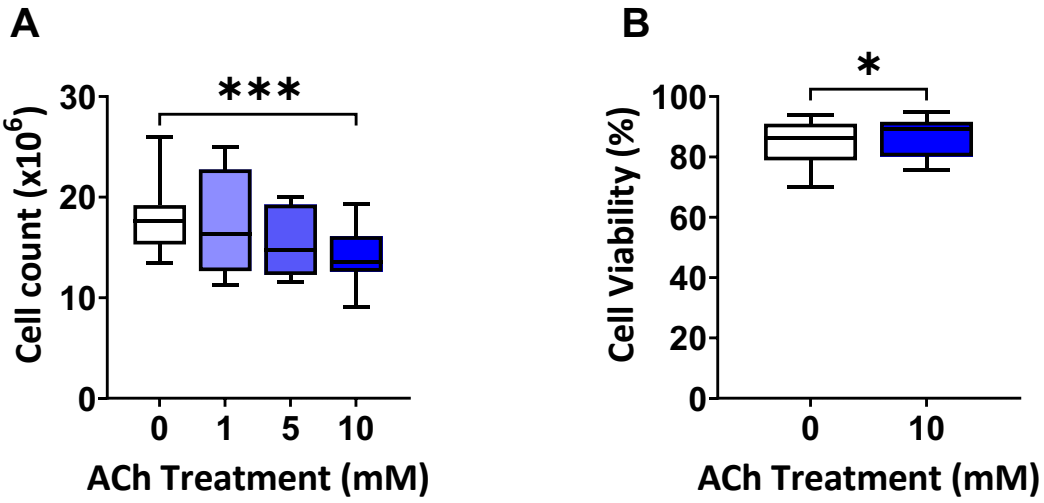

Figure 9. ACh suppresses T cell proliferation and slightly increases viability. Activated T cells were supplemented with IL-2 (300U/ml) in the presence or absence of ACh. (A) Cells were stained with trypan blue at a 1:1 dilution and counted with a hemocytometer and were supplemented with an ACh dose response from 0mM (white box) to 10mM (blue gradient boxes) 10mM ACh significantly reduced T cell counts at day 6. (B) Cell viability of the lowest (0mM, white) and highest (10mM, blue) ACh dose was measured through eBioscience™ efluor™ 450 fixable viability dye on the flow cytometer T cell viability was slightly increased by 10mM ACh. Graphs represent medians (lines), interquartile range (hinges), and minimum to maximum (whiskers). (n=17, Mixed-effects analysis, with Geisser-greenhouse correction and Dunnett's multiple comparisons test, with individual variances computed for each paired sample used for (A). Student's paired *t*-test used for (B). *p<0.05, ***p<0.001).

2.4.2 - ACh stimulation increases FOXP3 expression in both CD4+ and CD8+ T cells throughout expansion

To determine the effects of ACh signaling on FOXP3 expression during naïve T cell activation, healthy naïve T cells were isolated and activated using the methods described above. On day 6, T cells were stained with the flow cytometry cocktail described in the **Appendix** and run on the flow cytometer **(Figure 10A, B**).

FOXP3 expression was increased in both CD4+ and CD8+ ACh-T cell subtypes on day 6 and remained elevated on day 12 **(Figure 10C)**. FOXP3 expression in CD4+ ACh-T cells on day 6 was 11.14% (SE = 0.63%), compared to 6.99% (SE = 0.40%) in the Control-T cell population, a proportional increase of 59.42%. FOXP3 expression in CD8+ ACh-T cells on day 6 was 7.00% (SE = 0.62%), compared to 4.43% (SE = 0.37%) in the Control-T cell population, a proportional increase of 58.17%.

On day 12, FOXP3 expression in ACh-T cells remained elevated. FOXP3 expression in CD4+ ACh-T cells on day 12 was 17.38% (SE = 1.89%), compared to 13.06% (SE = 1.39%) in the Control-T cell population, a proportional increase of 33.08%. FOXP3 expression in CD8+ ACh-T cells on day 12 was 15.28% (SE = 1.50%), compared to 12.39% (SE = 1.45%) in the Control-T cell population, a proportional increase of 23.34%. These results were consistent with publications on murine cells, indicating that human and murine T cells respond to ACh and induce FoxP3/FOXP3 similarly[7,41].

To determine the length of ACh signaling time required to induce elevated FOXP3 expression in T cells, naïve T cells were isolated from healthy donor PBMCs and activated with anti-CD3/CD28 antibodies and IL-2 with no ACh for six days to recover from freezing. After 6 days, samples were then re-activated with plate-bound anti-CD3/CD28 antibodies, IL-2, and in

the presence or absence of 10mM ACh for 48 hours. After 24 or 48 hours, T cells were stained for flow cytometry. FOXP3 expression in re-activated CD4+ and CD8+ ACh-T cells was significantly elevated compared to controls at 48 hours **(Figure 10D).** FOXP3 expression was not significantly increased at 24 hours. FOXP3 expression in ACh-T cells was proportionally increased by 30.66% (CD4+ population) and 19.42% (CD8+ population) after 48 hours compared to control cells. FOXP3 expression in ACh-T cells was 9.39% (SE = 0.64%) (CD4+ population) and 9.38% (SE = 0.71%) (CD8+ population). FOXP3 expression in Control-T cells was 7.18% (SE = 0.28%) (CD4+ population) and 7.85% (SE = 0.43%) (CD8+ population). This indicates that ACh signaling on FOXP3 expression has a significant effect following T cell re-activation after 48 hours.

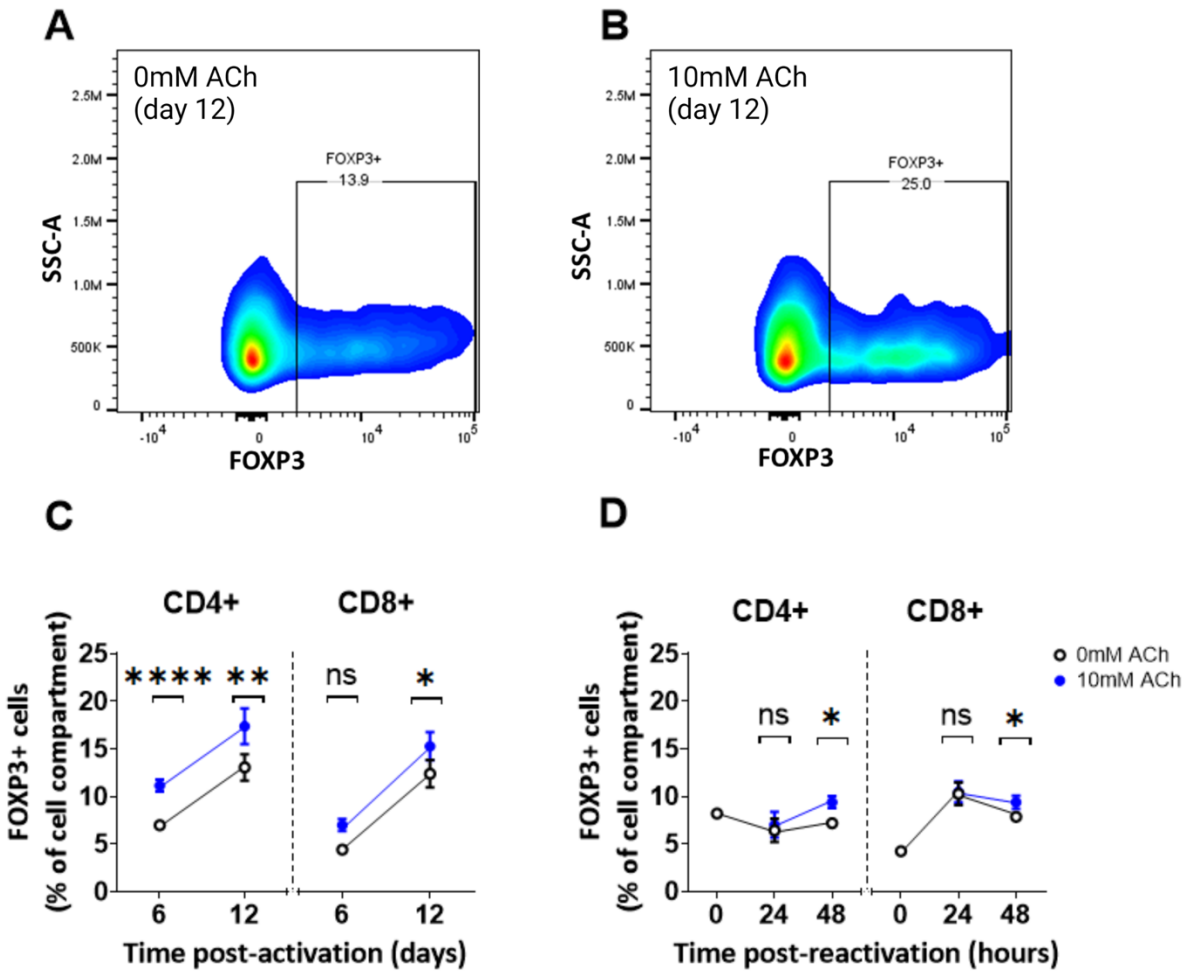

Figure 10. ACh increases FOXP3 expression in CD4+ and CD8+ T cells. Activated T cells were supplemented with IL-2 (300U/ml) in the absence (black lines, white points) or presence (blue) of 10mM ACh. Representative flow cytometry plots for FOXP3+ T cells in the absence (A) or presence (B) of 10mM ACh 12 days after activation. (C) Flow cytometry data representing the percentage of FOXP3-expressing CD4+ T cells (left) and CD8+ T cells (right) 6 days and 12 days following activation. (D) Flow cytometry data of samples that were activated for 6 days without ACh supplementation and then re-activated with plate-bound anti-CD3/CD28 antibodies in the presence or absence of 10mM ACh. Graphs represent average ± SE (n=5, each sample replicated in triplicate, multiple Student's paired *t*-tests used. ns = not significant, *p<0.05, **p<0.01, ****p<0.0001).

2.4.3 - ACh stimulation increases expression of both the full-length FOXP3 isoform and the FOXP3 isoform lacking exon 2 (FOXP3Δ2) in T cells

As discussed, FOXP3 is often alternatively spliced in human cells. Therefore, I determined if ACh preferentially induced the full-length FOXP3 isoform (FOXP3fl) or the FOXP3 isoform lacking exon 2 (FOXP3Δ2). To determine the effects of ACh on T cell expression of the two FOXP3 isoforms, T cells were isolated, activated, and cultured using the same methods outlined above. After 6 days of culturing, T cells were removed from the media and analyzed via western blot. Western blot analysis supports the previous FOXP3 flow cytometry data. The western blot revealed that ACh signaling increased both the FOXP3fl and FOXP3Δ2 isoforms equally **(Figure 11)**. Band intensity for ACh-T cells had about a 1.33-fold (Total FOXP3), 1.30-fold (FOXP3fl), and 1.30-fold (FOXP3Δ2) increase compared to controls. As such, it appears that ACh signaling does not influence the splicing of FOXP3 and increases the expression of both isoforms of FOXP3.

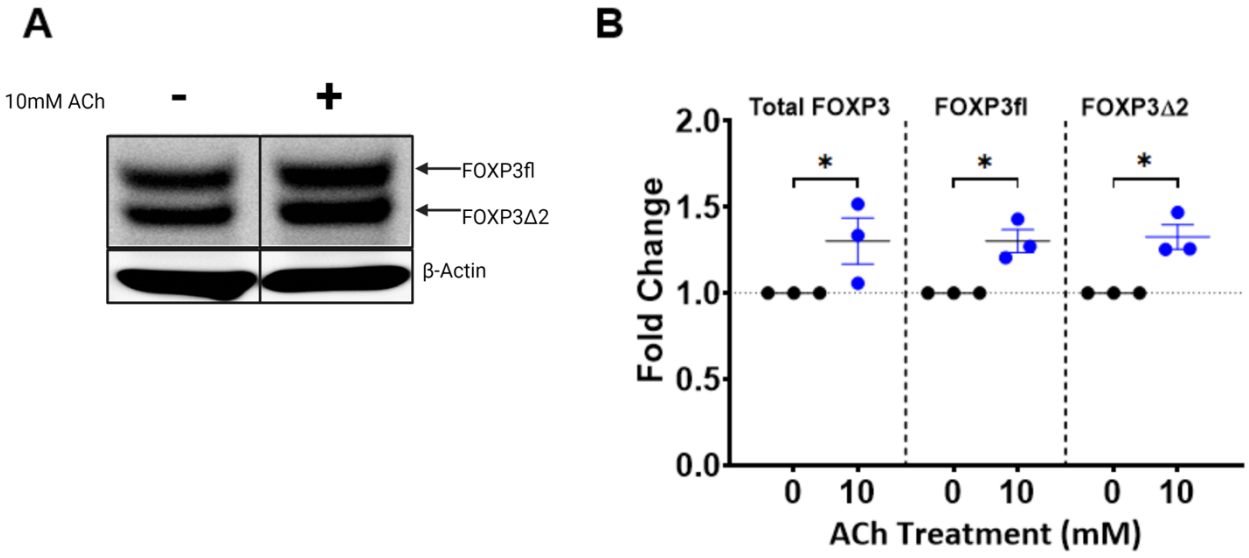

Figure 11. ACh increases the expression of the full-length FOXP3 isoform (FOXP3fl) and the FOXP3 isoform lacking exon two (FOXP3Δ2) equally in T cells. Activated T cells were supplemented with IL-2 (300U/ml) in the presence (blue) or absence (black lines, white points) of 10mM ACh for 6 days. (A) Representative western blot of FOXP3 isoforms in T cells (n=3). β-actin is used as the positive control. (B) Western blot data representing fold change in FOXP3fl, FOXP3Δ2, and total FOXP3 band intensity between T cells treated with (blue) or without (black) 10mM ACh for 6 days. Graphs represent average ± SE (n=3, Student's unpaired *t*-test. *$p<0.05$).

2.4.4 - ACh enhances FOXP3 expression in T cells supplemented with TGF-β.

After confirming that supplementation of ACh alone is sufficient to induce FOXP3 in T cells, my next goal was to determine if cholinergic signaling enhances TGF-β-induced FOXP3, as suggested from mouse data published by Mashimo *et al*[41]. Healthy naive T cells were isolated and activated using the methods described in *T cell isolation and Cell Culture* and *TGF-β administration* sections. External ACh + TGF-β supplementation increases FOXP3 expression in T cells compared to control cells that received TGF-β alone **(Figure 12).** FOXP3 expression in ACh + TGF-β samples (light blue) was proportionally increased by 54.34% compared to TGF-β-only control samples (grey) on day 6, and proportionally increased by 60.00% on day 12 **(Figure 12D and E, respectively)**. On day 6, FOXP3 expression in ACh + TGF-β T cells was 26.30% (SE = 7.97%) compared to TGF-β only T cells at 17.04% (SE = 8.93%). On day 12, FOXP3 expression in ACh + TGF-β T cells was 44.80% (SE = 3.70%) compared to TGF-β only T cells at 28.00% (SE = 2.97%). Differences in donor samples partially contributed to large standard error in samples on day 6, so statistics were computed using matched donor pairs for each sample. As predicted, my data suggest that ACh enhances TGF-β-induced FOXP3 expression in T cells. These results indicate that cholinergic signaling synergizes with TGF-β, producing a magnified effect on T cell proliferation and FOXP3 expression.

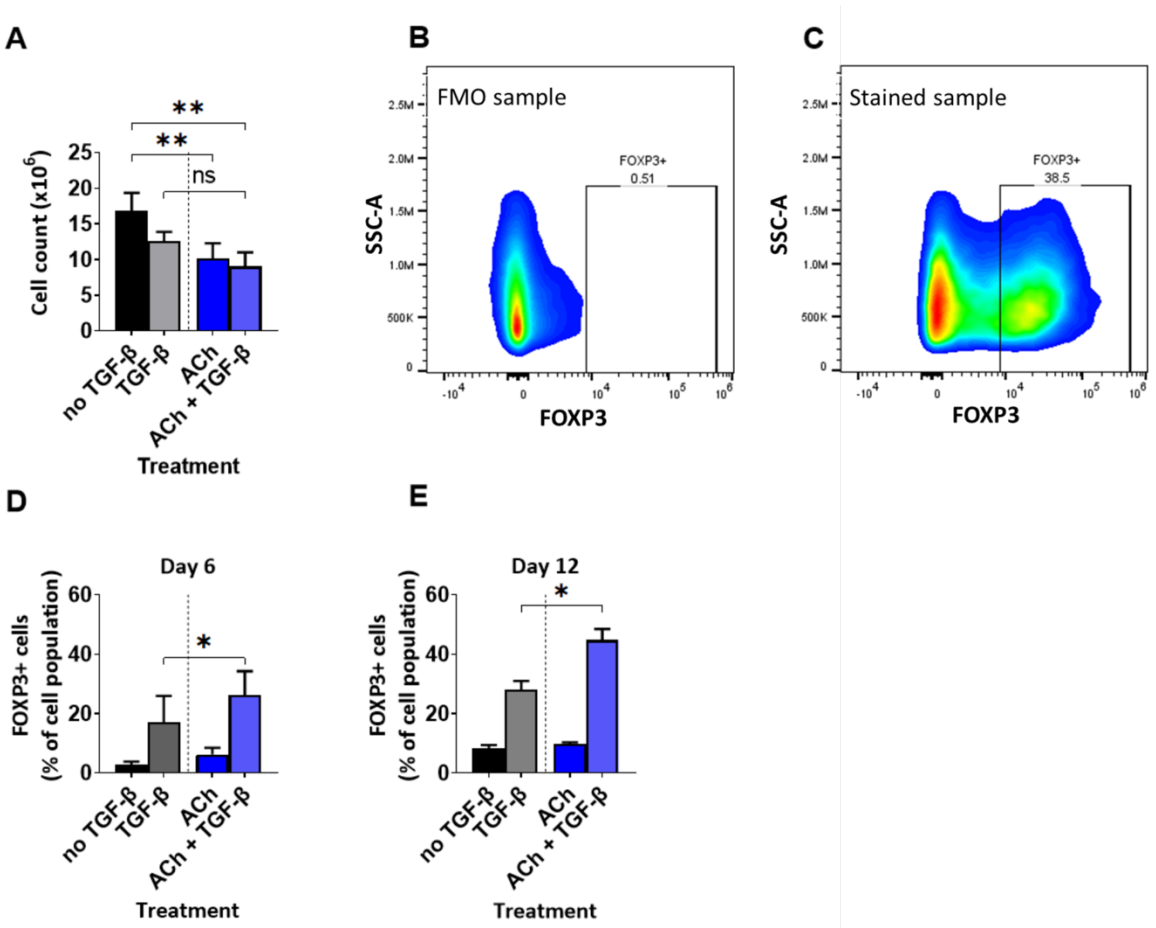

Figure 12. ACh magnifies FOXP3 expression induced by TGF-β in T cells. T cells were activated with anti-CD3/CD28 suspension antibodies with IL-2 in the presence (blue) or absence (black) of 10mM ACh and the presence (grey, light blue) or absence (black, dark blue) of 2.5ng/ml TGF-β. (A) Cells were stained with trypan blue at a 1:1 dilution and counted with a hemocytometer. (B) and (C) Representative flow cytometry plots for FOXP3+ gating on T cells using FMO sample as a negative. Flow cytometry data representing the percentage of FOXP3-expressing T cells 6 days (D) and 12 days (E) following activation. FMO – fluorescence minus one. Graphs represent average ± SE (n=3, One-way ANOVA, with Geisser-greenhouse correction and Dunnett's multiple comparisons test, with individual variances computed for each paired sample used. *p<0.05, **p<0.01).

2.4.5 - ACh increases IFN-γ production in both CD4+ and CD8+ T cells.

Now that I confirmed human FOXP3 was expressed in T cells from ACh signals, the next step was to assess the effector function of ACh-treated T cells. I measured the expression of IFN-γ in cultured T cells through flow cytometry to assess IFN-γ-expressing Teff induction by ACh. Naïve T cells were isolated, activated, and cultured as per the methods discussed above. On day 6, T cells were stained with the same antibody cocktail in the **Appendix** and run on the flow cytometer **(Figure 13A, B).** My data indicate a higher percentage of ACh-treated CD4+ and CD8+ human T cells express IFN-γ compared to Control-T cells **(Figure 13C).**

On day 6, IFN-γ expression in CD4+ ACh-T cells on day 6 was 33.21% (SE = 1.23%), compared to 27.91% (SE = 0.91%) in the Control-T cell population, a proportional increase of 18.17%. IFN-γ expression in CD8+ ACh-T cells on day 6 was 40.48% (SE = 2.42%), compared to 36.73% (SE = 2.57%) in the Control-T cell population, a proportional increase of 10.21%.

On day 12, IFN-γ expression in CD4+ ACh-T cells on day 6 was 52.66% (SE = 3.73%), compared to 45.46% (SE = 3.52%) in the Control-T cell population, a proportional increase of 15.82%. IFN-γ expression in CD8+ ACh-T cells on day 6 was 80.03% (SE = 2.90%), compared to 72.48% (SE = 4.19%) in the Control-T cell population, a proportional increase of 10.42%. Elevated IFN-γ expression in ACh-T cells remained by day 12, although statistical significance was lost. These results were consistent with previous publications on murine T cells[41], further indicating that human and mouse T cells respond to ACh similarly.

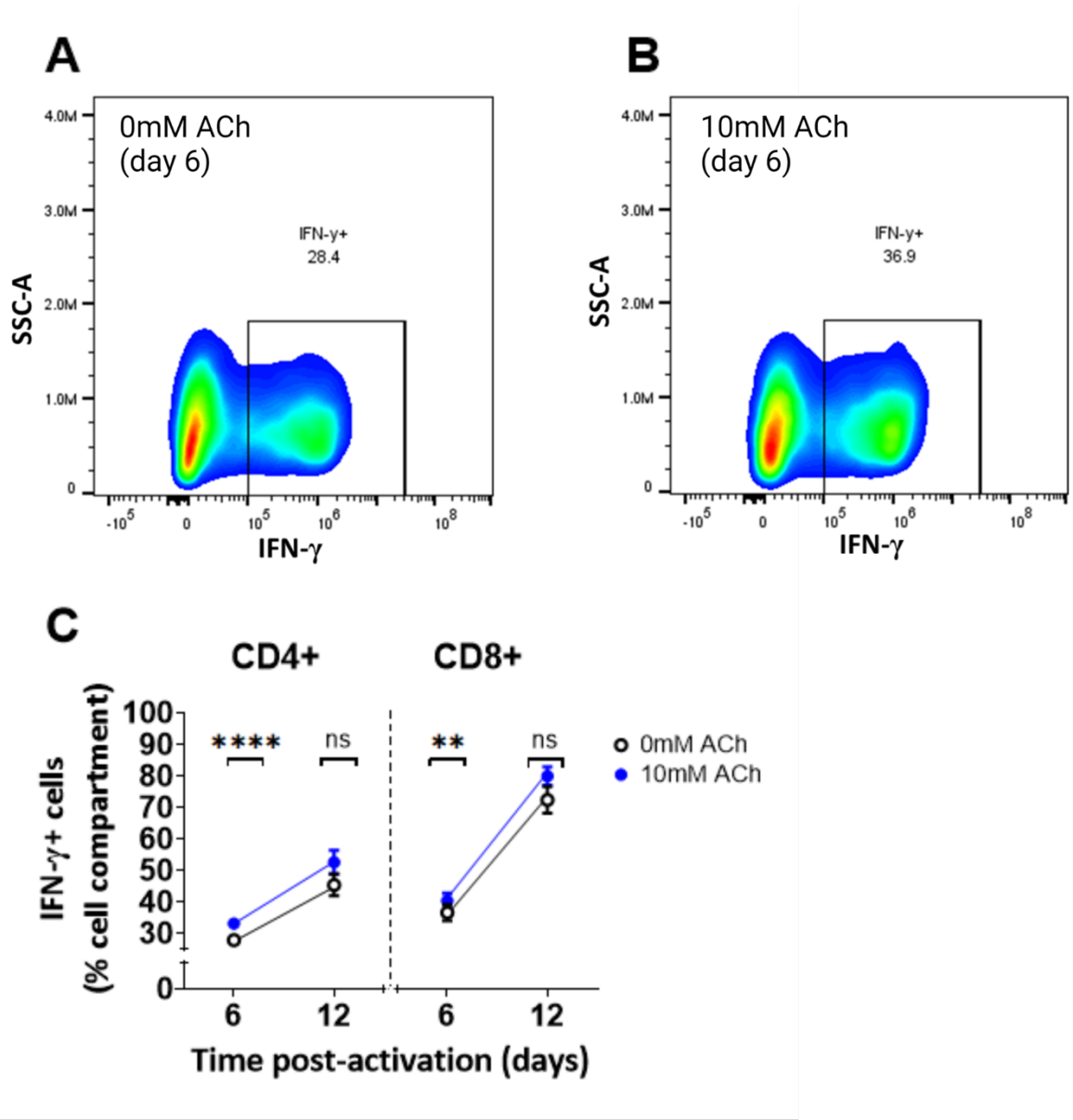

Figure 13. ACh increases IFN-γ expression in CD4+ and CD8+ T cells. Activated T cells were supplemented with IL-2 (300U/ml) in the absence (black lines, white points) or presence (blue) of 10mM ACh. Representative flow cytometry plots for IFN-γ+ T cells in the absence (A) or presence (B) of 10mM ACh 6 days after activation. (C) Flow cytometry data representing the percentage of IFN-γ-expressing CD4+ T cells (left) and CD8+ T cells (right) 6 days and 12 days following activation Graphs represent average ± SE (n=5, each sample replicated in triplicate. Multiple Student's paired t-tests used. ns = not significant, *p<0.05, **p<0.01, ***p<0.001).

2.4.6 - FOXP3+ T cells produce higher levels of IFN-γ compared to the total T cell population and the FOXP3- population

The findings above suggest that ACh increases both T cell effector function (IFN-γ) and FOXP3 expression. As these results initially suggest opposing phenotypic effects, I next determined if these FOXP3-expressing T cells are activated Teffs since FOXP3 can be transiently expressed in activated cultured Teffs[35,141]. To test whether FOXP3-expressing activated T cells represented an activated Teff population, cells within CD3+/CD4+/FOXP3+ and CD3+/CD8+/FOXP3+ flow cytometry gates were analyzed for IFN-γ expression. Gating strategy is represented in **Figure 14A.** The proportion of IFN-γ-expressing Teffs within FOXP3+ populations was measured and compared to expression from the total T cell population and FOXP3- populations**.** FOXP3+ T cells had a higher IFN-γ expression level compared both to the total population and FOXP3- T cells on day 6 **(Figure 14B)**. IFN-γ expression in ACh-CD4+/FOXP3+ T cells on day 6 was 44.16% (SE = 2.56), compared to 33.21% (SE = 1.23) in the ACh-Total CD4+ T cell population and 31.20% (SE = 1.61%) in the ACh-CD4+/FOXP3- population, a proportional increase of 32.97% and 41.54%, respectively. IFN-γ expression in ACh-CD8+/FOXP3+ T cells on day 6 was 62.82% (SE = 2.51) compared to 40.48% (SE = 4.42) in the ACh-Total CD8+ T cell population and 39.30% (SE = 2.28%) in the ACh-CD8+/FOXP3- population, a proportional increase of 35.56% and 59.85%, respectively.

Furthermore, ACh-T cell FOXP3+ populations had a higher proportion of IFN-γ-producing Teffs compared to Control-T cell FOXP3+ populations on day 6 (44.16% (SE = 2.56) (ACh-CD4+), 62.82% (SE = 2.51) (ACh-CD8+) and 36.97% (SE = 2.54) (control-CD4+), 55.53% (SE = 3.12)(control-CD8+), respectively). Elevated proportions of IFN-γ-expressing Teffs was still trending by day 12, although statistical significance was almost entirely lost.

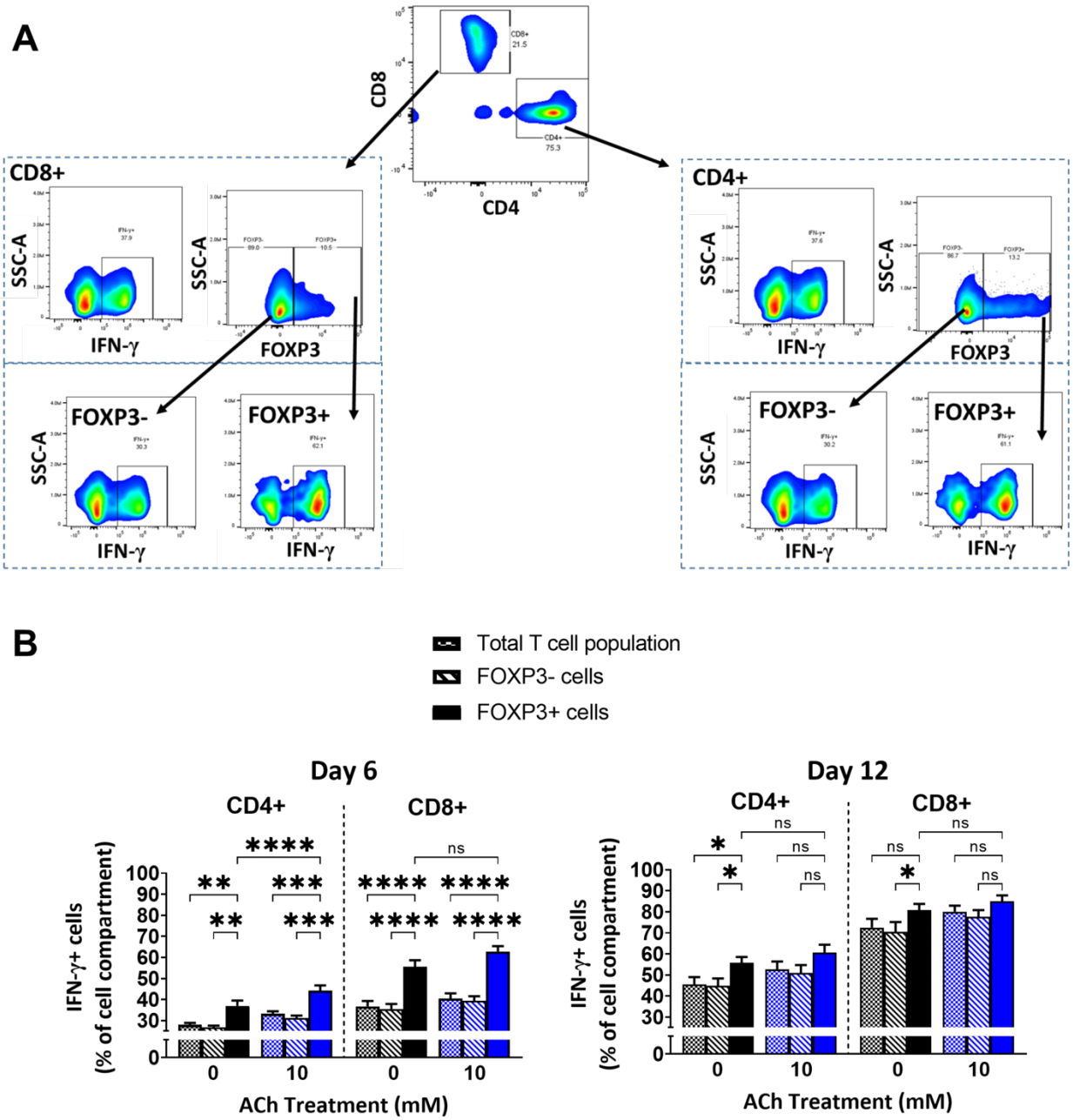

Figure 14. ACh increases IFN-γ expression in FOXP3-expressing CD4+ and CD8+ T cells. Activated T cells were supplemented with IL-2 (300U/ml) in the absence (black bars) or presence (blue bars) of 10mM ACh. (A) Representative flow cytometry gating strategy outlining separation of FOXP3+, FOXP3-, and total T cell population for both CD4+ and CD8+ compartments to measure IFN-γ expression. (B) Flow cytometry data representing the percentage of IFN-γ-expressing T cells 6 days (left) and 12 days (right) within the FOXP3+ (solid bars), FOXP3- (diagonal line bars) or total cell compartment (checkered bars) following activation. Graphs represent average ± SE (n=5, each sample replicated in triplicate. Mixed-effects analysis, with Geisser-greenhouse correction and Dunnett's multiple comparisons test, with individual variances computed for each paired sample used. ns = not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

2.4.7 - FRα CAR-T cells respond to 10mM ACh similarly to unedited T cells

Given that data suggest that ACh increases Teff differentiation, the next step was to test if ACh confers effector function onto CAR-T cells. To test whether FRα CAR-T cells respond to cholinergic signaling similarly to unedited T cells, naïve T cells were initially isolated and expanded under the methods described in *T cell isolation and Cell Culture* and *ACh administration* sections. Then, FRα CAR-T cells were generated following the methodology outlined in the *FRα-CAR-T Cell Generation* section, with half the samples receiving 0mM ACh and the other half 10mM ACh throughout all expansion. The FRα CAR-T cells and untransduced control samples were then stained for flow cytometry as per the previous experiments to measure responses to ACh.

FRα CAR-T cells responded similarly to unedited T cells. CAR-T cell counts were reduced in the 10mM ACh FRα CAR-T samples (1.20e7 cells (SE = 1.02e7 cells)) compared to Control-FRα CAR-T samples (1.95e7 cells (SE = 2.03e7 cells)), with no significant change in viability **(Figure 15A and B).** Both results were consistent with unedited T cells. Further, percent of T cells expressing the CAR (CAR+ cells) in transduced samples was unaffected by ACh administration, with a slight (but not statistically significant) increase in CAR expression (median fluorescent intensity, MFI) in the CAR+ population **(Figure 15C and D).**

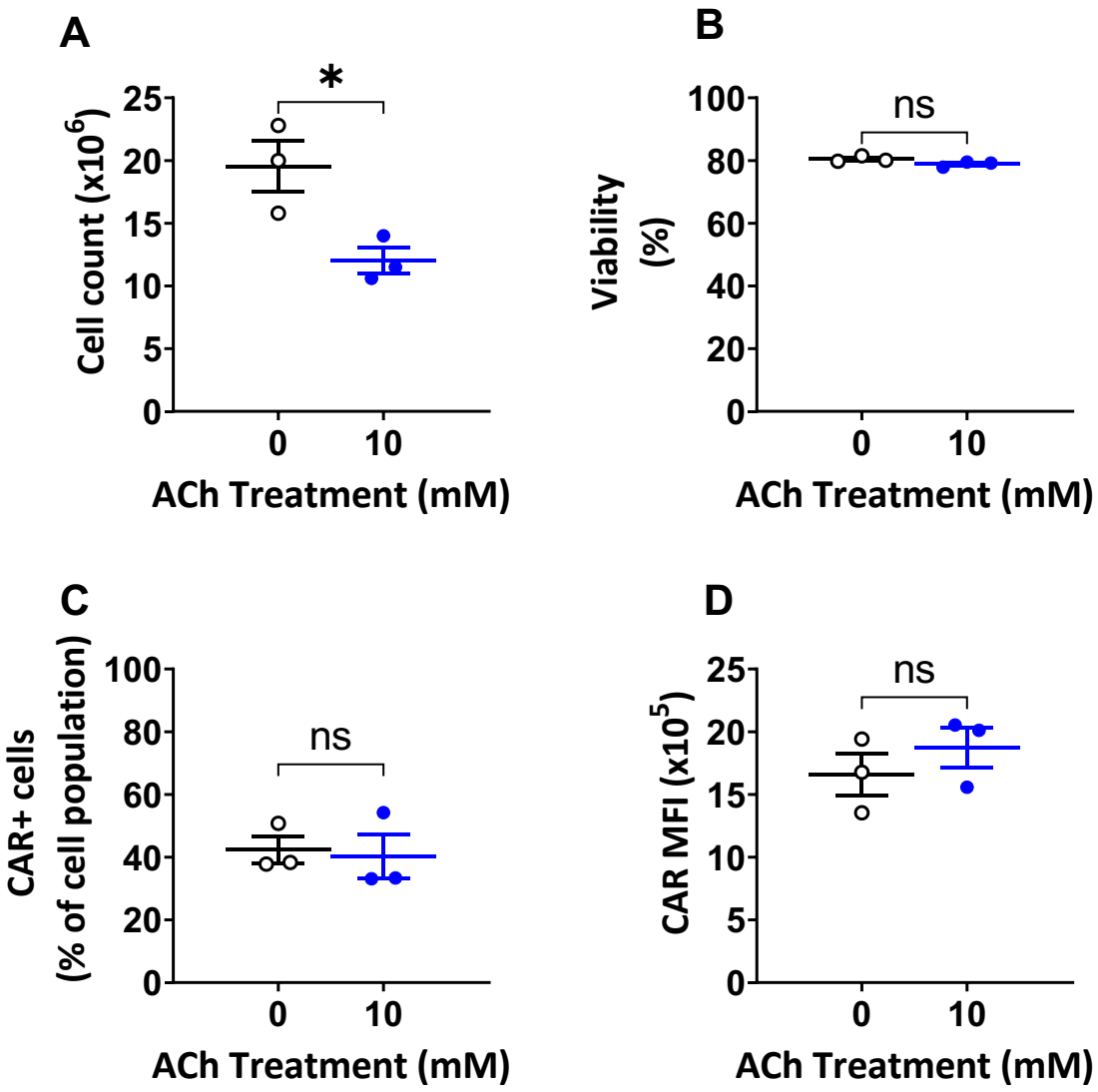

Figure 15. ACh suppresses the proliferation of CAR-T cells and does not impact viability or CAR expression. Activated T cells were supplemented with IL-2 (300U/ml) in the absence (white points) or presence (blue points) of 10mM ACh for 1 day before FRα lentivirus transduction. (A) T cell counts on day 6 of expansion before co-culture killing assay. (B) Flow cytometry data representing viable T cells using eBioscience™ efluor™ 450 fixable viability dye. (C) Flow cytometry data measuring CAR expression in T cells through the fluorescence of GFP promoter. (D) Median fluorescence intensity (MFI) of GFP promoter (representative of the level of CAR expression) in the CAR+ cells. Graphs represent average ± SE (n=3, Student's paired *t*-test. ns = not significant, *p<0.05).

FOXP3 expression in both CD4+ and CD8+ CAR-T cells were significantly increased in 10mM ACh FRα CAR-T cells compared to Control-FRα CAR-T cells **(Figure 16A).** FOXP3 expression in CD4+ 10mM ACh FRα CAR-T cells was 17.50% (SE = 1.06%), compared to 8.78% (SE = 0.31%) in the Control-FRα CAR-T cell population, a proportional increase of 78.90%. FOXP3 expression in CD8+ 10mM ACh FRα CAR-T cells was 12.01% (SE = 0.65%), compared to 7.65% (SE = 0.36%) in the Control-FRα CAR-T cell population, a proportional increase of 56.95%. This result was similar to unedited T cells, indicating that CAR-T cell generation with lentivirus does not impact ACh signaling and FOXP3 in T cells.

IFN-γ expression in 10mM ACh FRα CAR-T cells increased slightly compared to controls, however, statistical significance was not achieved **(Figure 16B**). IFN-γ expression in CD4+ 10mM ACh FRα CAR-T cells on day 6 was 32.80% (SE = 4.42%), compared to 30.33% (SE = 4.05%) in the Control-FRα CAR-T cell population. IFN-γ expression in CD8+ 10mM ACh FRα CAR-T cells on day 6 was 45.98% (SE = 2.04%), compared to 44.28% (SE = 1.97%) in the Control-FRα CAR-T cell population.

Like unedited T cells **(Figure 14)**, a significantly greater percentage of IFN-γ-producing cells were found in the FOXP3+ subsets compared to the total CAR-T cell population and the FOXP3- subset **(Figure 16C).** These results are consistent with healthy donor T cells tested previously.

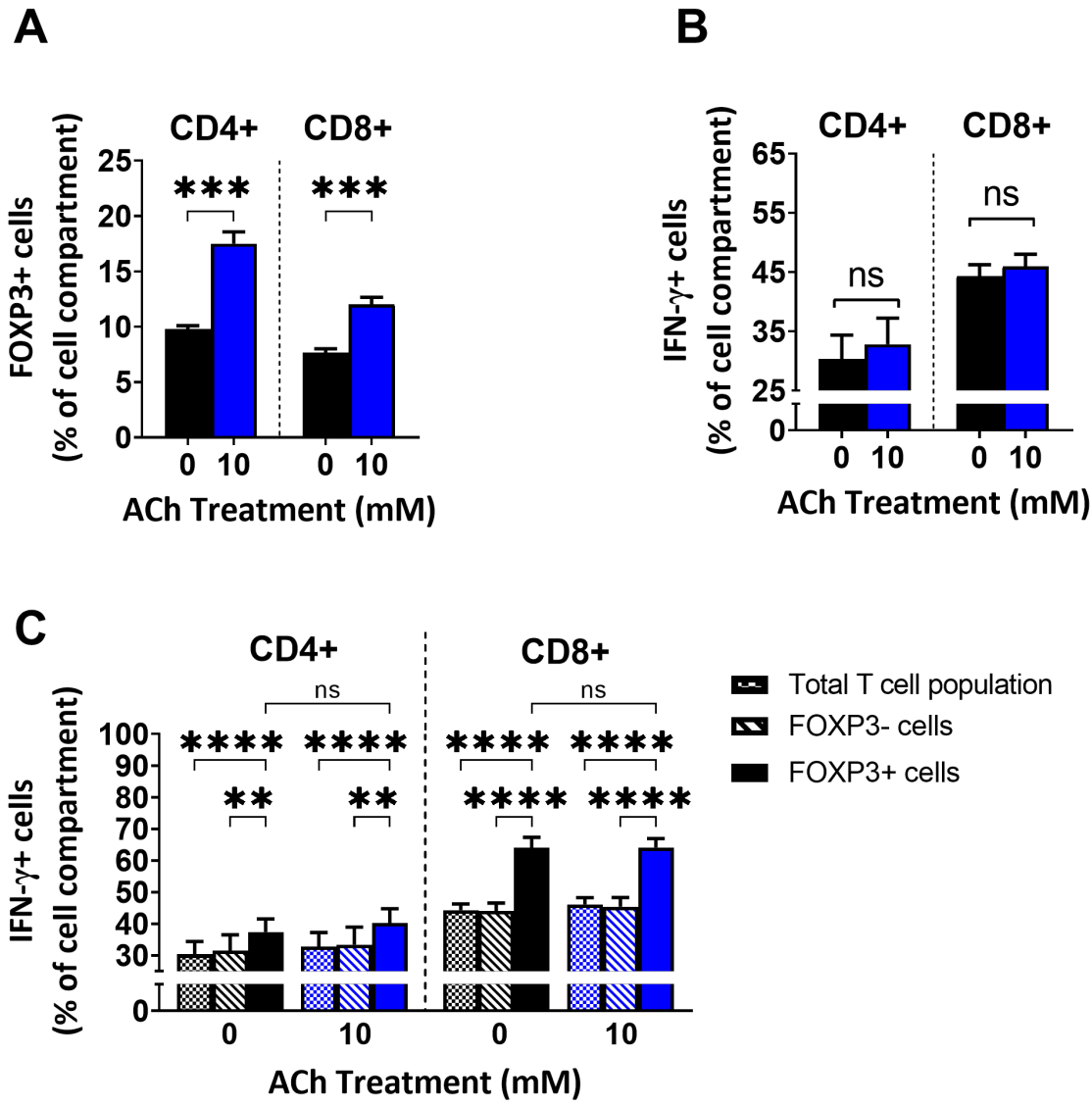

Figure 16. ACh stimulation increases FOXP3 and IFN-γ expression in FOXP3+ FRα CAR-T cells. Activated T cells were supplemented with IL-2 (300U/ml) in the absence (white points) or presence (blue points) of 10mM ACh for 1 day before FRα lentivirus transduction. (A) Flow cytometry data representing the percentage of FOXP3-expressing CD4+ T cells (left) and CD8+ T cells (right) on day 6 of expansion before co-culture killing assay. (B) Flow cytometry data representing the percentage of IFN-γ-expressing CD4+ T cells (left) and CD8+ T cells (right) on day 6 of expansion before co-culture killing assay. (C) Flow cytometry data representing the percentage of IFN-γ-expressing FOXP3+ T cells (solid bars) compared to the Total T cell population (checkered bars, from (B)) and the FOXP3- population (diagonal line bars). Graphs represent average ± SE (n=3, each sample replicated in triplicate. One-way paired ANOVA, with Geisser-greenhouse correction and Dunnett's multiple comparisons test, with individual variances computed for each paired sample used. ns = not significant, **p<0.01, ***p<0.001, ****p<0.0001).

2.4.8 - FRα CAR-T cells expanded in 10mM ACh kill FRα-expressing SKOV3 tumor cells more efficiently

Once I confirmed that CAR-T cells remain viable and respond to ACh similarly to naïve unedited T cells, the next step was to test the antigen-specific killing capacity. To test the cancer-killing efficiency of 10mM ACh FRα CAR-T cells, a portion of the same-day 6 CAR-T samples from the previous experiment were placed in co-culture conditions with FRα-expressing SKOV3 tumor cells.

Fluorescence of surviving SKOV3 tumor cells in the 10mM ACh FRα CAR-T population were significantly lower than Control-FRα CAR-T cells samples from 6-10% at all effector to target (E:T) ratios **(Figure 17)**. The reduction in fluorescence indicates that a smaller proportion of SKOV3 cancer cells are surviving after 48 hours. Therefore, CAR-specific cytotoxicity in the 10mM ACh FRα CAR-T samples was higher than in Control-FRα CAR-T cells. Cytotoxicity was increased in the 10mM ACh FRα CAR-T samples by about 6-10% when all samples were averaged. Of note, two donors (donor 16 and 18) received significant increases in cytotoxicity at all E:T ratios (about 8-17% ($p = 0.0031$) and 4-9% ($p = 0.0002$), respectively), while donor 17 only had elevated cytotoxicity at the highest two E:T ratios and was not statistically significant (see **Figure 26** in the Appendix). Therefore, two out of three donors responded strongly to ACh stimulation while the third only responded marginally (about a 1.5-7% increase in cytotoxicity).

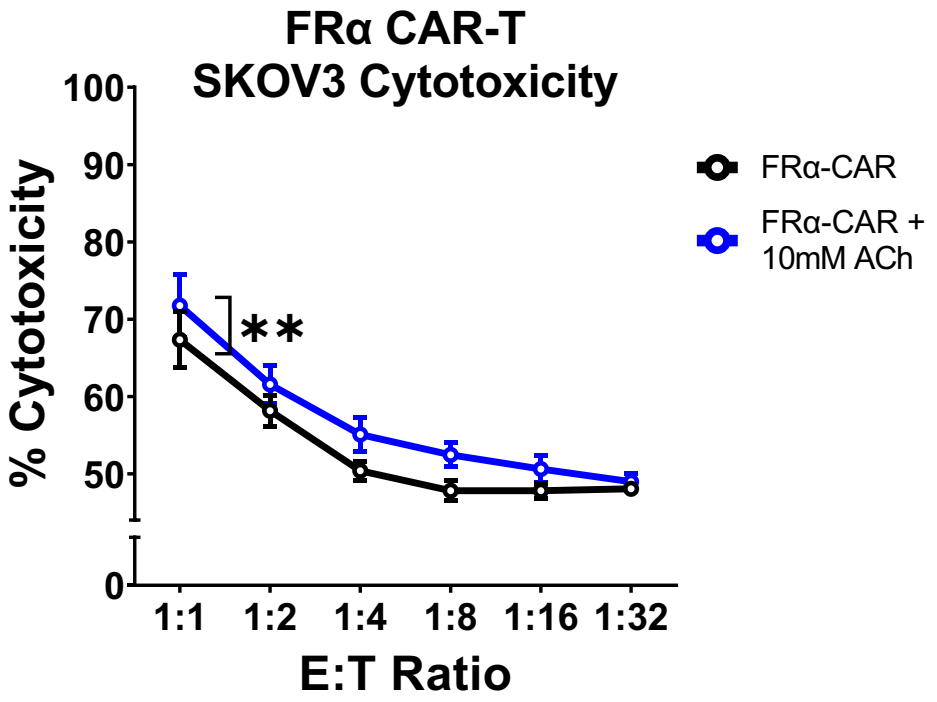

Figure 17. ACh-stimulated FRα CAR-T cells kill SKOV3 tumor cells more efficiently. FRα CAR-T cells and luciferase-expressing SKOV3 tumor cells were co-cultured in a flat opaque 96-well plate for 48 hours. After 48 hours, luciferin was added to co-culture for 5 minutes and luminescence was then immediately read using *Varioskan Lux* Plate Reader (ThermoFisher) with ScanIT RE 6.1 software. 20,000 SKOV3 tumor cells were seeded in each sample well. CAR-T cells were seeded in each sample at the given effector to target (E:T) ratio. The number of T cells seeded for each indicated CAR-T E:T ratio was calculated using the GFP/CAR-positivity expression rate measured through flow cytometry indicated in **Figure 15C**. CAR-T cells were activated with anti-CD3/CD28 suspension antibodies with IL-2 in the presence (blue) or absence (black) of 10mM ACh for 24 hours before CAR lentiviral transduction and cultured for 6 days before being transferred to 96 well plate containing SKOV3 cells. Percent cytotoxicity of FRα CAR-T cells from all donor samples measured by the change in relative luminescence (RLU) of the SKOV3 cells compared to no CAR-T cell controls. Fluorescence values were read using *Varioskan Lux* Plate Reader (ThermoFisher) with ScanIT RE 6.1 software. Graphs represent average ± SE (n=3, each sample replicated in triplicate. Student's paired *t*-test. ns = not significant, **p<0.01).

2.5 - Discussion

This data chapter indicates that ACh signaling regulates T cell differentiation, effector function, and activation state. T cells responding to external ACh stimulation proliferate less without a loss in viability **(Figure 9)**. It is reasonable to interpret that ACh is not toxic to the cells at a concentration of 10mM and is conferring a phenotypic change rather than killing the cells. ACh appears to be a supplemental signal that may control the degree of the immune response following activation.

Results are contrary to the initial hypothesis. As discussed at the end of Chapter 1, ACh was enriched in TILs in ovarian cancer TME in data published by Kilgour *et al*. from our lab **(Figure 5)**[109]. Previous literature suggested a possible link between ACh and immune suppression through the CAP and by promoting expression of the Treg marker FoxP3 in mice[7,41,62]. Since the TME is known to be highly immunosuppressive and ACh signaling suppresses immune function in some contexts, I hypothesized that ACh suppresses T cells and contributes to the immunosuppressive TME through polarization of Tregs that suppress Teffs.

However, upon completing this portion of research, the data seem to suggest that ACh signaling promotes ACh effector function. While low proliferation in ACh stimulated T cells suggests suppression, it is possible that Teffs re-prioritized energy towards enhanced effector function over proliferation, and so multiply slower. Further, as discussed in the introduction, *in vitro* expression of FOXP3 in human T cells has been shown to intrinsically reduce proliferation and modulate effectr cytokines without an iTreg phenotype on its own[141]. The reduction in proliferation observed in my data may suggest ACh causes T cells to reduce proliferation but maintain activation state and effector functions to control immune responses.

ACh's signaling role seems to be independent of the initial T cell activation state, given that naïve freshly activated T cells *and* re-activated T cells respond to external ACh signals in the same manner. Both freshly thawed, newly activated T cells and re-activated, actively growing human T cells increase the expression of FOXP3 when ACh is added **(Figure 10)**.

My data seems almost paradoxical at first examination – how can ACh influence human T cells to express FOXP3 and reduce proliferation, yet these same T cells appear to have increased effector functions? These results puzzled me for quite some time until I conducted further research and collaborative discussions with my colleagues inside and outside the Lum Laboratory.

While it is common for researchers to correlate FOXP3 expression with a regulatory T cell phenotype, there is more context needed. First, FOXP3 expression alone is not sufficient to confer a stable, functional human Treg phenotype *in vitro* or *vivo*[140,144,145]. A combination of FOXP3 expression, high CD25 expression, and low expression of CD127 is commonly used to identify circulating activated natural Treg (nTreg) cells in the body, since identifying the FOXP3+ T cells from bulk samples will not sufficiently locate suppressive Tregs[146]. Secondly, FOXP3 and CD25 expression is naturally expressed in human T cells when activated *in vitro* so sometimes is used as an indication of the overall T cell activation state instead of iTreg formation[35,136,141]. Therefore, simply associating FOXP3 expression with an iTreg phenotype under controlled *in vitro* cell culture conditions is not sufficient and requires further investigation into the cytokine profile and effector functions such as T cell-mediated cytotoxicity.

Expanding on these results, T cells stimulated with ACh do not preferentially express a specific FOXP3 isoform, instead increasing expression of both FOXP3fl and FOXP3Δ2 isoforms equally **(Figure 11)**. Equal upregulation of both isoforms suggests a transient overall FOXP3

expression from activation conditions rather than an iTreg phenotype. This is due to the conflicting and limited literature that correlates co-expression of FOXP3fl and FOXP3Δ2 isoforms with Treg stability and effector functions, but both FOXP3 isoforms have also been detected in activated Teffs and were not sufficient to suppress IFN-γ expression[143,144,147]. Given that my results show both isoforms are equally induced following ACh stimulation, this suggests that T cells are not preferentially splicing FOXP3 when stimulated with ACh.

Given the above statements and the remainder of the data, the results of this chapter strongly indicate that external ACh stimulation on T cells confers an elevated activation state and increased effector function for Teffs, instead of suppressive iTregs. Increased effector function of the T cell population is indicated by an elevated percentage of cells expressing IFN-γ in both CD4+ and CD8+ T cell compartments on day 6 **(Figure 13)**. These results indicate that helper CD4+ T cells respond to ACh by differentiating into IFN-γ+ Th1s (and/or other IFN-γ-producing subclasses), and cytotoxic CD8+ T cells likewise increase expression of IFN-γ. As IFN-γ expression is statistically significant on day 6 and loses significance by day 12, ACh stimulation may have a short-term, temporary effect on Teffs. T cells may require more signals other than ACh to maintain elevated production of IFN-γ over the long term, such as IL-12, and ACh alone loses effect on Teff differentiation after 6 days. T cells may become desensitized to prolonged ACh stimulation. Experiments that change ACh stimulation time throughout expansion would be useful to complete. Overall, ACh administration increased Teff production of IFN-γ regardless of cell class.

The above statements are further reinforced by the results from **Figure 14**. Above, I have discussed that FOXP3 expression in Teff cells under *in vitro* activation conditions are transiently increased and does not confer iTreg phenotypes. This statement is supported by my data showing

that the percentage of IFN-γ-expressing cells in the FOXP3+ populations is higher than the percentage of IFN-γ-expressing cells in both the overall T cell population and FOXP3- population **(Figure 14B)**. Since the FOXP3+ T cells are also have elevated IFN-γ expression, we can infer that a larger portion of these T cells are active Teffs instead of iTregs.

My results also suggest that external ACh may be a secondary signal to communicate and control immune responses. ACh's mechanism of action appears to be independent of external cytokine stimulation, as ACh alone was sufficient to increase FOXP3 and slightly increase IFN-γ expression **(Figures 10, 13, and 14)**. The experiments done by Mashimo *et al*. tested excised mouse spleen T cells and supplemented polarizing cytokines (IL-12 (Th1), and TGF-β (Treg)) alongside the cholinergic agonist GTS-21 and saw similar results to my experiments[41]. However, most of my experiments did not contain any additional cytokine supplementation and received similar results - increased FOXP3 and IFN-γ expression. Furthermore, ACh enhanced FOXP3 expression in the presence of 2.5ng/ml TGF-β **(Figure 12)**. Combining the results from my experiment and the work done by Mashimo *et al*. indicate that ACh can magnify signals from other cytokines to promote a stronger response in T cells. The publications by Fujii and Mashimo *et al*. corroborate my findings and suggest that ACh is instead a signal and activation magnifier used by T cells rather than a lineage-defining metabolite such as TGF-β.

Finally I developed FRα CAR T cells in the presence or absence of external 10Mm ACh stimulation to test if these findings can be extended to CAR-T cell immunotherapies. 10mM ACh FRα CARs were more efficient at killing SKOV3 tumor cells at all effector-to-target ratios **(Figure 17)**. FRα CAR-T cells only received ACh supplementation during activation and cell culture for 6 days. Since ACh was removed before co-killing assay and both 10mM ACh FRα CAR-Ts and Control-FRα CAR-Ts were administered at equal E:T ratios, this indicated that

ACh enhanced differentiation into generated CAR-Teffs that were more efficient at eliminating SKOV3 tumor cells. The 10mM ACh FRα CAR-T cells were more efficient regardless of the CAR T cell effector to target ratio. ACh stimulation reduced CAR-T proliferation and did not significantly affect CAR-T viability or CAR expression **(Figure 15).** 10mM ACh FRα CAR T cells, much like unedited healthy donor T cells, expressed elevated FOXP3 compared to controls **(Figure 16A)**. IFN-γ was slightly increased, but not statistically significant in the total CAR-T cell population **(Figure 16B)**. However, the FOXP3-expressing CAR-T cells had a higher proportion of IFN-γ-producing cells compared to the percentage of IFN-γ-producing cells in the total cell population or FOXP3- population **(Figure 16C).** Since 10mM ACh FRα CAR-T cells have a higher proportion of FOXP3-expressing cells and these FOXP3+ cells in turn have a higher proportion of IFN-γ expression, these are likely activated Teffs with transient FOXP3 expression. Therefore, it is possible that 10mM ACh FRα CAR-Ts had elevated effector function and this likely contributed to cancer cytotoxicity. However, since the actual proportion of IFN-γ-expressing cells in the total population was not statistically enriched (rather, there is a higher proportion of FOXP3+ CAR-T cells and these cells have high IFN-γ expression), it is possible that the increase in cytotoxicity was due to a different factor not measured.

These results suggest that ACh may increase Teff function rather than suppress it, directly opposing my initial hypothesis. Therefore, further testing was required to confirm the suppressive capacity of ACh-treated T cells and determine if elevated FOXP3 translated to suppressive iTregs. Furthermore, the mechanism that ACh acts on human T cells needs to be further understood. The following chapter will describe the change in perspective I experienced following my unexpected results that were in direct opposition to my hypothesis and the experiments I conducted to investigate these unexpected results.

Chapter 3: Mechanism of action of acetylcholine on T cells and investigation of Treg/Teff functions

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R.G. and J.J.L. conceptualized the project. R.G. conducted experiments after initial mentorship and training provided by laboratory members. G.C. mentored and assisted R.G. in CRISPR/Cas9 electroporation. Megan L. and Macyn L. provided advice and scientific input on Helios expression data.

R.G., J.J.L, G.C., Megan L., and Macyn L. contributed 40%, 40%, 10%, 5%, and 5% respectively in experimental design and scientific suggestions.

3.1 - Abstract

Chapter 2 demonstrated that cholinergic signaling in human T cells increases FOXP3 and IFN-γ expression and improves FRα CAR-T killing of SKOV3 tumor cells. FOXP3 is expressed by circulating Tregs but can also be transiently expressed by activated Teffs *in vitro.* it appears that ACh signaling may unexpectedly increase T cell effector function *in vitro* rather than suppress it. This chapter investigates the ACh's mechanism of action on T cells and further reinforces my results dictating that FOXP3+ ACh-T cells are activated Teffs. ACh-stimulated naïve T cells secrete less TGF-β, indicating reduced suppression by the Treg population. Next, the α7 nAChRs are linked to FOXP3 expression in T cells (like mice), but not IFN-γ. After, I use CRISPR/Cas9 to show that deletion of ChAT in human T cells reduces expression of FOXP3 and CD25, but has minimal effect on IFN-γ. I propose that T cells use cholinergic signaling to modulate activation state and control the degree of immune responses. I establish that ACh signaling does not create a stable iTreg population by demonstrating the expression of the transcription factor Helios. Stable Tregs have a stable expression of Helios, and a lack of Helios expression marks Treg cells that can convert to Teffs. Helios is temporarily increased in ACh-stimulated cells, but only high IFN-γ expressing, FOXP3+/Helios- T cells persist throughout expansion as they are the only population that increase in proportion by day 12. Therefore, I outline that cholinergic signaling enhances T cell activation state and promotes Teff differentiation. Given that increased Teff activity and decreased Treg suppression promotes cancer clearance, I propose a novel cell culture target to enhance the effector function of *in vitro*-produced CAR-T cells.

3.2 – Introduction

As discussed extensively, directly measuring iTregs *in vitro* is quite difficult. Both activated iTregs and Teffs express FOXP3 and CD25 transiently[35,141]. Flow cytometry is limited in its ability to determine iTreg/activated Teff populations reliably on its own. In Chapter 1, I highlighted publications indicating that cholinergic signaling upregulated FoxP3 expression in cultured mouse T cells[41]. In Chapter 2, my experimental results determined that ACh increased the expression of FOXP3 in cultured human T cells and suppressed T cell proliferation without reducing viability. As such, some researchers may conclude that suppressive Tregs are induced by ACh. However, as just discussed, FOXP3 (and CD25) expression is not sufficient to identify iTregs in culture. Further, IFN-γ was also increased by ACh and FRα CAR-T cells are more efficient at killing SKOV3 cancer cells when activated with ACh. It is likely that Teff functions are instead enhanced by ACh. It is unwise to reliably conclude if ACh is influencing T cells to differentiate into suppressive Tregs or instead activated Teffs using these results alone. To investigate this further, Chapter 3 will examine some of the mechanisms of ACh on T cells. I will conclude with my experiments measuring the expression of another Treg marker to elucidate ACh's effects more reliably.

3.2.1 – Difficulties identifying and isolating iTregs from Teffs in vitro by FACS sorting, microbead separation, and flow cytometry

Current experimental methods to isolate iTregs out of culture are limited. Fluorescent-activated cell sorting (FACS) uses flow cytometry staining techniques to separate cells into fractions based on fluorescently labelled antibodies[140,148]. Fluorescent antibodies can bind to extracellular markers without disturbing the cell membrane, allowing for the separation of viable

cells that can persist when re-cultured[149]. Staining for intracellular proteins disrupts the cell membrane and therefore cells cannot be re-cultured after sorting as they are effectively dead[148,150]. Magnetic bead-based isolation is another isolation procedure, but it likewise usually labels extracellular markers to separate cell types[151]. Therefore, the separation of cells using FACS sorting or magnetic beads requires a unique cell surface marker to differentiate cell populations and allow them to remain viable.

Natural Tregs (nTregs) circulating in the body express high levels of CD25 and low expression of CD127 (both extracellular cytokines) compared to circulating Teffs. Purification of nTregs is possible, but still difficult as flow cytometry gates need to be stringent to separate populations since clear positive/negative populations are not present[148,149,152]. Unfortunately, cultured activated T cells transiently express CD25 at high levels regardless of cell type and as such separation using CD25 is near impossible. While CD25 or CD127 are the current "best" extracellular markers for nTregs, staining for FACS or microbead sorting on cultured T cells do not produce pure iTreg populations. Furthermore, using flow cytometry techniques to stain intracellular cytokines (i.e. transcription factors like FOXP3) kills the T cells. Researchers are currently not able to assess the suppressive function of cultured iTregs after sorting since isolating based on an intracellular marker like FOXP3 kills the cells so they can't be re-cultured, and sorting iTregs based on surface markers is not consistent. Since FOXP3 is likewise transiently expressed by both culture-activated iTregs and Teffs, FACS sorting can not reliably define iTregs by FOXP3 expression anyways. All these factors indicate that novel iTreg extracellular markers need to be identified. To assess the function of cultured iTregs, other mechanisms than sorting such as expression of other transcription factors or production of unique cytokines need to be utilized.

3.2.1 - Transforming Growth Factor Beta (TGF-β) and Tregs

My goal was to determine if ACh signaling produces suppressive iTregs or Teffs. To test this, I determined if secretion of the cytokine TGF-β was modulated by ACh signaling. TGF-β is secreted by suppressive Tregs to suppress Teff function[75,76]. TGF-β is secreted in a soluble form and also exists in a membrane-bound form on the surface of Tregs where it participates in cell-cell contact-mediated suppression[153,154]. Mammalian TGF-β exists in three main isoforms: TGF-β1, β2, or β3, with the β1 isoform primarily used in the immune system[76]. TGF-β1 regulates transcription in T cells through high-affinity TGF-β receptor II, a type-II Ser/Thr kinase receptor, which then carries out phosphorylation steps on SMAD proteins 2, 3, and 4. The SMAD complex is then able to enter the nucleus and regulate the transcription of various T cell genes related to effector functions[155]. To bind to the TGF-β receptor, TGF-β needs to be activated beforehand, which is unlike many other cytokines produced by T cells[155]. TGF-β normally exists in an inactive form, complexed with latency associated peptide (LAP). LAP needs to be removed before TGF-β can bind to its receptor[156].

Given that TGF-β must be disassociated from LAP before it can begin signaling, it is difficult to directly measure active TGF-β levels through flow cytometry. Researchers can use antibodies that bind to LAP, but this does not accurately measure *active* TGF-β since it is not being directly measured[157]. I determined that the measurement of soluble TGF-β with BD Bioscience's BD OptEIA™ Human TGF-β1 ELISA Set was the optimal method to determine TGF-β production by Tregs in culture. This ELISA kit uses an acidification and neutralization step to disassociate soluble TGF-β from LAP before analysis, providing a clear accurate measurement of total TGF-β (active and inactive) secreted by Tregs in culture. Measurement of

supernatant TGF-β will support previous results and aid in determining if FOXP3+ ACh-T cells are suppressive TGF-β-producing Tregs, or if these T cells are instead activated Teffs.

3.2.2 - GTS-21 and alpha 7 nicotinic acetylcholine receptor (α7 nAChR) signaling

ACh receptors come in two classes, nicotinic acetylcholine receptors (nAChRs) and muscarinic acetylcholine receptors (mAChRs). These classes of AChRs are further subdivided by the subcomponents making up the receptors[27,158]. Of these subclasses, the most widely studied acetylcholine receptor in T cells is the nicotinic acetylcholine receptor made up entirely of alpha-7 subunits (α7 nAChR). As discussed in previous chapters, many publications regarding cholinergic signaling investigate the alpha 7 nicotinic acetylcholine receptor (α7 nAChR) due to its involvement in the cholinergic anti-inflammatory pathway (CAP) on macrophages[32,43,52]. Therefore, many researchers choose to investigate if α7 nAChR signaling likewise modulates T cell function. The research completed by Fujii and Mashimo *et al.*[7,41], discussed extensively throughout this thesis, primarily focused on α7 nAChR signaling in mouse T cells. The researchers determined that α7 nAChR signaling in mouse T cells modulated differentiation in culture[41]. They determined that the α7 nAChR cholinergic agonist drug GTS-21 increased mouse T cell differentiation into both FoxP3+/CD25+ Tregs and IFN-γ-producing Th1 populations when mouse T cells were given additional polarizing cytokines in culture (TGF-β for Tregs, and IL-12 and anti-IL-4 for Th1s, respectively). In Chapter 2, I determined that ACh signaling affects human T cells similarly to their mouse T cell results. Furthermore, I determined that ACh alone is sufficient in inducing FOXP3 and IFN-γ expression in human T cells, and additional cytokines

are not necessary but magnifies the effects in the case of TGF-β **(Figure 12)**. Therefore, I decided to test if the effects were due to α7 nAChR signaling.

GTS-21 (also known as DMXBA) is a synthetic drug developed for research purposes into clinical use to treat nicotine dependence and neurodegenerative diseases such as Alzheimer's disease and schizophrenia[159]. GTS-21 is a derivative of anabasine (a natural toxin produced by *Nemertines* and *Aphaenogaster ants*), similar in structure to nicotine[159]. GTS-21 preferentially binds to and activates α7 nAChRs[159]. Many synthetic cholinergic drugs are toxic and pose an elevated risk level when used in a laboratory, which limited the use in our lab as we did not want to bring in dangerous compounds[160–162]. However, GTS-21 poses less risk when used under controlled conditions of the lab and was therefore determined to be an ideal candidate to test α7 nAChR signaling in human T cells.

3.2.3 - Helios expression and T cells

Helios is a member of the Ikaros transcription factor family encoded by the *IKZF2* gene and is critical in the development of lymphocytes, as alterations in Ikaros family proteins result in defective lymphocyte development[163]. Helios is believed to suppress IL-2 expression in Tregs and is therefore important in the development of suppressive capabilities of Tregs[164]. Helios expression was initially associated with thymic-derived Tregs (tTregs) and not peripheral Tregs (pTreg) and was initially used as a distinguishing marker to determine tTregs from pTregs (their developmental origin in the body), but this has since become controversial[163,165,166].

Helios is typically highly expressed in about 70% or more of activated circulating Tregs[167,168]. Combined expression of Helios and FOXP3 can be used as a more robust metric to identify stable Tregs from Teffs[169]. Recent studies have shown that a lack of Helios expression in

Tregs does not necessarily indicate non-functional cells, but rather Helios is a marker, not a driving factor, in Treg function[169]. Further, FOXP3+ Tregs that do not express Helios (FOXP3+/Helios-) have shown the capacity to transition into Teff phenotypes, expressing high IFN-γ and increased antitumor activity[170]. This indicates that Helios expression may be a marker for stable suppressive Tregs and loss of this expression is a marker of unstable Tregs that can convert to Teffs under the right conditions[163,169]. The mechanisms behind the expression profile and effector functions of FOXP3+/Helios-, IFN-γ-producing, Treg-to-Teff converting cells are still unclear. However, Helios can be used as a marker to indicate cultured T cells that express conventional Treg-like markers such as FOXP3 but retain Teff functions[164,168,169].

Recall from Chapter 2 - my results indicate that ACh signaling increases FOXP3 *and* IFN-γ expression in human T cells, and the percentage of IFN-γ expression is especially high in the FOXP3+ population **(Figures 10, 13, and 14)**. In this chapter, I intend to show that the combined measurement of FOXP3, Helios, and IFN-γ is a measurement of T cell effector function and can be used to distinguish between stable suppressive iTregs, conventional Teffs, and unstable "iTregs" that can exhibit some Teff functionality. Here, my data will show that ACh increases differentiation and activation of Teff cells by separating cultured T cell populations into compartments based on FOXP3 and Helios expression and measuring IFN-γ production within each compartment.

3.3 - Methods

3.3.1 - ELISA testing

To measure TGF-β secretion by ACh-treated T cells, samples were activated and expanded according to methods outlined in Chapter 2's *T cell isolation and Cell Culture* and

ACh administration sections. Cell culture supernatant was removed from samples on day 6 and treated with 1N HCl for 1 hour at 4°C to disassociate the TGF-β/LAP complex. After 1 hour of incubation with HCl, the supernatant solution was neutralized with 1N NaCl and then TGF-β concentrations were immediately measured using BD OptEIA™ Human TGF-β1 ELISA Set (BD Biosciences) according to the manufacturer's instructions. Absorbance at 450nm was read using *Varioskan Lux* Plate Reader (ThermoFisher) with ScanIT RE 6.1 software.

3.3.2 - GTS-21 stimulation

To test whether the results from Chapter 2 are replicable with signaling through the α7 nAChR (in correlation to the published literature)[41,43], healthy human T cells were initially isolated and expanded using the same methods described in Chapter 2. However, tested samples were supplemented with 30μM GTS-21 (Cayman Chemical Co.) instead of 10mM ACh. GTS-21 concentration was set at a concentration of 30μM in correlation with published literature[7,41].

3.3.3 - ChAT Knockout

As discussed in Chapters 1 and 2, T cells not only react to external ACh stimulation but can produce their own ACh for autocrine signaling by expressing ChAT. To test the impact of autocrine ACh production on T cell differentiation, I worked together with my colleague in the Gillian Carleton to genetically alter ACh production by CRISPR-mediated deletion of the ChAT gene.

3.3.3.1 – Preparation of ribonucleoprotein (RNP) complexes

RNPs were prepared by combining single guide RNA (sgRNA: IDT, 100pmol/μl), poly-L-glutamic acid (PGS: Sigma-Aldrich, 100mg/ml), and recombinant spCas9 protein (IDT,

61pmol/µl) in a 2:1:1 molar ratio, followed by incubation for 10 minutes at 37ºC. RNPs were electroporated immediately after complexing.

3.3.3.2 – T cell electroporation

T cells were isolated and activated according to the same methodology outlined in Chapter 2. On day 3, T cells were centrifuged and resuspended in Nucleofection Buffer P3 (Lonza) at a concentration of 20µl buffer per 1 million cells. One million cells were electroporated with 2.5µl RNPs per well on the 4D-Nucleofector X Unit (Lonza) using pulse code EO115. Immediately following electroporation, 80µl of pre-warmed T cell medium (no cytokines) was gently added to each well, and the entire nucleovette strip was incubated for 15 minutes at 37ºC. After 15 minutes, the cells were transferred to a 48-well plate containing pre-warmed T cell medium (supplemented with 300U/ml IL-2) and returned to the incubator for regular culture.

3.3.3.3 – Analysis of insertions and deletions

Four days after electroporation, genomic DNA (gDNA) was extracted using DNA QuickExtract (Lucigen). In brief, 1 million cells were resuspended in 50µl QuickExtract solution, mixed by vigorous pipetting, then incubated on a thermocycler as per the following protocol: 15 minutes at 65ºC, 15 minutes at 68ºC, 10 minutes at 98ºC. gDNA concentration was quantified by NanoDrop and 100ng gDNA was used for PCR amplification of the region surrounding the expected cut site in the ChAT locus. PCR amplicons were purified and sequenced by Sanger sequencing (Sequencing Platform, Université Laval). Edited and non-edited sequence traces were compared using the online decomposition algorithm TIDE to determine the frequencing of insertions and deletions (indels) after RNA electroporation (see **Figure 21A**).

3.3.4 – Flow Cytometry and Statistical analysis

Flow Cytometry and statistical analysis were performed following the same protocol described in Chapter 2, with addition of Helios to the flow cytometry panel (see **Table 1** in the **Appendix** for the full antibody panel).

3.4 - Results

3.4.1 - ACh reduces TGF-β secretion by naïve T cells, but not FRα CAR-T cells

To examine the immune suppressive capability of ACh-treated T cells, I measured the secretion of TGF-β1 into the cell culture supernatant. As expected, cell supernatant extracted from ACh-T cells contained significantly less TGF-β compared to 0mM ACh T cells (Control-T cells). Supernatant from control T cells contained 2,394pg/ml TGF-β (SE = 58.15pg/ml) compared to 1686pg/ml (SE = 108.00pg/ml) in 10mM ACh T cell supernatant. Secreted TGF-β was proportionally reduced by about 29.57% **(Figure 18A)**. However, the same CAR-T cells generated in Chapter 2 secreted similar amounts of TGF-β in the 10mM ACh FRα CAR-T samples compared to the 0mM controls (Control-FRα CAR-T) **(Figure 18B)**.

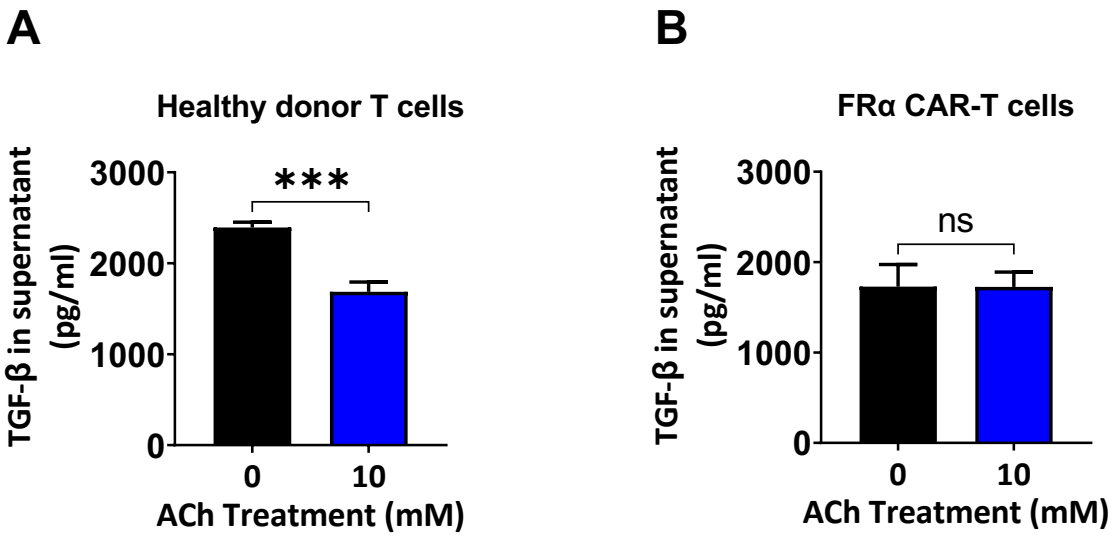

Figure 18. ACh decreases TGF-β secretion in healthy donor T cells but not FRα CAR-T cells. T cells were activated with anti-CD3/CD28 suspension antibodies with IL-2 in the presence (blue) or absence (black) of 10mM ACh. ELISA data representing the abundance of TGF-β secreted by A) Healthy donor T cells and B) FRα CAR-T cells into the culture supernatant 6 days following activation. Absorbance values were read using *Varioskan Lux* Plate Reader (ThermoFisher) with ScanIT RE 6.1 software. Graphs represent average ± SE (n=3, each sample replicated in triplicate. Student's paired *t*-test. ***$p<0.001$).

3.4.2 - FOXP3 induction, but not IFN-γ production, is induced by the α7 nicotinic acetylcholine receptor (α7 nAChR)

The next question addressed was if a7 nAChRs contribute to FOXP3 expression, reduced proliferation, and IFN-γ production as publications on mouse T cells suggest. As expected, GTS-21, and therefore α7 nAChR signaling, reduced human T cell counts and increased FOXP3 expression similarly to 10mM ACh stimulation **(Figure 19A)**. FOXP3 expression in GTS-21-T cells was 10.47% (SE = 1.71%), compared to 4.24% (SE = 0.85%) in the Control-T cell population, a proportional increase of 147.09% **(Figure 19B)**. In contrast, IFN-γ expression in GTS-21-T cells did not change significantly. IFN-γ expression in GTS-21-T cell samples was 25.80% (SE= 1.51%) compared to 24.77% (SE = 2.38%) in 0μM GTS-21 samples (Control-T cells) **(Figure 19C)**. This indicated that FOXP3 expression and suppression of T cell proliferation, but not IFN-γ production, is partially mediated by α7 nAChRs on human T cells. These results are consistent with some of Mashimo, Fujii, and Kawashima *et al*.'s mouse results, where they similarly found that GTS-21 stimulation alone on mouse T cells in culture with anti-CD3/CD28 antibodies resulted in increased expression of FoxP3 but no significant change in IFN-γ[7].

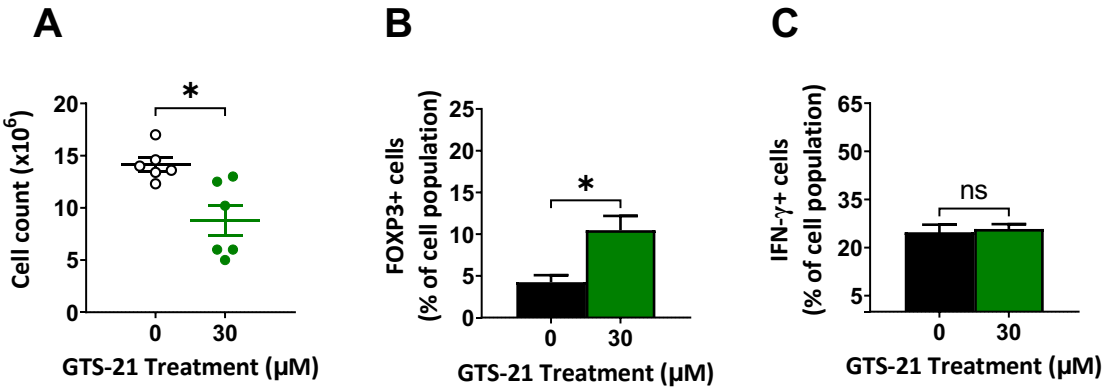

Figure 19. GTS-21 stimulation reduces proliferation, increases FOXP3 expression, and does not influence IFN-γ in healthy donor T cells. T cells were activated with anti-CD3/CD28 suspension antibodies with IL-2 in the presence (green) or absence (black) of 30μM GTS-21 for 6 days. (A) Cells were stained with trypan blue at a 1:1 dilution and counted with a hemocytometer. (B) Flow cytometry data representing the percentage of FOXP3-expressing T cells on day 6 of expansion. (C) Flow cytometry data representing the percentage of IFN-γ-expressing T cells on day 6 of expansion. Graphs represent average ± SE (n=3, each sample replicated in duplicate. Student's paired *t*-test. ns = not significant, *p<0.05).

3.4.3 - GTS-21 (α7 nAChRs) does not appear to synergistically interact with TGF-β to induce FOXP3 expression in human T cells, unlike ACh itself.

To determine if the same synergistic magnification of FOXP3 expression observed with ACh and TGF-β in Chapter 2 was replicable with GTS-21, and therefore α7 nAChRs, I repeated the experiment from Chapter 2, supplementing 30μM GTS-21 instead of 10mM ACh alongside TGF-β. Surprisingly, while GTS-21 and TGF-β both reduced T cell proliferation **(Figure 20A)**, the same effects were not observed in FOXP3 expression. T cells supplemented with 30μM GTS-21 and 2.5ng/ml TGF-β did not change the expression of FOXP3 to a statistically significant degree compared to TGF-β only controls **(Figure 20D and E).** These results are not consistent with the mouse results by Mashimo and Fujii *et al*., where they reported that GTS-21 + TGF-β supplementation on mouse T cells resulted in a significant increase in FoxP3 expression[41]

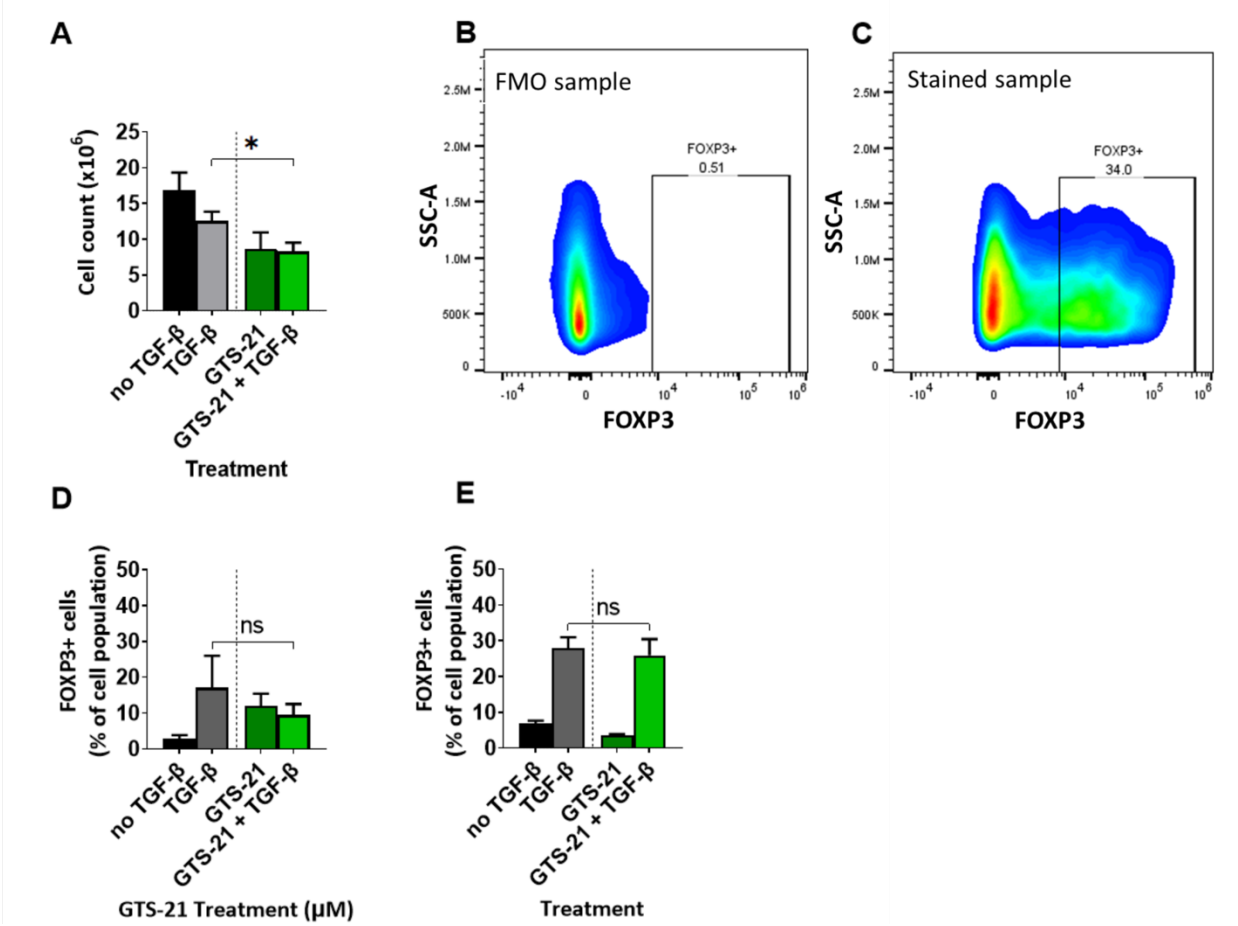

Figure 20. GTS-21 does not magnify FOXP3 expression induced by TGF-β in T cells, unlike ACh. T cells were activated with anti-CD3/CD28 suspension antibodies with IL-2 in the presence (blue) or absence (black) of 30μM GTS-21 and the presence (grey, light blue) or absence (black, dark blue) of 2.5ng/ml TGF-β. (A) Cells were stained with trypan blue at a 1:1 dilution and counted with a hemocytometer. (B) and (C) Representative flow cytometry plots for FOXP3+ gating on T cells using FMO sample as a negative. Flow cytometry data representing the percentage of FOXP3-expressing T cells 6 days (D) and 12 days (E) following activation. FMO – fluorescence minus one. Graphs represent average ± SE (n=3, One-way paired ANOVA, with Geisser-greenhouse correction and Dunnett's multiple comparisons test, with individual variances computed for each paired sample use . *$p<0.05$).

3.4.4 - Autocrine ACh production by T cells contributes to FOXP3 expression and T cell activation state

After α7 nAChRs were determined to induce FOXP3 expression in human T cells, the next question to answer was if autocrine ACh signal influenced human T cells like external ACh supplementation.

ChAT sgRNA electroporation successfully created 72.5% indels in the T cell samples (+ChAT sgRNA), for 72.5% impaired ACh production compared to control wild-type samples that had unimpaired ChAT activity (no ChAT sgRNA) **(Figure 21A).** +ChAT sgRNA resulted in lower FOXP3 expression compared control no ChAT sgRNA samples **(Figure 21B).** FOXP3 expression in CD4+ +ChAT sgRNA cells was 4.39% (SE = 0.13%), compared to 5.96% (SE = 0.60%) in the control CD4+ no ChAT sgRNA cell population, a proportional decrease of 26.39%. FOXP3 expression in CD8+ +ChAT sgRNA cells on was 5.40% (SE = 0.70%), compared to 6.83% (SE = 0.91%) in the control CD8+ no ChAT sgRNA cell population, a proportional decrease of 20.91%. These results indicate that T-cell-specific loss in ACh production is associated with lower FOXP3 expression.

Ablating ACh production did not significantly affect the proportion of IFN-γ+ CD4+ or CD8+ T cells **(Figure 21C),** nor did it influence IFN-γ expression of in FOXP3+ cells **(Figure 21D)**. +ChAT sgRNA samples expressed less CD25 compared to no ChAT sgRNA controls **(Figure 21E).** Median fluorescent intensity of CD25 in +ChAT sgRNA samples was proportionally decreased by 15.49% (CD4+ population) and 13.65% (CD8+ population) on day 6 compared to no ChAT sgRNA control cells **(Figure 21F)**. CD25 is strongly expressed by activated T cells in culture. These results support the findings outlined earlier in previous chapters which show cholinergic signaling in T cells is used to enhance T cell activation state.

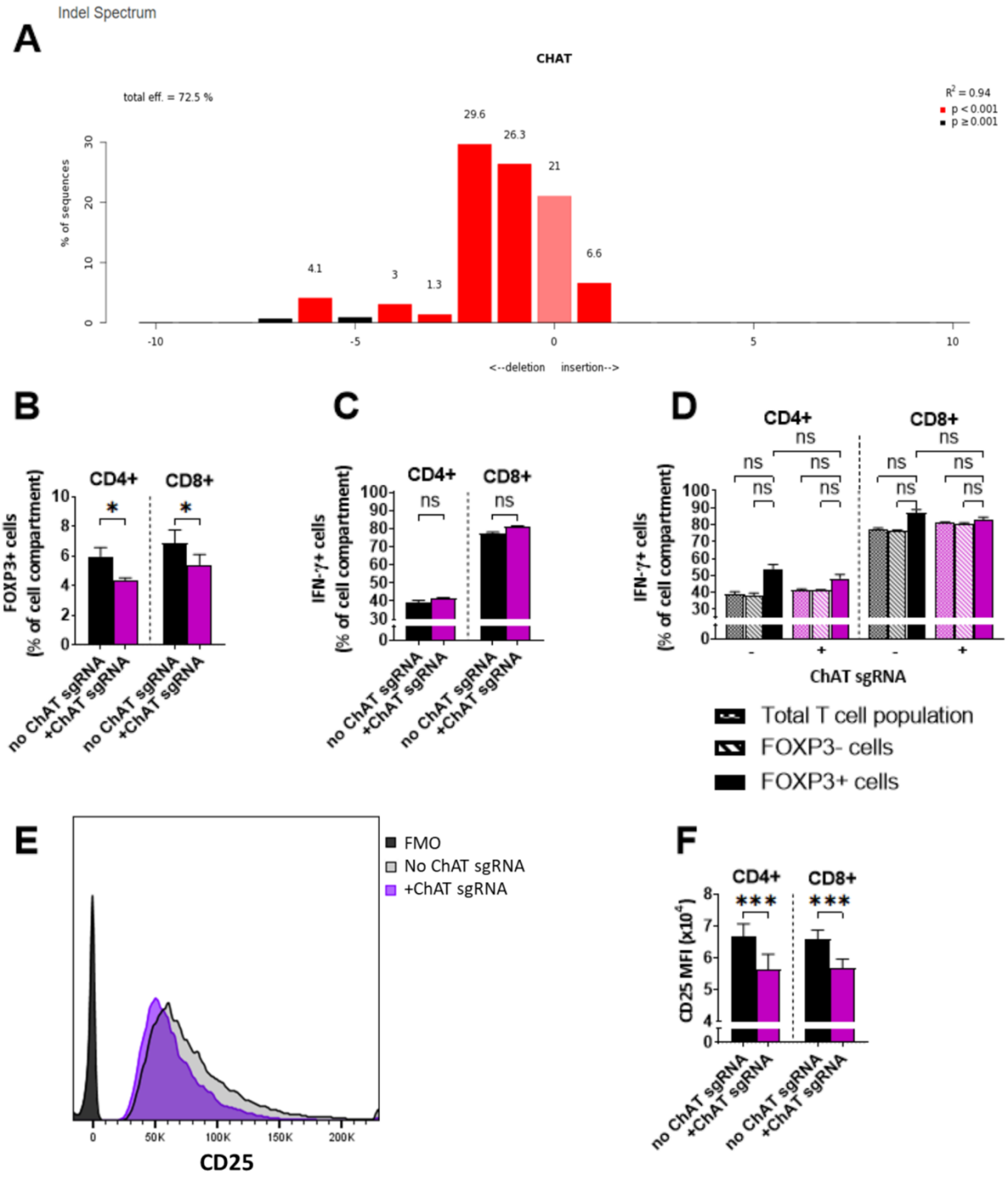

Figure 21. CRISPR-mediated disruption of the ChAT locus reduces FOXP3 and CD25 expression in T cells and does not significantly impact IFN-γ expression. Activated T cells were electroporated with 100pmol sgRNA, 50pmol recombinant Cas9 protein, and 80mg poly-L-glutamic acid. Four days post-electroporation, using pulse code EO115 (Lonza) (A) Four days post-electroporation, the ChAT locus was amplified, sequenced, and analyzed for indel formation using the online decomposition algorithm TIDE. Indels were successfully created in 72.5% of T cell samples. (B) T cells were re-stimulated with anti-CD3/CD28 antibodies and

cultured for an additional 6 days before flow cytometry analysis. Bar graph representing flow cytometry data of the percentage of FOXP3-expressing CD4+ T cells (left) and CD8+ T cells (right). (C) Bar graph representing flow cytometry data of the percentage of IFN-γ-expressing CD4+ T cells (left) and CD8+ T cells (right). (D) Flow cytometry data representing the percentage of IFN-γ-expressing T cells within the FOXP3+ (solid bars), FOXP3- (diagonal line bars) or total cell compartment (checkered bars) following activation. (E) Representative Flow Cytometry plots for CD25 MFI expression in T cells. (F) Flow cytometry data representing the percentage of CD25-expressing CD4+ T cells (left) and CD8+ T cells (right). Graphs represent average ± SE (n=3, each sample replicated in duplicate. One-way paired ANOVA, with Geisser-greenhouse correction and Dunnett's multiple comparisons test, with individual variances computed for each paired sample use. ns = not significant, *$p<0.05$, **$p<0.01$. ***$p<0.001$).

3.4.5 - ACh Signaling increases Helios expression in T cells, however, Helios expression decreases over time throughout expansion

After determining some of the mechanisms involved in ACh signaling, the next question to address was if ACh is creating stable Tregs or if the FOXP3+ T cell population is instead a result of transient expressing activated Teffs. To test this, I isolated and cultured T cells using the same methods discussed in the previous chapter. On days 6 and 12, I stained cells for flow cytometry using the same antibody cocktail discussed in the previous chapter, with the addition of an antibody to stain for Helios **(Figure 22A and B)**.

Helios expression in CD4+ and CD8+ T cells was increased in ACh-T cells, although expression was only statistically significant at day 12 of expansion **(Figure 22 C)**. Helios expression in CD4+ ACh-T cells on day 12 was 10.43% (SE = 1.03%), compared to 6.44% (SE = 6.44%) in the Control-T cell population, a proportional increase of 10.89%. Helios expression in CD8+ ACh-T cells on day 6 was 62.36% (SE = 1.27%), compared to 8.13% (SE = 0.52%) in the Control-T cell population, a proportional increase of 65.78%. While Helios expression was higher in ACh-treated samples compared to controls, the expression was not stable and reduced by day 12 compared to day 6, much like untreated controls.

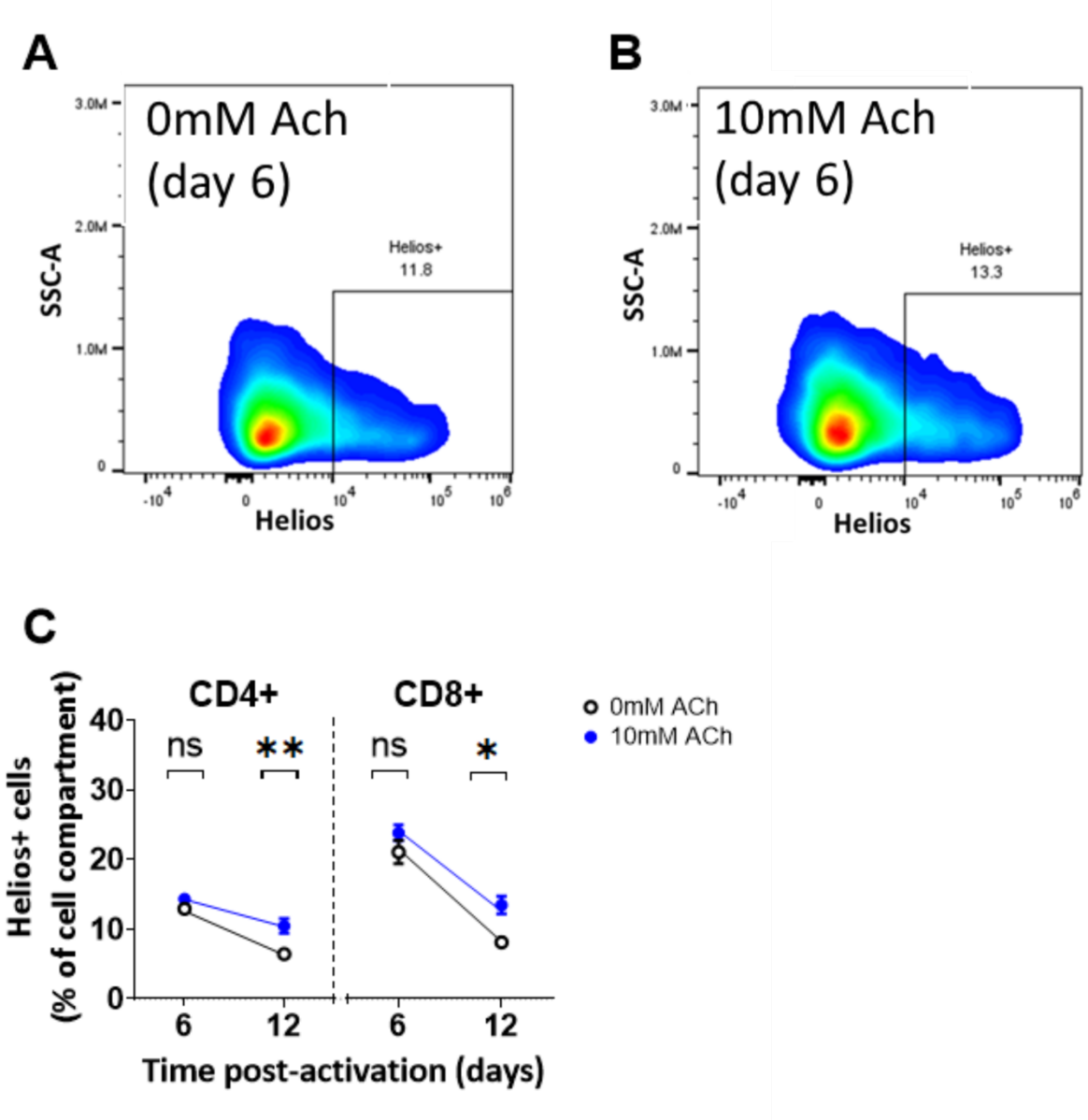

Figure 22. ACh-stimulated T cells express higher levels of Helios, however, the expression levels are not stable throughout expansion. T cells were activated with anti-CD3/CD28 suspension antibodies with IL-2 in the presence (blue) or absence (black lines, white points) of 10mM ACh. (A) and (B) Representative flow cytometry plots for Helios T cells. (C) Flow cytometry data representing the percentage of Helios-expressing CD4+ T cells (left) and CD8+ T cells (right) 6 days and 12 days following activation Graphs represent average ± SE (n=3, each sample replicated in triplicate, Multiple Student's paired *t*-tests used. ns = not significant, *p<0.05, **p<0.01).

3.4.6 – ACh treatment induces the development of FOXP3+/Helios- T cells in culture

Next, I analyzed the same samples for dual expression of FOXP3 and Helios, as shown in **Figure 23A**. A higher percentage of ACh-T cells expressed at least one of FOXP3 or Helios compared to Control-T cells **(Figure 23B-D).** Dual-positive (FOXP3+/Helios+) expression was increased in ACh-T cell samples, however, the expression in both ACh and control samples reduced by day 12 **(Figure 23C)**. Interestingly, T cells expressing FOXP3 and not expressing Helios (FOXP3+/Helios-) were the only subset of both ACh-T cells and Control-T cells that were stable and continued to increase by day 12 of expansion (**Figure 23E)**. Finally, FOXP3-/Helios+ cells were increased in ACh-T cell samples, but much like the other Helios-expressing subset, decreased at day 12 of expansion **(Figure 23B).** These results indicate that elevated Helios expression in T cells is not stable regardless of FOXP3 expression, and T cells maintain FOXP3 expression but not Helios.

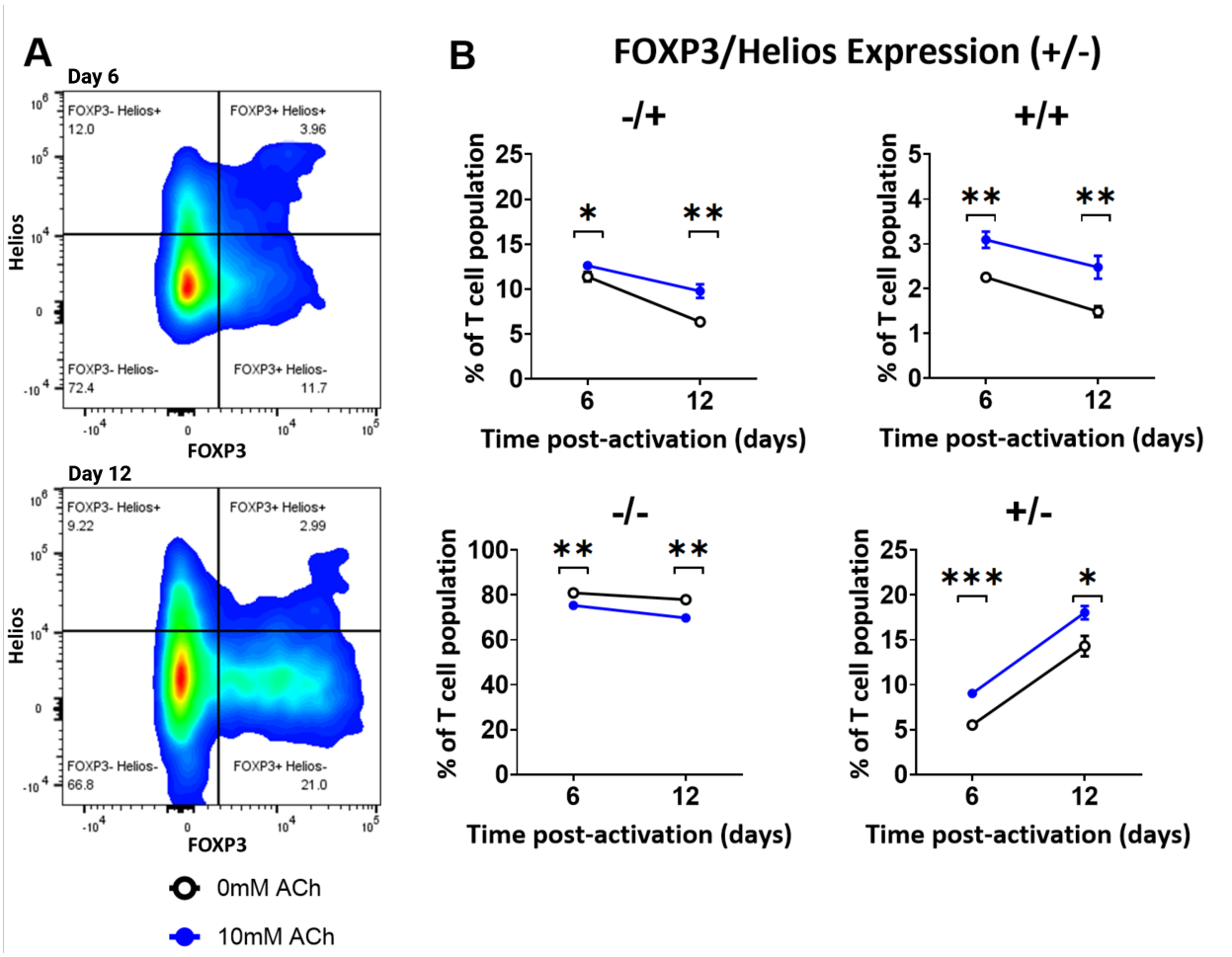

Figure 23. ACh-stimulated T cells stably express FOXP3 throughout expansion, however, Helios is not stable. (A) Representative flow cytometry plot for dual-stained FOXP3 (X-axis) and Helios (Y-axis) T cells, showing respective quadrants for positive and negative staining. Plots represent matched donor samples from day 6 (top) and day 12 (bottom). T cells were activated with anti-CD3/CD28 suspension antibodies with IL-2 in the presence (blue) or absence (black) of 10mM ACh. Flow cytometry data representing the percentage of (B) FOXP3-/Helios+, (C) FOXP3+/Helios-, (D) FOXP3-/Helios-, and (E) FOXP3+/Helios- T cells 6 days and 12 days following activation. Graphs represent average ± SE (n=3, each sample replicated in triplicate, Student's paired *t*-test. *p<0.05, **p<0.01, ***p<0.001).

3.4.7 – ACh treatment induces IFN-γ expression in T cells, especially in the FOXP3+/Helios+ and FOXP3+/Helios- populations

Finally, I measured the percentage of IFN-γ-expressing Teffs within all four FOXP3/Helios subsets **(Figure 24A)** to determine if IFN-γ expression was promoted or suppressed in the Helios+ populations. As expected and consistent with my previous results in chapter 2, IFN-γ-producing cells were increased or equal in all ACh-T cell subsets compared to the control **(Figure 24B)**. Only day 6 samples in FOXP3+ populations had statistical significance. As expected, FOXP3+ samples had the highest percentage of IFN-γ expression **(Figure 24B, first two plots)**, and Helios+ samples expressed less IFN-γ. IFN-γ expression was the lowest in FOXP3- samples (Helios+ and Helios-) **(Figure 24B, last two plots)** and the highest in FOXP3+/Helios- samples **(Figure 24B, second plot)**. The mean IFN-γ expression for each subset at both time points is outlined in **Table 3 in the Appendix**. The proportion of IFN-γ-expressing T cells in all four subsets trended to increase by day 12 compared to day 6 for both ACh-T cells and Control-T cells.

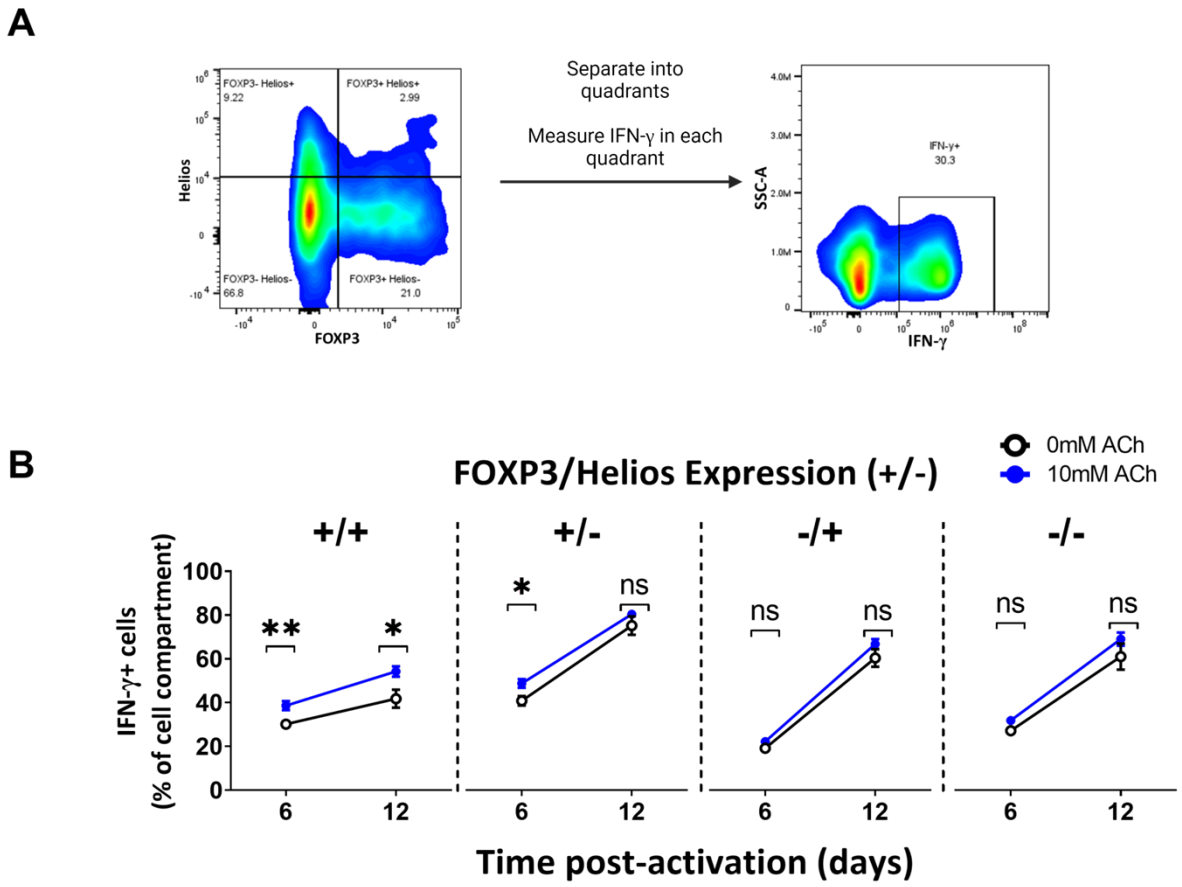

Figure 24. IFN-γ is increased in FOXP3+ T cells and not in Helios-only T cells, IFN-γ is highest in the FOXP3+/Helios- T cell population. T cells were activated with anti-CD3/CD28 suspension antibodies with IL-2 in the presence (blue) or absence (black) of 10mM ACh. (A) Representative flow cytometry plot for dual-stained FOXP3 (X-axis) and Helios (Y-axis) T cells, separated into 4 quadrants and then IFN-γ was measured for each quadrant. Flow cytometry data representing the percentage of IFN-γ-expressing cells within cell compartments: (B) FOXP3-/Helios+, (C) FOXP3+/Helios-, (D) FOXP3-/Helios-, and (E) FOXP3+/Helios-T cells 6 days and 12 days following activation. Graphs represent average ± SE (n=3, each sample replicated in triplicate, Student's paired *t*-test. *p<0.05, **p<0.01).

3.5 - Discussion

Based on the above results, ACh is utilized by T cells to control activation state and differentiation into IFN-γ-expressing Teffs. ACh appears to direct both CD4+ T cells and cytotoxic CD8+ T cells. The data from this chapter builds upon questions that arose from Chapter 2. The first question to address was if an increase in FOXP3 expression correlated with TGF-β production, an indication of suppressive iTregs. To test this, I measured TGF-β secreted into the culture supernatant by T cells stimulated with ACh. TGF-β secretion by ACh-T cells was remarkably lower than Control-T cells, indicating a reduction in TGF-β-secreting suppressive iTregs **(Figure 18A)**. These results indicate that ACh signaling induces FOXP3 during nonspecific T cell activation but does not create actively suppressing iTregs and instead likely reduces them. Previous literature suggests that ACh signaling is sufficient to induce active mouse iTregs[41], however, it appears that this is not sufficient in human T cells. These data presented in this chapter indicate that ACh-induced FOXP3 alone is not sufficient to produce a stable, suppressive Treg population.

Interestingly, while 10mM ACh FRα CAR-T cells were more efficient at eliminating SKOV3 tumor cells **(Figure 17)**, they did not reduce TGF-β secretion compared to controls as observed in naïve T cells **(Figure 18A and B)**. Furthermore, while there was a slight increase in the percentage of IFN-γ expressing cells in the total population of 10mM ACh FRα CAR T samples, statistical significance was not achieved **(Figure 16B)**. Nonetheless, cytotoxicity in 10mM ACh FRα CAR-Ts was higher compared to controls. Therefore, ACh stimulation produced more efficient CAR T cells. While this increase in efficiency may be in an IFN-γ-dependent manner and therefore due to the marginal increase in IFN-γ, there are likely more factors influencing CAR T-dependent tumor cytotoxicity that need to be elucidated. My data

suggests that ACh stimulation of T cells increases FOXP3 expression in CAR-T cells and these cultured FOXP3+ cells have a high percentage of IFN-γ-expression, which suggests they are activated Teffs. It is possible that the increase in tumor cytotoxicity was not due to a reduction in TGF-β by CAR-T cells but rather an increase in FOXP3-expressing active Teffs.

Next, I identified that α7 nAChRs are involved in T cell activation. As discussed, α7 nAChRs are the most well-studied cholinergic receptor in T cells[41,52,53,55]. The results from this chapter implicate the α7 nAChR signal pathway in reducing cell proliferation and FOXP3 induction, but not IFN-γ production in human T cells **(Figure 19)**. Therefore, it appears that signaling through the α7 nAChR alone contributes to FOXP3 production, but other cholinergic receptors or additional signals are likely involved in IFN-γ+ Teff differentiation. These results and previous literature indicate that different types of cholinergic receptors may have disparate effects on T cell functions.

Furthermore, it appears that GTS-21 did not synergistically enhance the effects of TGF-β-induced FOXP3 expression similar to ACh in Chapter 2 **(Figure 20 and 12, respectively)**. This may be due to a multitude of factors. Firstly, GTS-21 may bind to but not cause the exact same signaling cascade with cholinergic receptors that ACh does, resulting in no additional FOXP3 induction when paired with TGF-β. Secondly, while α7 nAChR stimulation alone induces FOXP3, it may be another cholinergic receptor that synergistically acts with TGF-β that was not tested and this receptor binds to ACh and not GTS-21. There may be a combined effort from multiple cholinergic receptor subclasses that work together with TGF-β to enhance FOXP3 expression. Alternatively, the results reported by Fujii *et al*.[7] show an increase in mouse FoxP3 following TGF-β and GTS-21 may not be transferrable to humans. Finally, my results may just be due to insufficient data points, and 3 biological replicates may not be sufficient to achieve a

significant result. Taken all together, α7 nAChR signaling appears to be involved in some of ACh's action on human T cells but does not account for all.

Next, I wanted to determine if external ACh stimulation and autocrine ACh by T cells have the same effect. T cells can receive ACh signals externally or produce their own ACh and it is crucial to identify if the source of ACh changes the phenotypic result. To elucidate this, I produced T cells that had impaired ChAT expression through sgRNA/CRISPR/Cas9 electroporation with the aid of my colleague Gillian Carleton. Indels created in the ChAT locus impair T cell populations from being able to synthesize ACh. T cells that received ChAT sgRNA (impaired ACh production) resulted in less FOXP3 and CD25 expression compared to T cells that did not receive ChAT sgRNA (unaltered ACh production) **(Figure 21)**. These results suggest autocrine ACh is involved in FOXP3 and CD25 expression in human T cells during activation *in vitro*. As CD25 expression is indicative of the T cell activation state and FOXP3 can be activation-induced, these results suggest that ChAT+ T cells control T cell activation state. Therefore, T cells appear to use a combination of autocrine ACh signaling and external ACh sources to control their activation state and potentially control the degree of the immune response.

Finally, I wished to determine if ACh stimulation correlated with a stable iTreg or activated Teff phenotype. To do this, I repeated previous experiments and measured the expression of the transcription factor Helios in combination with FOXP3 and IFN-γ. Combined Helios and FOXP3 expression are strong indicators of stable human Tregs[163,164,169], I wished to determine if Helios was stably induced by ACh *in vitro*. Interestingly, Helios expression overall was increased in ACh-T cells **(Figure 22)**. However, Helios expression did not appear to be stable, as Helios levels reduced from day 6 of culture to day 12 like Control-T cells **(Figure**

22C). Helios expression has been shown to reduce over time during cell culture in published data[169]. My data indicate ACh elevated Helios expression in T cells but the effect drops off by day 12 of expansion. It is likely that Helios is increased due to activation-induced effects and is transiently, but not stably expressed.

These results are further reinforced when T cells are separated by dual staining of FOXP3 and Helios **(Figure 23)**. FOXP3 expression is stably induced on day 6 and persists by day 12, but Helios expression does not. While there is an increase in FOXP3+/Helios+ T cells in 10mM ACh samples on day 6, the percentage of this subpopulation is reduced by day 12, indicating that prolonged stability in Treg cells is not reliably generated by ACh stimulation **(Figure 23B)**. Conversely, FOXP3+/Helios- T cells make up the bulk of the population expressing either transcription factor and the proportion of these cells increases even further between days 6 and 12 **(Figure 23B)**. This indicates that while T cells stimulated with ACh may initially express both FOXP3 and Helios due to transient activation-based effects, it is only the T cells that do not express Helios that persist. While Helios may be increased initially, these effects are only short-term and do not confer a true stable iTreg phenotype after longer cultures.

The above results are supported when I measured IFN-y expression by each T cell subset on days 6 and 12 **(Figure 24A).** IFN-γ expression in all four T cell subpopulations was higher in ACh-T cells compared to the control (with only FOXP3+ cells having statistical significance) **(Figure 24B, first two plots)**. This indicates that expression of Helios is likely a transient expression from Teffs. Furthermore, the highest percentage of IFN-γ-expressing cells were found in the FOXP3+/Helios- subpopulation **(Figure 24B, second plot)**. This indicates that the T cell subpopulation with the highest effector function were T cells expressing FOXP3 and not expressing Helios. As mentioned before, human Tregs expressing FOXP3 but not Helios are

capable of transitioning to Teff phenotypes[164,166,170]. Therefore, while ACh signaling may promote FOXP3, if iTregs are being formed, these cells can transition into effector T cells over time. My results suggest the need for more robust iTreg markers that can be utilized in *in vitro* conditions, as FOXP3 is the most common marker used in literature but does not sufficiently identify suppressive iTregs.

Chapter 4

4.1 - Summary and discussion of previous chapters

The objective of this thesis was to understand the complex signaling effects of the neurotransmitter acetylcholine on T cell activation to enhance T cell-based cancer immunotherapies. To this purpose, I investigated the effects of direct ACh stimulation of activated human T cells *in vitro*. My work provides context and a deeper understanding of current literature published, answering questions, and filling in gaps of knowledge.

In Chapter 1, I highlighted previous results published by Dr. Marisa Kilgour *et al*. from our lab that metabolically profiled the TME[109]. I re-analyzed this data and identified that ACh was significantly enriched in CD4+ TILs in ovarian cancer patients **(Figure 5)**. This discovery drove me to conduct an extensive literature search and to identify a multitude of ACh-related functions in the immune system: vasodilation and tissue infiltration[49], reduction of macrophage-produced TNF-α and inflammation[50,52], and mouse T cell class differentiation[41]. However, these publications have yet to fully elucidate all the effects and downstream mechanisms involved in human T cell/ACh interactivity, especially in the context of cancer.

In Chapter 2, I investigated the role of ACh on human T cell differentiation and CAR-T antitumor effector functions by supplementing naïve healthy donor human T cells with ACh during activation and throughout expansion. My first goal was to confirm that human T cells respond to ACh signaling by increasing FOXP3 and IFN-γ expression, much like mouse T cells published by Fujii and Mashimo *et al*.[41]. I confirmed that the link between ACh signaling and T cell differentiation observed in mice was consistent in humans **(Figures 9-14)**. Further, as their methods used additional cytokines and inhibitory antibodies alongside GTS-21 to promote specific class differentiation (i.e. IL-12 and anti-IL-4 antibody for Th1s and TGF-β for Tregs)[41],

this study did not confirm if ACh alone can produce these effects or if it is a supplementary magnification signal. My results indicated that ACh alone was sufficient to induce FOXP3 and increase in IFN-γ in human T cells following activation with anti-CD3/CD28 antibodies and IL-2 **(Figures 9, 13, and 14)**. I also confirmed that ACh supplementation further enhanced FOXP3 expression in T cells supplemented with TGF-β **(Figure 12)**. These data solidify the compounding effect ACh has on T cell differentiation.

Next, I investigated the effects of ACh on CAR-T cell generation and CAR-T-specific cytotoxicity by generating FRα CAR-T cells under ACh culture conditions and tested their antitumor efficiency. Flow cytometry and co-killing luciferase assay indicated that ACh increased FOXP3 and antitumor efficiency in CAR-T cells **(Figures 16 and 17)**. As FOXP3 expression in circulating T cells is commonly linked to suppressive Treg populations which should inhibit, not promote cancer killing, these results were quite surprising. Therefore, I decided to investigate if ACh signaling was creating "true" and "stable" iTregs in culture, or if FOXP3 was instead transiently expressed by highly active Teffs.

It is possible that FOXP3 expression in cultured T cells does not translate to a suppressive iTreg population[140]. Instead, activated T cells may transiently express FOXP3 within controlled cell culture environments[131,141,142]. Furthermore, it is possible that the increase in IFN-γ-producing CD4+ and CD8+ Teffs by ACh simply outweighs a comparatively weaker increase in iTreg activity, and this is the cause of increased CAR-T cell tumor clearance.

If ACh was creating suppressive iTregs, then TGF-β secretion should be increased. This change in TGF-β secretion may be a source for the enhanced CAR-T efficiency. In Chapter 3, I first investigated this production of TGF-β in ACh-T cells. ELISA examination of culture supernatant TGF-β concentration identified a significant reduction in TGF-β secreted by naïve T

cells stimulated with ACh, although 10mM ACh FRα CAR-T cells did not have a significant change **(Figure 18)**. A reduction in TGF-β secretion by ACh-stimulated T cells indicates that suppressive iTregs are reduced and Teffs predominate. Interestingly, the FRα CAR-T cells did not share the same result. This result requires further investigation.

Next, I investigated the mechanism of action of ACh on T cell expression of FOXP3 and IFN-γ. I confirmed that α7 nAChR signaling was involved in human T cell expression of FOXP3, similar to mouse T cell results published[41]. However, I found that α7 nAChR signaling alone was not sufficient to increase IFN-γ expression in human T cells. Human T cells may require additional cytokine signals aside from α7 nAChR signaling to induce IFN-γ, unlike FOXP3. It is also possible that a combination of different cholinergic receptor subclasses is required to induce IFN-γ in human T cells, rather than just α7 nAChRs.

Most of my experiments up until this point investigated external ACh supplementation on T cells. However, T cells can produce their own ACh by expressing ChAT[49,56]. My next investigation was the effect of inhibiting ChAT expression in T cells. I found that ablating the expression of ChAT through inserting or deleting nucleotides at the ChAT locus using CRISPR/Cas9 reduced the expression of FOXP3 and CD25 but did not significantly change IFN-γ **(Figure 21)**. Therefore, it is possible that both external and autocrine ACh signaling is responsible for FOXP3 expression, and that activation state (through CD25 expression) may be controlled by autocrine ACh in T cells. There may be further downstream effects of cholinergic signaling other than ChAT expression that influences IFN-γ-producing Teff differentiation that has not been elucidated yet.

Finally, I investigated the stability of the transcription factor Helios in ACh-stimulated cells. Since FOXP3 expression alone is not sufficient to identify stable, actively suppressing

iTregs in *in vitro* conditions, dual expression of Helios and FOXP3 is a more robust metric to test[164,169,170]. ACh-T cells increased Helios expression compared to controls **(Figure 22)**. However, Helios expression was reduced by day 12 of expansion compared to day 6, which indicates that Helios expression is transient. Furthermore, only FOXP3+/Helios- T cells persisted and increased by day 12 of expansion, while the percentage of FOXP3+/Helios+, FOXP3-/Helios+, and FOXP3-/Helios- populations reduced **(Figure 23)**.

The final important piece of my thesis was correlating IFN-γ expression to specific FOXP3/Helios-expressing subsets. The proportion of IFN-γ-producing cells was increased only in FOXP3+ cell populations and the highest expression was found in the actively expanding FOXP3+/Helios- population **(Figure 24)**. This indicated that stable iTregs are not induced by cholinergic signaling and it is instead active Teffs transiently expressing FOXP3 and Helios.

Here, I suggest that ACh signaling is used by human T cells to direct the activation state and control the degree of the immune response. Previous literature suggests that ACh may suppress functions of other immune cell types such as macrophages and dendritic cells[41,52,53], but my results suggest that T cells respond positively to ACh under *in vitro* activation conditions. T cells may use ACh to self-regulate and prioritize the activity of other immune cell types to control immune responses in coordination with the nervous system as an integrated network.

4.2 - Integration of theories from Chapters 2 and 3

In Chapters 2 and 3, I examined the link between cholinergic signaling, effector function, and activation state in human T cells. Chapter 2 identifies key outcomes from ACh supplementation on T cell effector function: induction of FOXP3, IFN-γ, and increased antitumor efficiency. Chapter 3 expands on these findings by investigating T cell functions

through secretion of TGF-β, autocrine ACh production, and unstable expression of Helios throughout the expansion. Together, I demonstrate that ACh regulates the degree of T cell differentiation and activation state, identifying this metabolite as a key modulator of effector T cell function.

The unexpected results from my experiments in Chapter 2 caused me to re-evaluate my methods and hypothesis for Chapter 3. My initial hypothesis at the start of my thesis was built upon the published data that highlighted two key effects: the cholinergic anti-inflammatory pathway in macrophages, and FoxP3 (the classical Treg marker) in activated mouse T cells[41,52]. I initially theorized that ACh suppressed T cell function and that it was a source of immunosuppression in the TME after I identified its abundance in ovarian cancer patients' tissues from preliminary data published from our lab[109]. However, my results from Chapter 2 led me to reconsider different methodologies. At the beginning of my thesis, I was focused on T cell suppression and iTregs so most of my experiments revolved around Treg functions. However, I now understand that elevated FOXP3 expression in cultured human T cells does not translate to elevated iTreg-based suppression. My experiments using ACh produced more efficient CAR-T cells rather than less efficient samples. Therefore, Chapter 3 was dedicated to elucidating the mechanism behind the cholinergic activation of T cells and determining why these 10mM ACh FRα CAR-T cells were *more* efficient rather than less. My first step in Chapter 3 was to investigate if the increase in FOXP3 expression in ACh-treated human T cells resulted in higher expression of TGF-β (a Treg cytokine). Next, I tested if autocrine production of ACh in human T cells influenced FOXP3, IFN-γ, and activation state. Then, I investigated if Helios was stably induced in coordination with FOXP3 to represent a stable iTreg population instead of transiently activated FOXP3+ Teffs. Chapter 3 showcases that ACh-stimulated naïve T cells have elevated

FOXP3 but lower TGF-β secretion, ChAT+ T cells are required for FOXP3 and CD25 expression in culture, and Helios is not stably induced by ACh. Together, these data from Chapters 2 and 3 strongly suggest that ACh signaling in human T cells controls activation state and promotes highly active IFN-y-producing Teffs, contrary to my initial hypothesis.

4.3 – Considerations, Complications, and Caveats

Acetylcholine is a relatively stable molecule, remaining stable in cell culture media for up to 84 days when stored at -20°C or 4°C, 28 days at 25°C, and one day at 50°C[171]. Since cell culture incubation conditions are set at 37°C, this sits in between published temperatures and my cell culture experiments do not exceed 12 days, so the stability of ACh should be expected. Furthermore, the cell culture media was replaced and ACh replenished on every day cells were split (days 0, 3, 6, 8, and 10). Since ACh is replenished every 2-3 days, it should be expected to be stable throughout all expansion.

One consideration is the action of acetylcholine esterase (AChE). As mentioned in Chapter 1, AChE is one of the fastest active enzymes in the human body, breaking down a molecule of ACh every 80μs[13,172]. This is required in the nervous system as nerve signals need to be activated and rapidly turned off for proper bodily function. This means that cholinergic signaling in T cells may be rapidly turned over following ACh supplementation in culture. To combat this, ACh was administered after anti-CD3/CD28 activator antibody and IL-2 was added to samples to ensure ACh signals are transmitted while T cells are receiving activation signals, not before. Furthermore, at high concentrations of 10mM ACh in culture, it is expected that T cells should be receiving strong, prolonged ACh stimulation throughout culture, rather than a brief signal right at administration. Furthermore, results from 10mM ACh experiments were replicated with the cholinergic drug GTS-21 (FOXP3, proliferation, but not IFN-γ). This

indicates that administration of 10mM ACh had similar phenotypic effects as GTS-21, indicating that ACh at this concentration is sufficient to elicit a phenotypic change on T cells. Using AChE inhibitor drugs such as pyridostigmine bromide to prevent AChE turnover of ACh were considered, but all drugs considered posed a significant health risk to our laboratory and as such, alternative methods were utilized.

T cells may also become desensitized to ACh stimulation after extended time in culture with ACh, much like nerve cells[173,174]. To my knowledge after an extensive literature review, there has not been much research into whether human T cells desensitization to ACh in culture. As nervous system desensitization equipment is not present in our lab, T cell desensitization tests could not be completed for this thesis. If T cells become desensitized to ACh after many days of exposure, this may be a source for my data losing statistical significance by day 12 of expansion. To investigate this, I suggest future experiments that administer ACh temporarily on T cells, adding and removing ACh stimulus and testing effects on T cell function.

As mentioned, ChAT+ T cells control vasodilation during some viral infections[45]. Since vasodilation increases blood flow to certain areas of the body and focuses nutrients and other inflammatory signals to the area of inflammation, this could contribute to cancer nutrient redirection and eventually tumor growth. On the other hand, increased vascular permeability can also assist in immune cell and immunotherapy drug infiltration into tumors. A handful of vasodilator drugs are undergoing clinical trials and are showing promising results in delivering chemotherapy and directing immune cells to tumor sites, promoting antiproliferative and apoptotic effects on tumors[175]. The effects of these drugs on cancer still need further elucidation before efficacy and safety can be fully confirmed. While the Teff enhancement results from my thesis are promising, further investigations into ACh signaling on T cells within the human body

system-like conditions need to be completed to ensure no unexpected negative, tumor-promoting effects are seen.

As discussed in Chapter 3, active TGF-β levels are difficult to measure directly, as it is commonly complexed with LAP in an immune-unreactive state and needs to be disassociated before analysis, which is difficult for flow cytometry. Therefore, direct measurement of immunoreactive TGF-β will not be accurate under standard flow cytometry conditions. I attempted to stain T cells for active TGF-β through flow cytometry, but all attempts to measure immunoreactive TGF-β through flow cytometry were not effective and data were not useable (results not shown). To account for this, secreted culture supernatant TGF-β was instead measured rather than intracellular levels through a TGF-β ELISA kit. The methods for the TGF-β ELISA kit incorporated an HCl incubation and NaOH neutralization step which disassociated TGF-β from the LAP complex, which allowed my experiments to quantify secreted amounts of TGF-β accurately.

4.4 - Future directions

The results of my thesis shed some light on the complexity of ACh signaling pathway in T cells. However, this field is still in its relative infancy and many more questions need to be answered. The downstream signaling mechanisms that cause FOXP3 and IFN-γ induction through ACh signaling need to be elucidated further. While the α7 nAChR has been implicated in FOXP3 induction, my results do not indicate that it significantly contributes to IFN-γ production. The literature published by Mashimo and Fujii *et al*. suggests that mouse T cells produce increased levels of IFN-γ when they are supplemented with the Th1-polarizing cytokine IL-12 and stimulated through α7 nAChRs, however, this may not be replicable in human T

cells[32]. Perhaps another cholinergic receptor subclass is responsible for IFN-γ in human T cells that were not tested.

As many cholinergic blocking chemicals are quite toxic and dangerous to bring into a lab, I suggest a laboratory with the proper equipment to handle neurotoxic drugs consider testing cholinergic receptor antagonists on T cell activation and antitumor efficacy. Since some cholinergic antagonists are used as current commercial drugs, perhaps there exists a drug cocktail that can be developed that can enhance T cell antitumor effector function through manipulation of specific cholinergic receptor subclasses. Ideally, there could be a method to specifically target the cholinergic receptors that are involved in IFN-γ expression and elevated Teff activity to design highly active and efficient CAR-T cells for therapies.

My research has been focused on naïve T cell activation; this is not always transferrable *in vivo* and clinical trials as there are often compounding variables in the body that is not accounted for in a controlled lab setting. Therefore, I suggest experiments designed to be as similar to *in vivo* conditions as possible. I would suggest experiments using T cell culture media designed to be more like human body conditions, such as the human plasma-like medium (HPLM), or culturing T cells with supplemented ACh in tumor-like suppressive conditions such as ascites supernatant. Completing experiments in conditions similar to the human body - especially cancer-like environments - will build significance before moving on to clinical trials.

While my results suggest that Teff stimulated with ACh are more active, as evidenced by the FOXP3/Helios, TGF-β, and IFN-γ data, as well as the CAR-T killing assay, all T cell functions have not been tested. There may be other off-target T cell mechanisms not investigated that have thus far gone unnoticed and could be a source of some of the unanswered questions. Some examples may be: why were the CAR-T cells stimulated with ACh more efficient at

killing SKOV3 tumor cells, if total IFN-γ was only slightly increased and TGF-β was not decreased? Why did external ACh-stimulated T cells produce more IFN-γ, but T cells with impaired ChAT expression did not have an altered IFN-γ production? It is possible that granzyme and perforin (chemicals secreted by cytotoxic T cells to kill tumor cells) are also affected by cholinergic signaling and this is the reason the CAR-T cells had higher SKOV3 cytotoxicity?

Furthermore, while my co-killing assay data may suggest a novel therapy development in the future, there are more conditions required to test. First, I suggest future researchers complete a co-killing assay using FRα CAR-T cells from day 6, day 12, and longer expansion times to identify if ACh-CAR-T cells maintain their elevated effector functions through an extended period and not just on day 6 of expansion. Since my data may suggest that the increase in T cell effector function may be short-term, these tests would identify if cholinergic signaling is a viable long-term therapy option. Next, my co-killing assay experiments were designed to only test ACh supplementation on T cell activation and development *before* co-culture cancer-killing, and as such all traces of external ACh was removed before the co-culture with SKOV3 cancer cells. Due to this, I suggest a repeat of the killing assay experiment with additional supplementation of ACh in the media during the 48-hour co-culturing period to identify if ACh signaling impacts Teff function during tumor cell recognition in unexpected ways. There is some limited research published that may suggest ACh signaling promotes tumor cell proliferation and metastasis[176–178]. This recommended ACh-supplemented co-culturing experiment will be determine if ACh's enhancement of CAR-T cells outweigh any potential negative effects it has on promoting tumor growth. Finally, I also suggest the generation of CAR-T cells with impaired ChAT expression through the same ChAT sgRNA CRISPR/Cas9 experiment performed in this thesis. I expect that

CAR-T cells with impaired ChAT activity should be less efficient at killing tumor cells. This will reinforce my results indicating that ACh is an important signal used by T cells to enhance effector function following activation. Therefore, techniques to take advantage of enhanced T cell effector functions through cholinergic signaling will hopefully be a promising therapeutic option in the future.

In conclusion, my findings in this thesis have shed some light on the complex and fascinating interconnectivity between the immune and nervous systems. T cells have a far deeper interconnectedness in other body systems than just the immune system. I hope that my results will provide the groundwork for novel therapies in the future, and I hope researchers follow down this pathway and investigate some of the unanswered questions. Hopefully, this thesis has identified a previously untested method to improve the efficacy of therapies designed to target difficult-to-treat cancers and save the lives of patients in the future.

Appendix, Abbreviations, and Supplementary Materials

Abbreviations

ACh – acetylcholine

AChE – acetylcholinesterase

AChR – acetylcholine receptor

APCs – antigen presenting cells

CAP – cholinergic anti-inflammatory pathway

CAR-T cell – chimeric antigen receptor T cell

Cas9 – CRISPR-associated protein nuclease

ChAT – choline acetyltransferase

CRISPR – clustered regularly interspaced short palindromic repeats

CD – cluster of differentiation

CoA – coenzyme A

CRS – cytokine release syndrome

ELISA – enzyme-linked immunosorbent assay

E:T ratio – effector to target ratio

FOXP3 – forkhead box P3

FMO – fluorescence minus one

FRα – folate receptor alpha

GFP – green fluorescent protein

ICANS – immune effector cell-associated neurotoxicity syndrome

IFN-γ – interferon gamma

IL – interleukin

IL2RA – IL-2 receptor alpha

iTreg – induced Treg

LAP – latency associated peptide

MACS – magnetic-activated cell sorting

nAChR – nicotinic acetylcholine receptor

NE – norepinephrine

NK cell – natural killer cell

nTreg – natural Treg

PBMC – peripheral blood mononuclear cells

RLU – relative luminescence

sgRNA – single guide RNA

SKOV3 – ovarian cancer cell line derived from a 64-year-old Caucasian female

TCR – T cell receptor

Tconv – conventional T cell

Teff – effector T cell

Th1 – T cell helper class 1

TILs – tumor infiltrating lymphocytes

TGF-β – transforming growth factor beta

TME – tumor microenvironment

TNF-α – tumor necrosis factor alpha

Treg – regulatory T cell

Table 1: Antibody and fluorochrome mix for flow cytometry

Marker	Fluorochrome	Relative brightness (1 to 4)	Supplier name
Viability dye	eFluor 450	2	ThermoFischer
CD3	BrilliantViolet 750	3	Biolegend
CD4	Alexa Fluor 700	2	Biolegend
CD8	PerCP	1	Biolegend
CD25	APC-Cy7	2	BD Biosciences
FOXP3	Alexa Fluor 647	4	BD Biosciences
IFN-y	PE	4	Biolegend
Helios	Alexa Fluor488	3	BD Biosciences

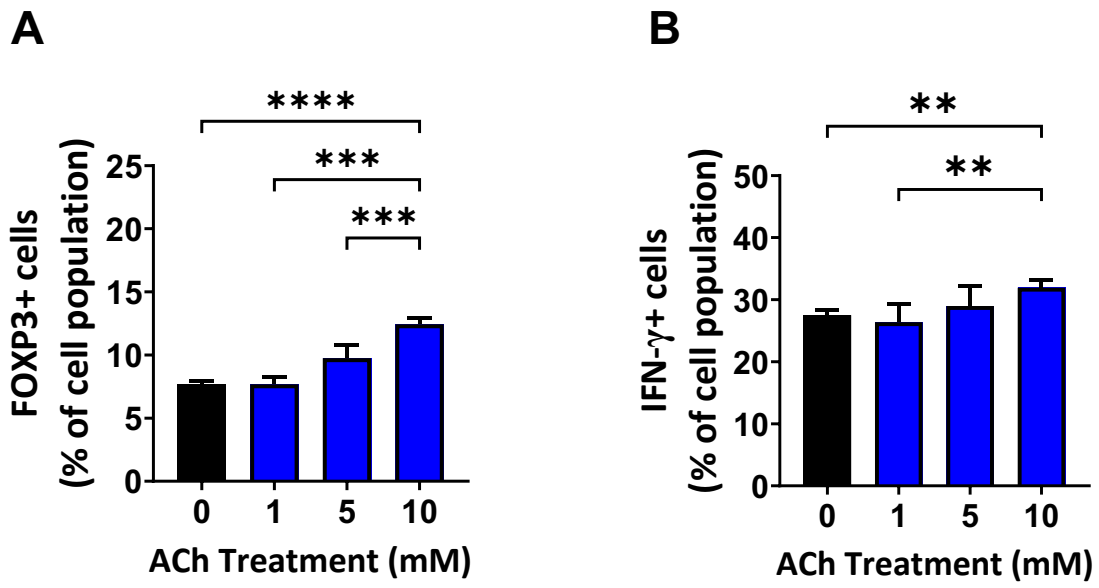

Figure 25. ACh increases FOXP3 and IFN-γ expression dose-responsively. Activated T cells were supplemented with IL-2 (300U/ml) in the presence or absence of ACh for 6 days. Flow cytometry data representing the percentage of FOXP3-expressing (A) and IFN-γ-expressing (B). Graphs represent average ± SE (n=6, Mixed-effects analysis, with Geisser-greenhouse correction and Dunnett's multiple comparisons test, with individual variances computed for each paired sample used for (A). Student's paired *t*-test used for (B). **p<0.01, ***p<0.001, ****p<0001).

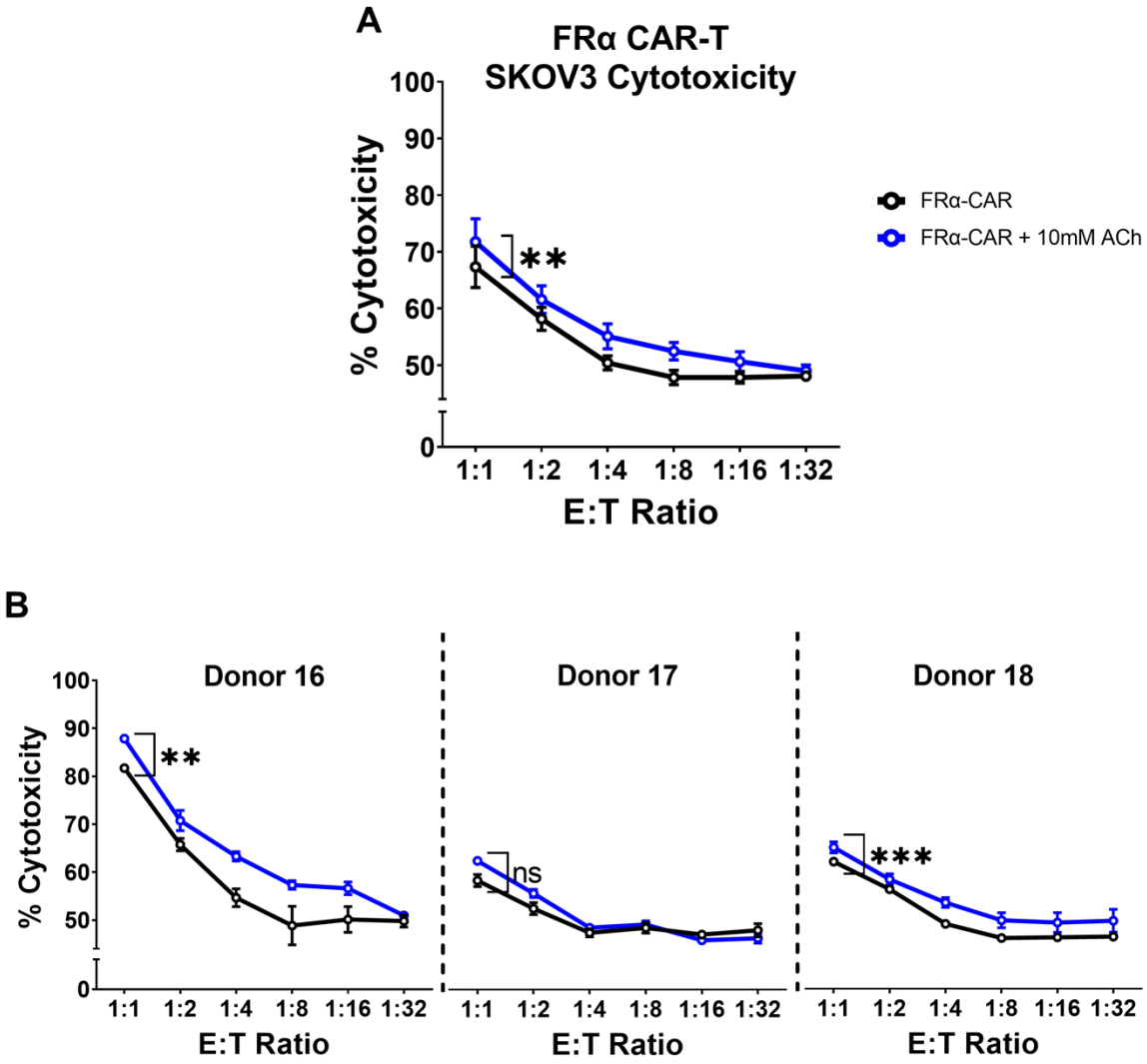

Figure 26. FRα CAR-T SKOV3 killing assay results separated by donor populations. Percent cytotoxicity of FRα CAR-T cells from averaged donor samples (A) and separated donor samples (B) measured by the change in relative luminescence (RLU) of the SKOV3 cells compared to no CAR-T cell controls. Fluorescence values were read using *Varioskan Lux* Plate Reader (ThermoFisher) with ScanIT RE 6.1 software. Graphs represent average ± SE (n=3, each sample replicated in triplicate. Student's paired *t*-test. ns = not significant, **p<0.01, ***p<0.001).

Raw statistical values

Table 2: Corresponding raw data for Chapter 2

Naïve T cells – ACh stimulation

		Healthy donor CD3+ T cells											
	Cell compartment	CD4+ Cells						CD8+ Cells					
	ACh supplementation	0mM			10mM			0mM			10mM		
		Mean	SE	N	Mean	SE	N	Mean	SE	N	Mean	SE	N
Metric	Time point												
Cell Count (Total T cell pop.)	6 Days	1.76E+07	7.20E+05	17	1.43E+07	6.20E+05	17	-	-	-	-	-	-
% Viability (Total T cell pop.)	6 Days	84.78	1.60	23	86.64	1.28	23	-	-	-	-	-	-
% FOXP3+ cells	6 Days	6.99	0.40	27	11.14	0.63	26	4.43	0.37	18	7.00	0.62	14
	12 Days	13.06	1.39	14	17.38	1.89	14	12.39	1.45	14	15.28	1.50	14
	24 Hours	6.42	1.22	8	7.03	1.34	9	10.26	1.19	5	10.47	1.11	6
	48 Hours	7.18	0.28	5	9.39	0.64	6	7.85	0.43	5	9.38	0.71	6
% IFN-γ+ cells	6 Days	27.97	0.91	22	33.21	1.23	21	36.73	2.57	18	40.48	2.42	17
	12 Days	45.46	3.52	14	52.66	3.73	14	72.48	4.19	14	80.03	2.90	14
% IFN-γ+ cells (FOXP3+ pop.)	6 Days	36.97	2.54	22	44.16	2.56	21	55.53	3.12	15	62.82	2.51	15
	12 Days	57.25	2.87	12	62.34	3.66	12	82.61	3.10	12	86.47	2.75	12
% IFN-γ+ cells (FOXP3- pop.)	6 Days	26.75	0.87	22	31.20	1.16	21	35.49	2.40	18	39.30	2.28	17
	12 Days	44.87	3.46	12	50.91	3.80	12	70.51	4.64	12	77.63	3.19	12

Naïve T cells – ACh + TGF-β stimulation

		Healthy donor CD3+ T cells											
	Supplementation	No TGF-β			2.5 ng/ml TGF-β			10mM ACh			ACh + TGF-β		
		Mean	SE	N	Mean	SE	N	Mean	SE	N	Mean	SE	N
Metric	Time point												
Cell Count (Total T cell pop.)	6 Days	1.68E+07	2.50E+00	5	1.25E+07	1.34E+06	5	1.02E+07	2.11E+06	5	9.00E+06	1.99E+06	5
% FOXP3+ cells	6 Days	2.74	1.09	3	17.04	8.93	3	6.19	2.29	3	26.30	7.97	3
	12 Days	6.91	0.72	3	28.00	2.97	3	9.71	0.62	3	44.80	3.70	3

FRα CAR-T cells – ACh stimulation

		FRα CAR-T cells											
	Cell compartment	CD4+ Cells						CD8+ Cells					
	ACh supplementation	0mM			10mM			0mM			10mM		
		Mean	SE	N	Mean	SE	N	Mean	SE	N	Mean	SE	N
Metric	Time point												
Cell Count (Total T cell pop.)	6 Days	1.95E+07	2.03E+06	3	1.20E+07	1.02E+06	3	-	-	-	-	-	-
% Viability (Total T cell pop.)	6 Days	80.50	0.52	3	79.00	0.50	3	-	-	-	-	-	-
% CAR Expression (GFP+)	5 Days	42.30	4.25	3	40.23	6.98	3	-	-	-	-	-	-
% FOXP3+ cells	6 Days	9.78	0.31	3	17.50	1.06	3	7.65	0.36	3	12.01	0.65	3
% IFN-γ+ cells	6 Days	30.33	4.05	3	32.80	4.42	3	44.28	1.97	3	45.98	2.04	3
% IFN-γ+ cells (FOXP3+ pop.)	6 Days	37.39	4.11	3	40.24	4.49	3	63.98	3.37	3	64.14	2.79	3
% IFN-γ+ cells (FOXP3- pop.)	6 Days	31.48	5.04	3	33.34	5.59	3	44.01	2.54	3	45.39	2.90	3

FRα CAR-T cells – SKOV3 cytotoxicity assay

		FRα CAR-T cells																	
	Cell compartment	L16						L17						L18					
	ACh supplementation	0mM			10mM			0mM			10mM			0mM			10mM		
		Mean	SE	N	Mean	SE	N	Mean	SE	N	Mean	SE	N	Mean	SE	N	Mean	SE	N
E:T Ratio	Metric																		
1:1	% Cytotoxicity	81.70	0.62	3	87.85	0.48	3	58.25	1.26	3	62.34	0.32	3	62.08	0.42	3	65.05	1.15	3
1:2		65.74	1.30	3	70.75	2.13	3	52.43	1.25	3	55.56	0.84	3	56.31	0.08	3	58.37	1.14	3
1:4		54.66	1.87	3	63.28	0.99	3	47.38	0.85	3	48.43	0.20	3	49.09	0.59	3	53.53	1.03	3
1:8		48.86	4.03	3	57.31	0.89	3	48.41	1.11	3	49.09	0.78	2	46.15	0.17	3	49.84	1.57	3
1:16		50.13	2.68	3	56.62	1.31	3	47.01	0.60	3	45.83	0.62	3	46.30	0.45	3	49.38	2.07	3
1:32		49.79	1.27	3	50.96	0.70	3	47.90	1.36	3	46.25	1.02	3	46.45	0.57	3	49.74	2.38	3

Table 3: Corresponding raw data for Chapter 3

TGF-β supernatant concentration

	Cell compartment	Healthy Donor T Cells						FRα CAR-T Cells					
	ACh supplementation	0mM			10mM			0mM			10mM		
		Mean	SE	N	Mean	SE	N	Mean	SE	N	Mean	SE	N
Metric	Time point												
TGF-β concentration (pg/ml)	6 Days	2,394.00	58.15	3	1,686.00	108.00	3	1,729.00	244.30	3	1,725.00	164.80	3

Naïve T cells – GTS-21 stimulation

	Cell compartment	Healthy donor CD3+ T cells					
	GTS-21 supplementation	0μM			30μM		
		Mean	SE	N	Mean	SE	N
Metric	Time point						
Cell Count (Total T cell pop.)	6 Days	1.42E+07	6.50E+05	3	8.78E+06	1.45E+06	3
% FOXP3+ cells	6 Days	4.24	0.85	3	10.47	1.71	3
% IFN-γ+ cells	6 Days	24.77	2.38	3	25.80	1.51	3

Naïve T cells – GTS-21 + TGF-β stimulation

		Healthy donor CD3+ T cells											
	Supplementation	No TGF-β			2.5 ng/ml TGF-β			30μM GTS-21			ng/ml TGF-β + 30μM GTS		
		Mean	SE	N	Mean	SE	N	Mean	SE	N	Mean	SE	N
Metric	Time point												
Cell Count (Total T cell pop.)	6 Days	1.68E+07	2.50E+06	3	1.25E+07	1.34E+06	3	8.60E+06	2.34E+06	3	8.28E+06	1.25E+06	3
% FOXP3+ cells	6 Days	2.74	1.09	3	17.04	8.93	3	11.96	3.44	3	9.52	2.99	3
	12 Days	6.91	0.73	3	28.00	2.97	3	3.53	0.34	3	25.93	4.54	3

ChAT knockout T cells

		CRISPR/Cas9 ChAT K/O Helathy Donor T Cells											
	Cell compartment	CD4+ Cells						CD8+ Cells					
	ChAT sgRNA addition	No ChAT sgRNA			+ ChAT sgRNA			No ChAT sgRNA			+ ChAT sgRNA		
		Mean	SE	N	Mean	SE	N	Mean	SE	N	Mean	SE	N
Metric	Time point												
% FOXP3+ cells	6 Days	5.96	0.60	3	4.39	0.13	3	6.83	0.91	3	5.40	0.70	3
% IFN-γ+ cells	6 Days	38.97	1.36	3	41.53	0.46	3	77.30	0.70	3	81.17	0.37	3
% CD25+ cells	6 Days	84.58	1.50	3	73.40	3.25	3	87.48	2.18	3	76.93	2.74	3

Naïve T cells – Helios expression

		Healthy donor CD3+ T cells											
	Cell compartment	CD4+ Cells						CD8+ Cells					
	ACh supplementation	0mM			10mM			0mM			10mM		
		Mean	SE	N	Mean	SE	N	Mean	SE	N	Mean	SE	N
Metric	Time point												
% Helios+ cells	6 Days	12.96	0.64	3	14.37	0.44	3	21.10	1.67	3	23.86	1.15	3
	12 Days	6.44	0.35	3	10.43	1.03	3	8.13	0.52	3	13.47	1.27	3

Naïve T cells – Helios vs FOXP3 expression

	Cell compartment	Healthy donor CD3+ T cells					
	ACh supplementation	0mM			10mM		
		Mean	SE	N	Mean	SE	N
Metric	Time point						
% FOXP3-/Helios+ cells	6 Days	11.38	0.53	3	1.62	0.45	3
	12 Days	6.35	0.23	3	9.78	0.77	3
% FOXP3+/Helios+ cells	6 Days	2.26	0.09	3	3.09	0.18	3
	12 Days	1.49	0.12	3	2.48	0.26	3
% FOXP3-/Helios- cells	6 Days	80.86	0.90	3	75.28	0.64	3
	12 Days	77.86	1.16	3	69.74	1.36	3
% FOXP3+/Helios- cells	6 Days	5.52	0.43	3	9.03	0.44	3
	12 Days	14.30	1.13	3	18.02	0.74	3

Naïve T cells – IFN-γ in Helios vs FOXP3 cells

	Cell compartment	Healthy donor CD3+ T cells					
	ACh supplementation	0mM			10mM		
		Mean	SE	N	Mean	SE	N
Metric	Time point						
% IFN-γ+ cells (FOXP3-/Helios+ cells)	6 Days	19.12	0.71	3	22.16	1.15	3
	12 Days	60.36	3.98	3	66.71	2.29	3
% IFN-γ+ cells (FOXP3+/Helios+ cells)	6 Days	30.10	0.86	3	38.57	2.05	3
	12 Days	41.77	4.15	3	54.20	2.40	3
% IFN-γ+ cells (FOXP3-/Helios- cells)	6 Days	27.12	1.55	3	31.79	1.27	3
	12 Days	60.99	5.93	3	68.99	3.01	3
% IFN-γ+ cells (FOXP3+/Helios- cells)	6 Days	40.81	2.17	3	48.73	2.01	3
	12 Days	75.12	4.26	3	80.38	1.85	3

References

1. Dale, H. H. The Action of Certain Esters and Ethers of Choline, and Their Relation to Muscarine. *J Pharmacol Exp Ther* **6**, 147–190 (1914).
2. Cox, M. A. *et al.* Beyond neurotransmission: acetylcholine in immunity and inflammation. *Journal of Internal Medicine* **287**, 120–133 (2020).
3. Akaike, A. & Izumi, Y. Overview. in *Nicotinic Acetylcholine Receptor Signaling in Neuroprotection* (eds. Akaike, A., Shimohama, S. & Misu, Y.) (Springer, 2018).
4. Pahud, G. *et al.* Study of subcellular localization of membrane-bound choline acetyltransferase in Drosophila central nervous system and its association with membranes. *European Journal of Neuroscience* **10**, 1644–1653 (1998).
5. Taylor, P. & Brown, J. H. Synthesis, Storage and Release of Acetylcholine. *Basic Neurochemistry: Molecular, Cellular and Medical Aspects. 6th edition* (1999).
6. Choline | The Nutrition Source | Harvard T.H. Chan School of Public Health. https://www.hsph.harvard.edu/nutritionsource/choline/.
7. Fujii, T. *et al.* Expression and Function of the Cholinergic System in Immune Cells. *Front. Immunol.* **8**, (2017).
8. Haam, J. & Yakel, J. L. Cholinergic modulation of the hippocampal region and memory function. *J Neurochem* **142**, 111–121 (2017).
9. George, J. A. *et al.* Cholinergic Stimulation Prevents the Development of Autoimmune Diabetes: Evidence for the Modulation of Th17 Effector Cells via an IFNγ-Dependent Mechanism. *Frontiers in Immunology* **7**, (2016).

10. Noureddine, H., Carvalho, S., Schmitt, C., Massoulié, J. & Bon, S. Acetylcholinesterase associates differently with its anchoring proteins ColQ and PRiMA. *J Biol Chem* **283**, 20722–20732 (2008).
11. Purves, D. *et al.* Acetylcholine. *Neuroscience. 2nd edition* (2001).
12. Acetylcholine Release - an overview | ScienceDirect Topics. https://www.sciencedirect.com/topics/neuroscience/acetylcholine-release.
13. Taylor, P. The cholinesterases. *J Biol Chem* **266**, 4025–4028 (1991).
14. Gysler, S. M. & Drapkin, R. Tumor innervation: peripheral nerves take control of the tumor microenvironment. *J Clin Invest* **131**, e147276.
15. Kamiya, A. *et al.* Genetic manipulation of autonomic nerve fiber innervation and activity and its effect on breast cancer progression. *Nat Neurosci* **22**, 1289–1305 (2019).
16. Li, X. *et al.* Targeting tumor innervation: premises, promises, and challenges. *Cell Death Discov.* **8**, 1–12 (2022).
17. Baraldi, J. H., Martyn, G. V., Shurin, G. V. & Shurin, M. R. Tumor Innervation: History, Methodologies, and Significance. *Cancers (Basel)* **14**, 1979 (2022).
18. Hayakawa, Y. *et al.* Nerve growth factor promotes gastric tumorigenesis through aberrant cholinergic signaling. *Cancer Cell* **31**, 21–34 (2017).
19. Shang, B., Liu, Y., Jiang, S. & Liu, Y. Prognostic value of tumor-infiltrating FoxP3+ regulatory T cells in cancers: a systematic review and meta-analysis. *Sci Rep* **5**, 15179 (2015).
20. Saleh, R. & Elkord, E. FoxP3+ T regulatory cells in cancer: Prognostic biomarkers and therapeutic targets. *Cancer Letters* **490**, 174–185 (2020).

21. Wang, C. *et al.* PD-1, FOXP3, and CSF-1R expression in patients with Hodgkin lymphoma and their prognostic value. *Int J Clin Exp Pathol* **11**, 1923–1934 (2018).
22. Hosoi, T. & Nomura, Y. Functional role of acetylcholine in the immune system. *Frontiers in bioscience : a journal and virtual library* **9**, 2414–9 (2004).
23. Mashimo, M., Moriwaki, Y., Misawa, H., Kawashima, K. & Fujii, T. Regulation of Immune Functions by Non-Neuronal Acetylcholine (ACh) via Muscarinic and Nicotinic ACh Receptors. *International Journal of Molecular Sciences* **22**, 6818 (2021).
24. Rosas-Ballina, M. *et al.* Acetylcholine-Synthesizing T Cells Relay Neural Signals in a Vagus Nerve Circuit. *Science* **334**, 98–101 (2011).
25. Auer, K. *et al.* Role of the immune system in the peritoneal tumor spread of high grade serous ovarian cancer. *Oncotarget* **7**, 61336–61354 (2016).
26. Fernández-Cabezudo, M. J. *et al.* Involvement of Acetylcholine Receptors in Cholinergic Pathway-Mediated Protection Against Autoimmune Diabetes. *Front Immunol* **10**, 1038 (2019).
27. Mashimo, M., Moriwaki, Y., Misawa, H., Kawashima, K. & Fujii, T. Regulation of Immune Functions by Non-Neuronal Acetylcholine (ACh) via Muscarinic and Nicotinic ACh Receptors. *International Journal of Molecular Sciences* **22**, 6818 (2021).
28. Traced on the Timeline: Discovery of Acetylcholine and the Components of the Human Cholinergic System in a Primitive Unicellular Eukaryote Acanthamoeba spp. | ACS Chemical Neuroscience. https://pubs.acs.org/doi/10.1021/acschemneuro.7b00254.
29. Komal, P. & Nashmi, R. T-cell receptors modify neuronal function in the central nervous system. *Biochemical Pharmacology* **97**, 512–517 (2015).

30. Komal, P., Manjari, S. K. V. & Nashmi, R. An opinion on the debatable function of brain resident immune protein, T-cell receptor beta subunit in the central nervous system. *IBRO Neurosci Rep* **13**, 235–242 (2022).
31. Halder, N. & Lal, G. Cholinergic System and Its Therapeutic Importance in Inflammation and Autoimmunity. *Front Immunol* **12**, 660342 (2021).
32. Lv, J., Ji, X., Li, Z. & Hao, H. The role of the cholinergic anti-inflammatory pathway in autoimmune rheumatic diseases. *Scandinavian Journal of Immunology* **94**, e13092 (2021).
33. Toker, A. *et al.* Regulatory T Cells in Ovarian Cancer Are Characterized by a Highly Activated Phenotype Distinct from that in Melanoma. *Clin Cancer Res* **24**, 5685–5696 (2018).
34. Domblides, C., Lartigue, L. & Faustin, B. Control of the Antitumor Immune Response by Cancer Metabolism. *Cells* **8**, (2019).
35. Reinhardt, J. *et al.* Distinguishing activated T regulatory cell and T conventional cells by single-cell technologies. *Immunology* **166**, 121–137 (2022).
36. CLUSTER OF DIFFERENTIATION (CD) ANTIGENS. *Immunology Guidebook* 47–124 (2004) doi:10.1016/B978-012198382-6/50027-3.
37. Helper and Cytotoxic T Cells | British Society for Immunology. https://www.immunology.org/public-information/bitesized-immunology/cells/helper-and-cytotoxic-t-cells.
38. Yu, W. *et al.* Neuroimmune Crosstalk Between the Peripheral and the Central Immune System in Amyotrophic Lateral Sclerosis. *Frontiers in Aging Neuroscience* **14**, (2022).

39. Limanaqi, F., Biagioni, F., Gaglione, A., Busceti, C. L. & Fornai, F. A Sentinel in the Crosstalk Between the Nervous and Immune System: The (Immuno)-Proteasome. *Front Immunol* **10**, 628 (2019).
40. Tian, L., Ma, L., Kaarela, T. & Li, Z. Neuroimmune crosstalk in the central nervous system and its significance for neurological diseases. *Journal of Neuroinflammation* **9**, 155 (2012).
41. Mashimo, M. *et al.* Distinct Roles of α7 nAChRs in Antigen-Presenting Cells and CD4+ T Cells in the Regulation of T Cell Differentiation. *Frontiers in Immunology* **10**, 1102 (2019).
42. Sanders, V. M. The Beta2-Adrenergic Receptor on T and B Lymphocytes: Do We Understand It Yet? *Brain Behav Immun* **26**, 195–200 (2012).
43. Pavlov, V. A., Wang, H., Czura, C. J., Friedman, S. G. & Tracey, K. J. The Cholinergic Anti-inflammatory Pathway: A Missing Link in Neuroimmunomodulation. *Mol Med* **9**, 125–134 (2003).
44. Slota, C., Shi, A., Chen, G., Bevans, M. & Weng, N. Norepinephrine preferentially modulates memory CD8 T cell function inducing inflammatory cytokine production and reducing proliferation in response to activation. *Brain Behav Immun* **46**, 168–179 (2015).
45. Cox, M. A. Acetylcholine-producing CD4 T cells regulate vasculature in humans. *Proceedings of the National Academy of Sciences* **120**, e2303525120 (2023).
46. Malin, S. G., Shavva, V. S., Tarnawski, L. & Olofsson, P. S. Functions of acetylcholine-producing lymphocytes in immunobiology. *Current Opinion in Neurobiology* **62**, 115–121 (2020).

47. Willemze, R. A. *et al.* Acetylcholine-producing T cells augment innate immune-driven colitis but are redundant in T cell-driven colitis. *Am J Physiol Gastrointest Liver Physiol* **317**, G557–G568 (2019).
48. Tyagi, E., Agrawal, R., Nath, C. & Shukla, R. Cholinergic protection via alpha7 nicotinic acetylcholine receptors and PI3K-Akt pathway in LPS-induced neuroinflammation. *Neurochem Int* **56**, 135–142 (2010).
49. Cox, M. A. *et al.* Choline acetyltransferase-expressing T cells are required to control chronic viral infection. *Science* **363**, 639–644 (2019).
50. Borovikova, L. V. *et al.* Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. *Nature* **405**, 458–462 (2000).
51. Frontiers | Activation of the Cholinergic Anti-Inflammatory Pathway as a Novel Therapeutic Strategy for COVID-19. https://www.frontiersin.org/articles/10.3389/fimmu.2020.595342/full.
52. Simon, T. *et al.* The cholinergic anti-inflammatory pathway inhibits inflammation without lymphocyte relay. *Frontiers in Neuroscience* **17**, (2023).
53. Liu, E. Y. L. *et al.* Interacting with α7 nAChR is a new mechanism for AChE to enhance the inflammatory response in macrophages. *Acta Pharm Sin B* **10**, 1926–1942 (2020).
54. Olofsson, P. S. *et al.* Blood pressure regulation by CD4+ lymphocytes expressing choline acetyltransferase. *Nat Biotechnol* **34**, 1066–1071 (2016).
55. Mashimo, M. [Dual Roles of α7 Nicotinic Acetylcholine Receptors Expressed in Immune Cells in T Cell Differentiation -α7 nAChRs Exert Different Actions between Antigen-presenting Cells and CD4+ T Cells]. *Yakugaku Zasshi* **140**, 1421–1425 (2020).

56. Fujii, T. *et al.* Localization and synthesis of acetylcholine in human leukemic T cell lines. *Journal of Neuroscience Research* **44**, 66–72 (1996).

57. Blat, D., Zigmond, E., Alteber, Z., Waks, T. & Eshhar, Z. Suppression of Murine Colitis and its Associated Cancer by Carcinoembryonic Antigen-Specific Regulatory T Cells. *Mol Ther* **22**, 1018–1028 (2014).

58. Facciabene, A., Motz, G. T. & Coukos, G. T Regulatory Cells: Key Players in Tumor Immune Escape and Angiogenesis. *Cancer Res* **72**, 2162–2171 (2012).

59. Han, S., Toker, A., Liu, Z. Q. & Ohashi, P. S. Turning the Tide Against Regulatory T Cells. *Front Oncol* **9**, (2019).

60. Ovarian Tumor Tissue Analysis (OTTA) Consortium *et al.* Dose-Response Association of CD8+ Tumor-Infiltrating Lymphocytes and Survival Time in High-Grade Serous Ovarian Cancer. *JAMA Oncol* **3**, e173290 (2017).

61. Sato, E. *et al.* Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. *PNAS* **102**, 18538–18543 (2005).

62. Mashimo, M. *et al.* Minireview: Divergent roles of α7 nicotinic acetylcholine receptors expressed on antigen-presenting cells and CD4+ T cells in the regulation of T cell differentiation. *International immunopharmacology* **82**, 106306 (2020).

63. Powell, M. D., Read, K. A., Sreekumar, B. K. & Oestreich, K. J. Ikaros Zinc Finger Transcription Factors: Regulators of Cytokine Signaling Pathways and CD4+ T Helper Cell Differentiation. *Front Immunol* **10**, 1299 (2019).

64. Differentiation of Effector CD4 T Cell Populations. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3502616/.
65. Pagès, F. *et al.* Effector Memory T Cells, Early Metastasis, and Survival in Colorectal Cancer. *New England Journal of Medicine* **353**, 2654–2666 (2005).
66. Oh, D. Y. & Fong, L. Cytotoxic CD4+ T cells in cancer: Expanding the immune effector toolbox. *Immunity* **54**, 2701–2711 (2021).
67. Levescot, A. & Cerf-Bensussan, N. Regulatory CD8+ T cells suppress disease. *Science* **376**, 243–244 (2022).
68. Yu, Y. *et al.* Recent advances in CD8+ regulatory T cell research. *Oncol Lett* **15**, 8187–8194 (2018).
69. Mishra, S., Srinivasan, S., Ma, C. & Zhang, N. CD8+ Regulatory T Cell – A Mystery to Be Revealed. *Frontiers in Immunology* **12**, (2021).
70. Takeuchi, A. & Saito, T. CD4 CTL, a Cytotoxic Subset of CD4+ T Cells, Their Differentiation and Function. *Frontiers in Immunology* **8**, (2017).
71. Propper, D. J. *et al.* Low-dose IFN-gamma induces tumor MHC expression in metastatic malignant melanoma. *Clin Cancer Res* **9**, 84–92 (2003).
72. Jorgovanovic, D., Song, M., Wang, L. & Zhang, Y. Roles of IFN-γ in tumor progression and regression: a review. *Biomarker Research* **8**, 49 (2020).
73. Zhang, S. *et al.* Systemic Interferon-γ Increases MHC Class I Expression and T-cell Infiltration in Cold Tumors: Results of a Phase 0 Clinical Trial. *Cancer Immunology Research* **7**, 1237–1243 (2019).

74. Zhou, F. Molecular mechanisms of IFN-gamma to up-regulate MHC class I antigen processing and presentation. *Int Rev Immunol* **28**, 239–260 (2009).
75. Tran, D. Q. TGF-β: the sword, the wand, and the shield of FOXP3+ regulatory T cells. *Journal of Molecular Cell Biology* **4**, 29–37 (2012).
76. Wan, Y. Y. & Flavell, R. A. 'Yin-Yang' functions of TGF-β and Tregs in immune regulation. *Immunol Rev* **220**, 199–213 (2007).
77. Höfer, T., Krichevsky, O. & Altan-Bonnet, G. Competition for IL-2 between Regulatory and Effector T Cells to Chisel Immune Responses. *Front Immunol* **3**, 268 (2012).
78. Ghelani, A. *et al.* Defining the Threshold IL-2 Signal Required for Induction of Selective Treg Cell Responses Using Engineered IL-2 Muteins. *Frontiers in Immunology* **11**, (2020).
79. TAKENAKA, M. *et al.* FOXP3 expression in tumor cells and tumor-infiltrating lymphocytes is associated with breast cancer prognosis. *Mol Clin Oncol* **1**, 625–632 (2013).
80. Wang, Y. *et al.* Regulatory T cells are an important prognostic factor in breast cancer: a systematic review and meta-analysis. *Neoplasma* **63**, 789–798 (2016).
81. Stenström, J., Hedenfalk, I. & Hagerling, C. Regulatory T lymphocyte infiltration in metastatic breast cancer—an independent prognostic factor that changes with tumor progression. *Breast Cancer Research* **23**, 27 (2021).
82. Shuvalov, O., Daks, A., Fedorova, O., Petukhov, A. & Barlev, N. Linking Metabolic Reprogramming, Plasticity and Tumor Progression. *Cancers* **13**, 762 (2021).
83. Li, X. *et al.* Navigating metabolic pathways to enhance antitumour immunity and immunotherapy. *Nature Reviews Clinical Oncology* **16**, 425–441 (2019).

84. Koundouros, N. & Poulogiannis, G. Reprogramming of fatty acid metabolism in cancer. *British Journal of Cancer* **122**, 4–22 (2020).
85. Metabolic reprogramming and cancer progression | Science. https://www.science.org/doi/abs/10.1126/science.aaw5473.
86. Arner, E. N. & Rathmell, J. C. Metabolic programming and immune suppression in the tumor microenvironment. *Cancer Cell* **41**, 421–433 (2023).
87. Farc, O. & Cristea, V. An overview of the tumor microenvironment, from cells to complex networks (Review). *Experimental and Therapeutic Medicine* **21**, 1–1 (2021).
88. Lactate in the Tumor Microenvironment: An Essential Molecule in Cancer Progression and Treatment - PMC. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7693872/.
89. Wu, Y. *et al.* Lactate, a Neglected Factor for Diabetes and Cancer Interaction. *Mediators Inflamm* **2016**, 6456018 (2016).
90. Liberti, M. V. & Locasale, J. W. The Warburg Effect: How Does it Benefit Cancer Cells? *Trends Biochem Sci* **41**, 211–218 (2016).
91. Bubeník, J. Tumour MHC class I downregulation and immunotherapy (Review). *Oncol Rep* **10**, 2005–2008 (2003).
92. Taylor, B. C. & Balko, J. M. Mechanisms of MHC-I Downregulation and Role in Immunotherapy Response. *Frontiers in Immunology* **13**, (2022).
93. Escors, D. *et al.* The intracellular signalosome of PD-L1 in cancer cells. *Signal Transduction and Targeted Therapy* **3**, 1–9 (2018).
94. Parry, R. V. *et al.* CTLA-4 and PD-1 Receptors Inhibit T-Cell Activation by Distinct Mechanisms. *Mol Cell Biol* **25**, 9543–9553 (2005).

95. Yi, M., Niu, M., Xu, L., Luo, S. & Wu, K. Regulation of PD-L1 expression in the tumor microenvironment. *Journal of Hematology & Oncology* **14**, 10 (2021).

96. Ma, S. *et al.* Current Progress in CAR-T Cell Therapy for Solid Tumors. *Int J Biol Sci* **15**, 2548–2560 (2019).

97. Sterner, R. C. & Sterner, R. M. CAR-T cell therapy: current limitations and potential strategies. *Blood Cancer J.* **11**, 1–11 (2021).

98. Fujiwara, K. *et al.* Hinge and Transmembrane Domains of Chimeric Antigen Receptor Regulate Receptor Expression and Signaling Threshold. *Cells* **9**, 1182 (2020).

99. Weinkove, R., George, P., Dasyam, N. & McLellan, A. D. Selecting costimulatory domains for chimeric antigen receptors: functional and clinical considerations. *Clinical & Translational Immunology* **8**, e1049 (2019).

100. Wang, H. *et al.* A simple and effective method to purify and activate T cells for successful generation of chimeric antigen receptor T (CAR-T) cells from patients with high monocyte count. *Journal of Translational Medicine* **20**, 608 (2022).

101. Sengsayadeth, S., Savani, B. N., Oluwole, O. & Dholaria, B. Overview of approved CAR-T therapies, ongoing clinical trials, and its impact on clinical practice. *EJHaem* **3**, 6–10 (2021).

102. FDA-approved CAR T-cell Therapies | UPMC Hillman. *UPMC Hillman Cancer Center* https://hillman.upmc.com/mario-lemieux-center/treatment/car-t-cell-therapy/fda-approved-therapies.

103. Kyte, J. A. Strategies for Improving the Efficacy of CAR T Cells in Solid Cancers. *Cancers (Basel)* **14**, 571 (2022).

104. Marofi, F. *et al.* CAR T cells in solid tumors: challenges and opportunities. *Stem Cell Research & Therapy* **12**, 81 (2021).

105. Safarzadeh Kozani, P. *et al.* Recent Advances in Solid Tumor CAR-T Cell Therapy: Driving Tumor Cells From Hero to Zero? *Front Immunol* **13**, 795164 (2022).

106. Remodeled CAR T-Cell Therapy Causes Fewer Side Effects - NCI. https://www.cancer.gov/news-events/cancer-currents-blog/2020/car-t-cell-therapy-lymphoma-reduced-side-effects (2020).

107. The Other Side of CAR T-Cell Therapy: Cytokine Release Syndrome, Neurologic Toxicity, and Financial Burden | American Society of Clinical Oncology Educational Book. https://ascopubs.org/doi/full/10.1200/EDBK_238691.

108. Mechanisms of cytokine release syndrome and neurotoxicity of CAR T-cell therapy and associated prevention and management strategies | Journal of Experimental & Clinical Cancer Research | Full Text. https://jeccr.biomedcentral.com/articles/10.1186/s13046-021-02148-6.

109. Kilgour, M. K. *et al.* 1-Methylnicotinamide is an immune regulatory metabolite in human ovarian cancer. *Science Advances* **7**, eabe1174 (2021).

110. Ahmed, N. & Stenvers, K. L. Getting to Know Ovarian Cancer Ascites: Opportunities for Targeted Therapy-Based Translational Research. *Front Oncol* **3**, (2013).

111. Rickard, B. P. *et al.* Malignant Ascites in Ovarian Cancer: Cellular, Acellular, and Biophysical Determinants of Molecular Characteristics and Therapy Response. *Cancers (Basel)* **13**, 4318 (2021).

112. Dhankhar, R. *et al.* Diagnostic significance of adenosine deaminase, uric acid and C-reactive protein levels in patients of head and neck carcinoma. *Clin Lab* **57**, 795–798 (2011).

113. Koyas, A. *et al.* Interleukin-7 protects CD8+ T cells from adenosine-mediated immunosuppression. *Sci. Signal.* **14**, (2021).

114. Leone, R. D. & Emens, L. A. Targeting adenosine for cancer immunotherapy. *J Immunother Cancer* **6**, 57 (2018).

115. Vijayan, D., Young, A., Teng, M. W. L. & Smyth, M. J. Targeting immunosuppressive adenosine in cancer. *Nat Rev Cancer* **17**, 709–724 (2017).

116. Gouasmi, R. *et al.* The Kynurenine Pathway and Cancer: Why Keep It Simple When You Can Make It Complicated. *Cancers (Basel)* **14**, 2793 (2022).

117. Routy, J.-P., Routy, B., Graziani, G. M. & Mehraj, V. The Kynurenine Pathway Is a Double-Edged Sword in Immune-Privileged Sites and in Cancer: Implications for Immunotherapy. *Int J Tryptophan Res* **9**, 67–77 (2016).

118. Venkateswaran, N. & Conacci-Sorrell, M. Kynurenine: an oncometabolite in colon cancer. *Cell Stress* **4**, 24–26.

119. Stone, J. D., Chervin, A. S. & Kranz, D. M. T-cell receptor binding affinities and kinetics: impact on T-cell activity and specificity. *Immunology* **126**, 165–176 (2009).

120. Goswami, R. & Awasthi, A. Editorial: T Cell Differentiation and Function in Tissue Inflammation. *Front Immunol* **11**, 289 (2020).

121. Zhu, J., Yamane, H. & Paul, W. E. Differentiation of Effector CD4 T Cell Populations. *Annu Rev Immunol* **28**, 445–489 (2010).

122. Dong, C. & Flavell, R. A. Cell fate decision: T-helper 1 and 2 subsets in immune responses. *Arthritis Research & Therapy* **2**, 179 (2000).

123. Saravia, J., Chapman, N. M. & Chi, H. Helper T cell differentiation. *Cell Mol Immunol* **16**, 634–643 (2019).

124. Gonzalez, H., Hagerling, C. & Werb, Z. Roles of the immune system in cancer: from tumor initiation to metastatic progression. *Genes Dev* **32**, 1267–1284 (2018).

125. Cicchese, J. M. *et al.* Dynamic balance of pro- and anti-inflammatory signals controls disease and limits pathology. *Immunol Rev* **285**, 147–167 (2018).

126. Wu, C. *et al.* IFN-γ Primes Macrophage Activation by Increasing Phosphatase and Tensin Homolog via Downregulation of miR-3473b. *The Journal of Immunology* **193**, 3036–3044 (2014).

127. Ivashkiv, L. B. IFNγ: signalling, epigenetics and roles in immunity, metabolism, disease and cancer immunotherapy. *Nat Rev Immunol* **18**, 545–558 (2018).

128. Tau, G. Z. *et al.* Interferon γ Signaling Alters the Function of T Helper Type 1 Cells. *J Exp Med* **192**, 977–986 (2000).

129. Beatty, G. & Paterson, Y. IFN-gamma-dependent inhibition of tumor angiogenesis by tumor-infiltrating CD4+ T cells requires tumor responsiveness to IFN-gamma. *J Immunol* **166**, 2276–2282 (2001).

130. Bhat, P., Leggatt, G., Waterhouse, N. & Frazer, I. H. Interferon-γ derived from cytotoxic lymphocytes directly enhances their motility and cytotoxicity. *Cell Death Dis* **8**, e2836–e2836 (2017).

131. Ziegler, S. F. FOXP3: of mice and men. *Annu Rev Immunol* **24**, 209–226 (2006).

132. Lindqvist, C. A. *et al.* T regulatory cells control T-cell proliferation partly by the release of soluble CD25 in patients with B-cell malignancies. *Immunology* **131**, 371–376 (2010).

133. Khan, S. & Gerber, D. E. Autoimmunity, checkpoint inhibitor therapy and immune-related adverse events: A review. *Seminars in Cancer Biology* **64**, 93–101 (2020).

134. Ge, X., Zhao, Y., Chen, C., Wang, J. & Sun, L. Cancer Immunotherapies Targeting Tumor-Associated Regulatory T Cells. *Onco Targets Ther* **12**, 11033–11044 (2019).

135. Yu, Y. *et al.* Cancer-associated fibroblasts induce epithelial–mesenchymal transition of breast cancer cells through paracrine TGF-β signalling. *Br J Cancer* **110**, 724–732 (2014).

136. Mailer, R. K. W. Alternative Splicing of FOXP3—Virtue and Vice. *Frontiers in Immunology* **9**, (2018).

137. Mercer, F. & Unutmaz, D. The Biology of FoxP3: A Key Player in Immune Suppression during Infections, Autoimmune Diseases and Cancer. *Adv Exp Med Biol* **665**, 47–59 (2009).

138. van der Vliet, H. J. J. & Nieuwenhuis, E. E. IPEX as a Result of Mutations in FOXP3. *Clin Dev Immunol* **2007**, 89017 (2007).

139. Frontiers | Foxp3 Post-translational Modifications and Treg Suppressive Activity. https://www.frontiersin.org/articles/10.3389/fimmu.2019.02486/full.

140. Wang, J., Ioan-Facsinay, A., van der Voort, E. I. H., Huizinga, T. W. J. & Toes, R. E. M. Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. *Eur J Immunol* **37**, 129–138 (2007).

141. McMurchy, A. N. *et al.* A novel function for FOXP3 in humans: intrinsic regulation of conventional T cells. *Blood* **121**, 1265–1275 (2013).

142. Magg, T., Mannert, J., Ellwart, J. W., Schmid, I. & Albert, M. H. Subcellular localization of FOXP3 in human regulatory and nonregulatory T cells. *European Journal of Immunology* **42**, 1627–1638 (2012).

143. Sato, Y. *et al.* Co-Expression of FOXP3FL and FOXP3Δ2 Isoforms Is Required for Optimal Treg-Like Cell Phenotypes and Suppressive Function. *Frontiers in Immunology* **12**, (2021).

144. Allan, S. E. *et al.* The role of 2 FOXP3 isoforms in the generation of human CD4+ Tregs. *J Clin Invest* **115**, 3276–3284 (2005).

145. Schmidt, A. *et al.* Time-resolved transcriptome and proteome landscape of human regulatory T cell (Treg) differentiation reveals novel regulators of FOXP3. *BMC Biol* **16**, 1–35 (2018).

146. Magnuson, A. M. *et al.* Identification and validation of a tumor-infiltrating Treg transcriptional signature conserved across species and tumor types. *Proceedings of the National Academy of Sciences* **115**, E10672–E10681 (2018).

147. Allan, S. E. *et al.* Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production. *International Immunology* **19**, 345–354 (2007).

148. Zhou, X. *et al.* Isolation of Purified and Live Foxp3+ Regulatory T Cells using FACS Sorting on Scatter Plot. *J Mol Cell Biol* **2**, 164–169 (2010).

149. Liao, X., Makris, M. & Luo, X. M. Fluorescence-activated Cell Sorting for Purification of Plasmacytoid Dendritic Cells from the Mouse Bone Marrow. *J Vis Exp* 54641 (2016) doi:10.3791/54641.

150. Kochurani, K. J. *et al.* Live detection and purification of cells based on the expression of a histone chaperone, HIRA, using a binding peptide. *Sci Rep* **5**, 17218 (2015).

151. Tajti, G. *et al.* Immunomagnetic separation is a suitable method for electrophysiology and ion channel pharmacology studies on T cells. *Channels* **15**, 53–66 (2021).

152. Baru, A. M. *et al.* Optimal Isolation of Functional Foxp3+ Induced Regulatory T Cells Using DEREG Mice. *PLOS ONE* **7**, e44760 (2012).

153. Yang, Z.-Z. *et al.* Soluble and Membrane-Bound TGF-β-Mediated Regulation of Intratumoral T Cell Differentiation and Function in B-Cell Non-Hodgkin Lymphoma. *PLoS One* **8**, e59456 (2013).

154. Ostroukhova, M. *et al.* Treg-mediated immunosuppression involves activation of the Notch-HES1 axis by membrane-bound TGF-beta. *J Clin Invest* **116**, 996–1004 (2006).

155. Tzavlaki, K. & Moustakas, A. TGF-β Signaling. *Biomolecules* **10**, 487 (2020).

156. Ahn, Y.-O., Lee, J.-C., Sung, M.-W. & Heo, D. S. Presence of Membrane-Bound TGF-β1 and Its Regulation by IL-2-Activated Immune Cell-Derived IFN-γ in Head and Neck Squamous Cell Carcinoma Cell Lines1. *The Journal of Immunology* **182**, 6114–6120 (2009).

157. Areström, I., Zuber, B., Bengtsson, T. & Ahlborg, N. Measurement of human latent Transforming Growth Factor-β1 using a latency associated protein-reactive ELISA. *J Immunol Methods* **379**, 23–29 (2012).

158. Carlson, A. B. & Kraus, G. P. Physiology, Cholinergic Receptors. in *StatPearls* (StatPearls Publishing, 2023).

159. Kem, W. *et al.* The Nemertine Toxin Anabaseine and Its Derivative DMXBA (GTS-21): Chemical and Pharmacological Properties. *Marine Drugs* **4**, 255–273 (2006).

160. Aldossary, S. A. Review on Pharmacology of Atropine, Clinical Use and Toxicity. *Biomedical and Pharmacology Journal* **15**, 691–697 (2022).

161. Lott, E. L. & Jones, E. B. Cholinergic Toxicity. in *StatPearls* (StatPearls Publishing, 2023).

162. Bugay, V., Gregory, S. R., Belanger-Coast, M. G., Zhao, R. & Brenner, R. Effects of Sublethal Organophosphate Toxicity and Anti-cholinergics on Electroencephalogram and Respiratory Mechanics in Mice. *Frontiers in Neuroscience* **16**, (2022).

163. Chougnet, C. & Hildeman, D. Helios—controller of Treg stability and function. *Transl Cancer Res* **5**, S338–S341 (2016).

164. Kim, H.-J. *et al.* Stable inhibitory activity of regulatory T cells requires the transcription factor Helios. *Science* **350**, 334–339 (2015).

165. Elkord, E. Helios Should Not Be Cited as a Marker of Human Thymus-Derived Tregs. Commentary: Helios+ and Helios− Cells Coexist within the Natural FOXP3+ T Regulatory Cell Subset in Humans. *Frontiers in Immunology* **7**, (2016).

166. Thornton, A. M. *et al.* Helios+ and Helios- Treg subpopulations are phenotypically and functionally distinct and express dissimilar TCR repertoires. *Eur J Immunol* **49**, 398–412 (2019).

167. Thornton, A. M. & Shevach, E. M. Helios: still behind the clouds. *Immunology* **158**, 161–170 (2019).

168. Sebastian, M. *et al.* Helios controls a limited subset of regulatory T cell functions. *J Immunol* **196**, 144–155 (2016).

169. Lam, A. J., Uday, P., Gillies, J. K. & Levings, M. K. Helios is a marker, not a driver, of human Treg stability. *Eur J Immunol* **52**, 75–84 (2022).

170. Nakagawa, H. *et al.* Instability of Helios-deficient Tregs is associated with conversion to a T-effector phenotype and enhanced antitumor immunity. *Proceedings of the National Academy of Sciences* **113**, 6248–6253 (2016).

171. Sletten, D. M., Nickander, K. K. & Low, P. A. Stability of acetylcholine chloride solution in autonomic testing. *J Neurol Sci* **234**, 1–3 (2005).

172. Trang, A. & Khandhar, P. B. Physiology, Acetylcholinesterase. in *StatPearls* (StatPearls Publishing, 2023).

173. Sokolova, E., Matteoni, C. & Nistri, A. Desensitization of neuronal nicotinic receptors of human neuroblastoma SH-SY5Y cells during short or long exposure to nicotine. *Br J Pharmacol* **146**, 1087–1095 (2005).

174. Ren, C., Tong, Y., Li, J., Lu, Z. & Yao, Y. The Protective Effect of Alpha 7 Nicotinic Acetylcholine Receptor Activation on Critical Illness and Its Mechanism. *International Journal of Biological Sciences* **13**, 46–56 (2017).

175. Ribeiro, E., Costa, B., Vasques-Nóvoa, F. & Vale, N. In Vitro Drug Repurposing: Focus on Vasodilators. *Cells* **12**, 671 (2023).

176. Cheng, W.-L., Chen, K.-Y., Lee, K.-Y., Feng, P.-H. & Wu, S.-M. Nicotinic-nAChR signaling mediates drug resistance in lung cancer. *J Cancer* **11**, 1125–1140 (2020).

177. Singh, S., Pillai, S. & Chellappan, S. Nicotinic acetylcholine receptor signaling in tumor growth and metastasis. *J Oncol* **2011**, 456743 (2011).

178. Zhang, H. *et al.* FOXP3 expression in FOXP3 + tumor cells promotes hepatocellular cells metastasis. *Translational Cancer Research* **9**, (2020).