Investigating the Germinal Center Localization of T Follicular Regulatory Cells in PTEN- ΔTReg Mice

Investigating the Germinal Center Localization of T Follicular Regulatory Cells in PTEN-ΔT_{Reg} Mice

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Abstract: Germinal center (GC) formation is essential for orchestrating antigen specific B cell-mediated immune responses. During the GC reaction, T follicular helper (T_{fh}) cells provide the limiting source of survival signals to GC B cells which ensures that only B cells of the highest affinity survive to differentiate into plasma cells and memory B cells. Recently, a subpopulation of effector regulatory T cells (T_{Reg}) known as T follicular regulatory (T_{fr}) cells have been identified that home to the B cell follicle and regulate GC size and activity. Although their precise mechanism remains unclear, it has been shown that T_{fr} cells promote the production of high affinity antigen-specific antibodies. Published and unpublished work suggest that control of phosphoinositide 3-kinase (PI3K) by the phosphatase PTEN is crucial for T_{fr} suppressive function, as mice lacking PTEN specifically in the T_{Reg} compartment have larger GCs and impaired production of high affinity antigen-specific antibodies. The PI3K signaling pathway is known to be important for cell migration, and we wondered if impaired suppressive capacity is due to mis-localization of PTEN-deficient T_{fr} cells. It is suggested that T_{fr} cells can make direct contact with GC B cells or T_{fh} cells, therefore mis-localized PTEN-deficient T_{fr} cells that are unable to physically interact with their targets may explain their suppressive defect. Using immunofluorescence with multispectral imaging techniques, we quantified T_{fr} cell localization in the GC and B cell follicle in both control and PTEN-ΔT_{Reg} mice. We have observed a decrease in the T_{fr} to T_{fh} cell ratio within the germinal centers of PTEN-ΔT_{Reg} mice compared to Foxp3 Cre mice. However, these data also suggest that T_{fr} cells lacking PTEN have no absolute defect in localizing to the GC, so reasons other than a defect in localization likely contribute to the dysfunction of T_{fr} cells in PTEN-ΔT_{Reg} mice.

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Chapter One: Background

Section 1.1: Introduction

Formation of high affinity bodies is a crucial process that plays a role in our body's defense and protection against foreign pathogens. This necessitates a delicate balance between mounting a robust response to foreign pathogens while screening against self-antigens to prevent against harmful immune responses. The adaptive immune system requires collaboration between antigen presenting cells, B cells and T cells and provides a highly specific antigen-directed response against foreign pathogens while also providing memory for a more rapid response to future repeated exposures.[1] Normally, the germinal center reaction is dependent on the T-cell collaboration resulting in a highly dynamic process that leads to the production of highly selective B cells to virtually any antigen. Germinal center importance has been established in numerous animal studies and can be inferred with respect to the phenotypical characteristics observed in germinal center dysfunction including systemic lupus erythematosus (SLE), rheumatoid arthritis, multiple sclerosis, autoimmune lymphoproliferative syndrome, Sjogren's syndrome, and Type 1 diabetes in which we see elevated levels of autoantibodies.[2] As such, the germinal center is widely appreciated as a vital component for proper adaptive immunity with major emerging therapeutic implications centered on eliciting the body's adaptive immune system for the treatment of infectious disease.

Section 1.2: Germinal Center Dynamics

Germinal center (GC) formation is a dynamic process that occurs in secondary lymphoid organs and requires the participation of various immune cell players.[2]

This begins in the extrafollicular focus where T cells activated in the T cell zone (TCZ) interact with B cells through peptide-MHC and T cell receptor (TCR) engagement. This interaction leads to the activation of B cells through CD40 ligand and CD40 signaling and subsequent B cell expansion and the formation of the germinal center. Notably, CD40 signaling in B cells lead to expression of the enzyme activation induced deaminase (AID), which is essential in the induction of class switching and affinity maturation via somatic hypermutation within the dark zone. Within the light zone are additional cellular players involved including follicular dendritic cells (FDC) and follicular helper T cell (T_{fh}) which contribute to the selection of only high affinity B cell receptors (BCR) (note that affinity is relative and that GC B cells with very low affinity can be selected if there is no competition). This entire process is somewhat cyclical in nature - germinal center B cells traffic back-and-forth between the dark and light zone where they undergo BCR editing through the induction of somatic hypermutation and class switching to then have their BCR affinity tested in the light zone through interactions with follicular dendritic cells (FDC) and T_{fh} cells. B cells that are able to successfully capture antigen from FDCs and with respective antigen presentation to T_{fh} cells is positively selected and receives signals promoting the differentiation of germinal center B cells into memory B cells and plasmablasts. These resulting B cells then perform their effector functions or serve as memory providing a more rapid response to a repeated encounter. [3,4]

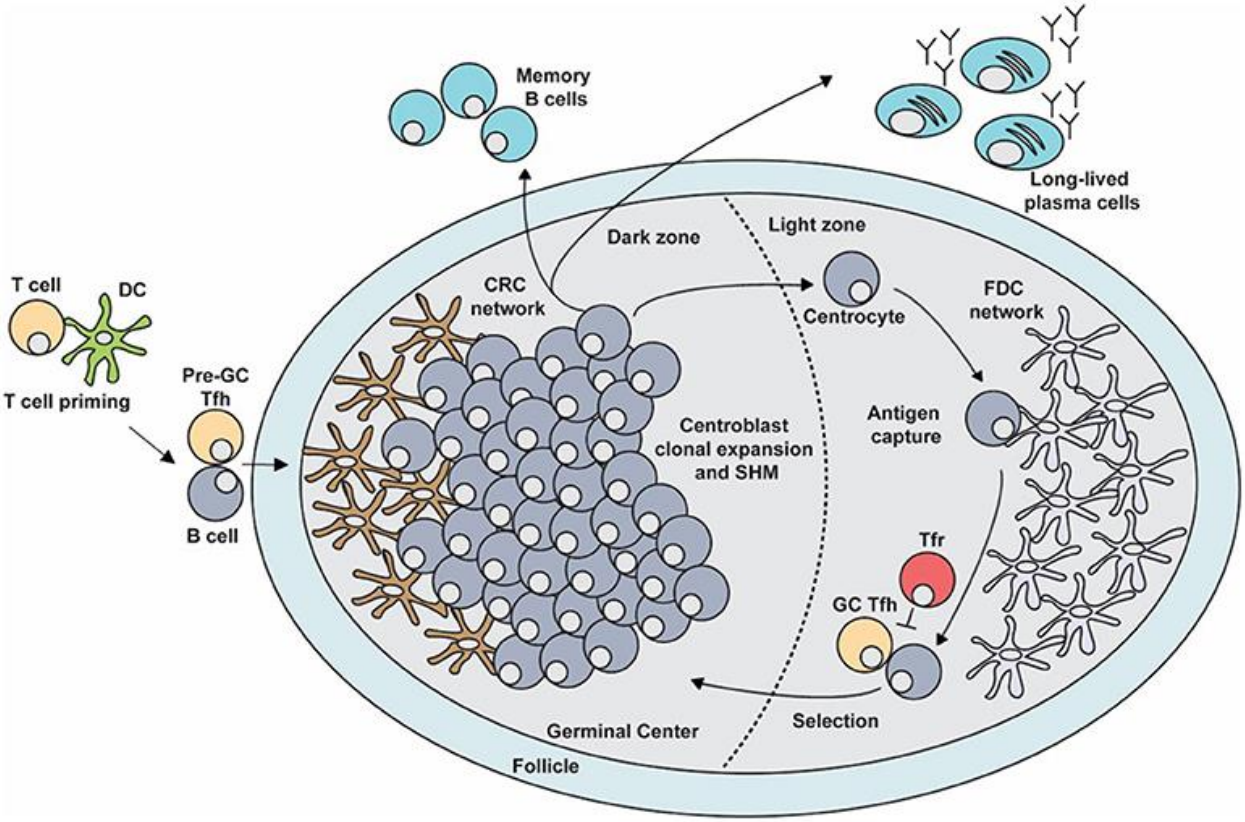

Figure 1.1: The Germinal Center Response. The GC is a specialized microenvironment formed within the B cell follicles of secondary lymphoid tissues upon infection or immunization. The GC is divided into two distinct compartments. The dark zone (DZ) that contains a network of CXCL12-producing reticular cells (CRCs) and is the site of GC B cell proliferation and somatic hypermutation (SHM). Centroblasts then follow a CXCL13 gradient to enter the light zone (LZ) as centrocytes through their expression of CXCR5. In the LZ, centrocytes capture antigen presented on follicular dendritic cells (FDCs) which they internalize, process and subsequently present to T follicular helper (T_{fh}) cells in order to undergo selection. This process is regulated by T follicular regulatory (T_{fr}) cells which are also present in the LZ. Upon receiving survival signals from T_{fh} cells, centrocytes re-enter the DZ for further rounds of proliferation and SHM after which they exit the GC as memory B cells or high-affinity antibody-secreting plasma cells. Adapted from Stebegg et al., 2018.

Section 1.3: Defining T_{fh} Cells and the Cleverly Designed B Cell Screening Process

The germinal center was engineered with several layers of selection criteria as a safety check to ensure that only B cells that are high affinity in nature can receive the necessary survival and/or proliferation signals. A key contributor to this screening process are T_{fh} cells. T_{fh} cells are initially activated by dendritic cells presenting peptide MHCII (pMHCII) complex and later by activated B cells providing ICOS ligand which results in the full differentiation into the T_{fh} cell phenotype and the trafficking into the germinal center where they perform their designated function. Simply put, T_{fh} cells are a gatekeeper for the initiation of the germinal center reaction, screening activated B cells through the interaction of TCR and peptide MHCII complex from the T_{fh} and germinal center B cells, respectively. This interaction between activated B cells and T_{fh} cells specific to the same antigen result in the upregulation of CD40 ligand by T_{fh} cells. Subsequently, the interaction of CD40 on activated B cells with CD40 ligand result in B cell proliferation and differentiation. These findings were validated in prior studies which found that blocking of CD40/CD40 ligand resulted in the loss of germinal center B cells.[5–8]

T_{fh} cells are crucial for a normally functioning adaptive immune response and despite a defined role, T_{fh} cells have stark differences to other CD4+ helper T cells with similarities to CD4+ T_{fr} cells. At the surface, T_{fh} cells notably express the markers CXCR5, programmed death-1 (PD-1), ICOS, SLAM adaptor protein (SAP), B and T-lymphocyte attenuator (BTLA), and interleukin-21. At the gene profiling level, T_{fh} cells are unique to other CD4+ T cell subtypes including Th1, Th2, and Th17 cells which are dependent on T-BET, GATA3, and RORγt, respectively. Different from the CD4+ helper T cells family, T_{fh} cells have been shown to rely on the master regulator transcription factor Bcl6.[9] Its importance in T_{fh} cell development has been

demonstrated in previous studies which reported impaired T_{fh} cell development with Bcl6 deficiency both in vitro and in vivo. Additionally, impaired germinal center formation results from lack of Bcl6 function.[10–12] T_{fh} cells have a unique and defined role within the CD4+ helper T cell family; however, interestingly these cells share similarities to a subset of CD4+ T cells from the T_{Reg} cell family which are responsible for maintaining a balanced immune response.

Section 1.4: Defining Follicular Regulatory T Cells

Despite the robustness and precision of the humoral response, preventing a damaging and excessive response is essential in balancing this process. There are key processes in place that serve to prevent against an overactive immune response. These processes include central tolerance which result in the deletion of self-reactive T and B cells. Additionally, the requirement for additional signals beyond signal 1 serve as a screening system ensures adaptive immune responses directed at the appropriate danger/foreign targets.[13] Despite these biological systems in place, a key question that remains is how humoral immunity is regulated during the commencement of the germinal center reaction. Interestingly, one of the approaches is through a subset of effector regulatory T cells (T_{Reg}) called follicular regulatory T cells (T_{fr}). T_{fr} cells differentiate from thymic T_{Reg} precursors and have shared characteristics with T_{Reg} albeit with unique characteristics. T_{fr} cells share T_{Reg}-specific characteristics including the expression of Foxp3, CTLA-4, and CD25. T_{fr} cells also have T_{fh}-like characteristics, including expression of Bcl6 which is the master transcriptional regulator for T_{fh} cells. Like T_{fh} cells, T_{fr} cells express ICOS, PD-1, and CXCR5 which allow them to traffic to the germinal center.[14,15] Interestingly, unlike conventional T_{Reg} cells, a population of $CD25^{lo}$ T_{fr} have been identified within the germinal center. [16,17] It is possible that the low interleukin-25 environment within the germinal

center led to the selection of the Foxp3+CD25$_{lo}$ phenotype that is reported within the germinal center of immunized mice.

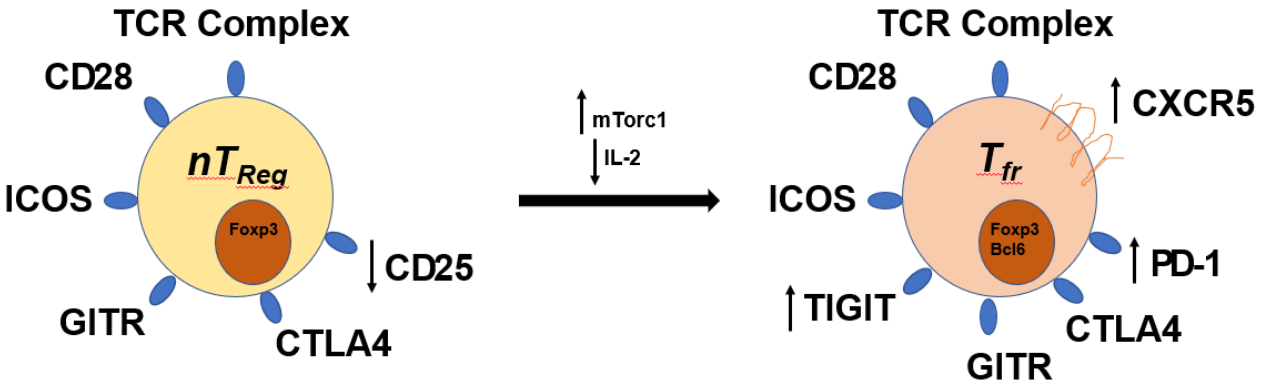

Figure 1.2: nT_{Reg} Cells Versus T_{fr} cells - Cell Surface Receptors and Transcription Factors Involved in T Follicular Regulatory Cell Differentiation and Function. CD25 is downregulated while CXCR5, PD-1, and TIGIT are upregulated during T_{fr} cell differentiation. Figure adapted from Xie and Dent, 2018.

Section 1.5: PTEN Regulates PI3K

Interestingly, an enzyme that is uniquely expressed in T_{Reg} but downregulated in conventional T cells is phosphatase and tensin homolog (PTEN).[18] PTEN is a key negative regulator for Phosphoinositide 3-kinase (PI3K). PI3K activation occurs through activation of various receptors including T cell receptor (TCR), G protein coupled receptor (GPCR), integrins, cytokine receptors, and B cell receptors (BCR).[19,20] PI3K catalyzes the phosphorylation of carbon position three on membrane bound phosphatidylinositol 4,5-biphosphate (PIP2) leading to its conversion to phosphatidylinositol-3,4,5-triphosphate (PIP3). The formation of PIP3 leads to the recruitment and activation of Akt via PH domain interactions, which leads to changes in cell survival, proliferation and metabolism.

Section 1.6: Control of PI3K by PTEN is Critical for T_{Reg} Homeostasis

Our understanding of Foxp3+ T cells is evolving; however, exactly how these immunosuppressive cells perform their effector function at the molecular level remains to be elucidated. Prior studies have shown a causative relationship between termination of TCR, PI3K, Akt, and mammalian target of rapamycin (mTOR) signaling conferring a Foxp3+ phenotype.[21] Additionally, prior studies conducted by Fortenot/Huynh showed that PTEN is important for T_{Reg} differentiation. However, the importance of PTEN in T_{Reg} cells was established in a study conducted by Huynh et al which compared PTEN-ΔT_{Reg} mice to Foxp3 Cre which was selected as the control to rule out any physiological impacts from Cre recombinase expression. The study reported autoimmine-lymphoproliferative disease, reduced expression of CD25, the buildup of Foxp3+CD25- cells, and the eventual loss of Foxp3 expression in PTEN-ΔT_{Reg} mice. Additionally, PTEN-ΔT_{Reg} mice experienced a lupus-like autoimmune-lymphoproliferative

disease at 17-20 weeks of age characterized by excessive levels of T helper type 1 (T_H1) and B cell activation. Diseased mice exhibited increases in serum c and creatinine and auto-antibody production, including anti-dsDNA antibodies.[22] (cite). Interestingly, humoral defects were observed prior to obvious signs of autoimmune disease, which consisted of increased levels of GC B cells, T_{fh} cells, and T_{fr} cells within lymph node tissue, as well as increased levels of serum immunoglobulins. This suggests that the pathological features of PTEN-ΔT_{Reg} mice could potentially be driven by a defect related to deregulation of the humoral compartment. In-line with prior studies focused on colitis and experimental autoimmune encephalomyelitis, these findings strongly suggest a link between the onset of an overactive autoimmune disease with T_{Reg} cell defects.

Despite an overactive humoral immune response, PTEN-ΔT_{Reg} mice were unable to produce high affinity antibodies. In a study conducted by Kelsey Finn, PhD Candidate, mice serum antibody from NP-OVA immunized PTEN-ΔT_{Reg} and Foxp3-YFP-CRE mice were tested for binding affinity to NP bound bovine serum albumin (BSA). Strikingly, antibody binding affinity was differentiated between the two mouse types with strong binding associated only in the Foxp3-YFP-CRE mice. This was different from the similar binding affinity between the two mouse types that were reported in the low affinity conditions. These findings strongly suggest that PTEN-sufficient Foxp3+ cells play a key role in the formation of high affinity antibodies.

Although there are increasing evidence supporting the role of T_{fr} cells in humoral immunity, it is challenging to design an in vivo experiment to knockout PTEN specifically in T_{fr} cells without disrupting the normal physiological/biological environment. The hybrid nature of T_{fr} cells with T_{Reg} and T_{fr} cells regarding transcription factor dependence and cellular surface markers make it challenging to design experiments to delete PTEN exclusively in T_{fr} cells. These

hybrid elements pose a challenge in creating a controlled experiment environment targeting PTEN expression in T_{fr} cells without disrupting the normal biological/physiological environment. In-vitro and in-vivo. Consequently, application of the cre-lox experimental system to induce deletion of PTEN in in specific cells will inherently have its caveats including the inability to prevent the deletion of PTEN in conventional T_{Reg} cells within our experimental mouse model. Additionally, deletion of PTEN will impact other cell types including T_{fh} cells which rely heavily on PI3K signaling.[23]

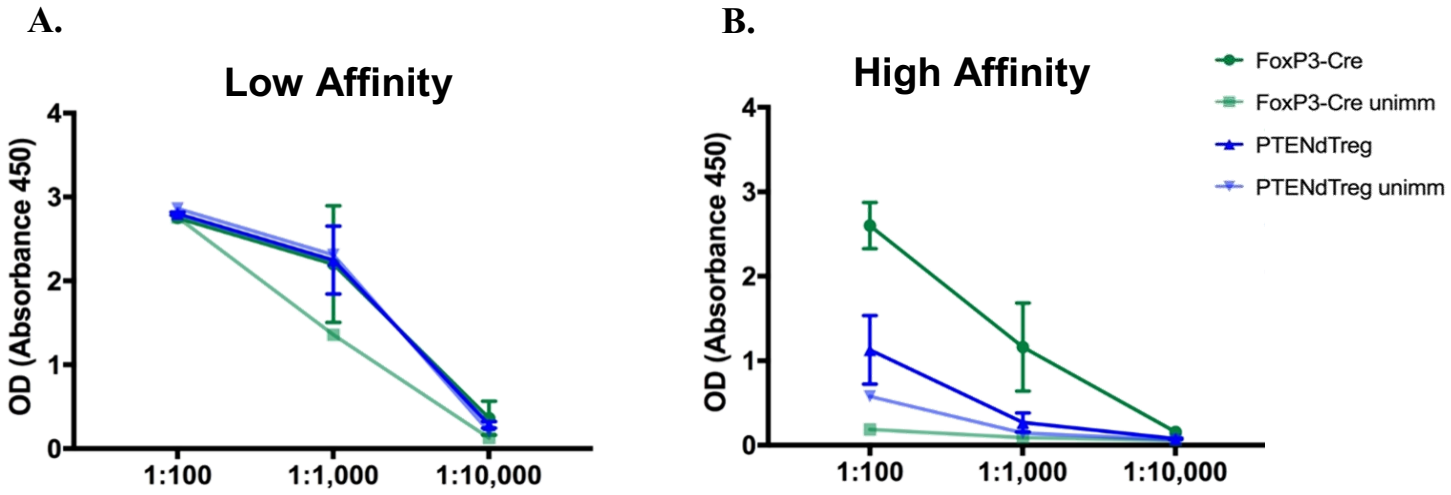

Figure 1.3: Comparison of Antibody Affinity Between Foxp3-Cre and PTEN-Δ-T_{Reg} Unimmunized and Immunized Mice. Serial dilutions of serum obtained from NP-OVA immunized or unimmunized mice were obtained via cardiac puncture and incubated with NP_8-BSA or NP_{23}-BSA. (A) FoxP3-Cre and PTEN-ΔT_{Reg} mice produced similar levels of low-affinity anti-NP antibody (B) FoxP3-Cre mice produced more high affinity anti-NP antibody compared to PTEN-ΔT_{Reg} mice. Adapted from unpublished data obtained by Kelsey Finn.

Section 1.7: Locating and Determining the Function of T_{fr} Cells

The contribution of T_{fr} cells to the germinal center reaction has been reported by multiple studies suggesting differing roles, albeit not mutually exclusive in nature. An in-vivo immunofluorescence study conducted in mesenteric human lymph node tissue staining for CD25 and Foxp3 to positively identify the presence of T_{fr} cells within the B cell follicle identified greater numbers of Foxp3+CD25+ cells within the region of the B and T cell border. Additionally, they note less numbers of T_{fr} cells within the germinal center. Based on these findings with respect to their gating strategy, they conclude T_{fr} cells mostly reside within the B and T cell border where they mainly exert their effector function.[24] Although the function of T_{fr} cells has yet to be shown experimentally in-vivo, it is likely that they are playing a role as a regulator of B and T cell collaboration.

In a separate in-vivo immunofluorescence experiment conducted in BCL6 mice lymph nodes, T_{fr} cells were reported within the germinal center that were $CD25^{lo}$. Specifically, CD25 expression levels on lymph node T_{fr} cells appeared to negatively correlate with CXCR5 expression in these Foxp3+ T cells and were naturally greater in number within the germinal center as opposed to $CD25^{hi}$ T_{fr} cells. Although these findings suggest a different angle of interpretation regarding the role and location of T_{fr} cells within the lymph node, they do show greater T_{fr} levels that were $CD25^{hi}$ outside the germinal center which were in-line with conclusions from prior studies. However, these findings are significant to the field of T_{fr} cells because they provide evidence towards the view that T_{fr} cells play an important role in regulation of the germinal center reaction.[16]

Despite these studies revealing findings about the location of T_{fr} cells within the lymph node, they do not show experiments regarding the biological role that T_{fr} cells play within the

lymph node and germinal center reaction. Interestingly, an experiment studying the function of T_{fr} cells determined that T_{fr} cells may exert its effector function by interacting with both T_{fh} and germinal center B cells, thus physically preventing an interaction between the two cells.[25] Despite these findings, it is important to note that this experiment was conducted in-vitro and the role of T_{fr} cells at the molecular level remains to be shown in-vivo. It is unknown exactly what T_{fr} cells are doing, and which cells are directly interacting with T_{fr} cells from an effector stance.

Section 1.8: The Unmet Need and My Hypothesis

Despite the growing body of evidence around T_{fr} cells having a significant role in humoral responses, it is yet to be shown how T_{fr} and T_{fh} levels are altered specifically in within the germinal center in T_{fr} cells with PTEN deletion. Here we investigated the question of whether there is a change in T_{fr} to T_{fh} levels with conditional PTEN deletion specifically in Foxp3+ cells. By demonstrating changes in germinal center T_{fr} to T_{fh} cell ratios in PTEN-ΔT_{Reg} mice, our results suggest that part of the reason T_{fr} cells are dysfunctional in the absence of PTEN could be linked to mislocalization. However, the results also indicate that T_{fr} cells lacking PTEN have no absolute defect in localizing to the GC, so reasons other than localization likely contribute more significantly to the dysfunction of T_{fr} cells in PTEN-ΔT_{Reg} mice.

Chapter Two: Data

Section 2.1: Materials and Methods

Mice. PTEN $^{fl/fl}$ were obtained and bred with Foxp3-YFP-Cre mice, which express the YFP sequence, into the Foxp3 locus, with Cre recombinase to produce PTEN-ΔT_{Reg} mice or Foxp3-YFP-cre which served as our control mice. Mice were age matched at 7 weeks of age.

Harvesting Lymph Nodes. Mice were sacrificed through exposure to carbon dioxide (CO_2) gas. Lymph nodes from various sites including cervical, mesenteric, peripheral, and draining were surgically removed through usage of scissors and scalpel. All procedures were conducted in accordance with protocols approved by each Animal Care and Use Committee.

Tissue Processing. Lymph nodes were placed 10 mL of 4% paraformaldehyde (PFA) for a 1-hour duration than transferred into a 30% sucrose solution for overnight incubation in 4^{o}C. Lymph nodes were frozen in OTC in cryomold, by Tissue-Tek using liquid nitrogen. Frozen lymph node samples were stored in the -80^{o}C fridge. Sections were obtained at a thickness of 12 μm using a Leica Cryostat

CD4 Alexa Fluor 750 Antibody Conjugation. 1 mg CD4 monoclonal antibody clone GK1.5 (Catalog # 14-0041-86; ThermoFisher Scientific) were utilized for the AF750 conjugation protocol. CD4 antibody was concentrated into a 500 ug volume (~2 mg/mL volume concentration) through a 5-minute centrifugation using the Amicon Ultra-15 centrifugal filter tube. CD4 antibody was conjugated to AF750 through application of the SAIVI Rapid Antibody Labeling Kit protocol (Catalog # S30046; Invitrogen/Thermofisher Scientific): 1) 500 uL of CD4 was combined with 50 uL of sodium bicarbonate and 10 uL of regulator solution in a 1.5 mL microcentrifuge tube. 2) The mixture from Step 1 was transferred into a reaction vial and mixed with the lyophilized AF750 dye, dissolved, and incubated at room temperature and without

exposure to light for a duration of 60 minutes. 3) Following the 60-minute incubation, the CD4 antibody and AF750 mixed solution was run through a resin column. 4) Eluate was collected, and filtration was performed through using a syringe filter and a plunger tool. 5) CD4-AF750 conjugated antibody was stored in an Eppendorf tube at 4 degrees Celsius.

Validation of CD4 conjugation. Resulting conjugated CD4 antibody concentration was measured through use of NanoDrop manufactured by ThermoFisher Scientific. Degree of Labeling was measured at ~2.5 which was in-line with the targeted range specified by the SAIVI Rapid Antibody Labeling Kit protocol.

Immunofluorescence Stain. Permeabilizing and blocking solution was made consisting of 2% bovine serum albumin, 2% donkey serum, 0.5% triton X, and 1x phosphate buffer solution (PBS). Lymph node sections from PTEN-ΔT_{Reg} and Foxp3-YFP-Cre mice were then dried for 30 minutes over a layer of dry Kimwipes. Fixation was conducted by covering tissues for ten minutes at room temperature in 4% PFA solution. Following fixation, tissues were washed 5 times for 9 minutes per wash in 1x PBS. A hydrophobic pen was used to form a hydrophobic barrier around the lymph node tissue. Permeabilization and blocking were conducted by incubating the lymph node tissue in permeabilizing and blocking solution for 30 minutes at room temperature. CD4, GFP (Catalog # ab290; Abcam), Gl7-AF647 (Catalog # 144606; BioLegend), and IgD-AF594 (Catalog # 405740; BioLegend) antibodies were diluted to their respective optimized dilution factors using the permeabilizing and blocking solution as the designated diluent. Primary antibodies were incubated on respective mice lymph node tissue covered for one hour at room temperature. Following primary antibody incubation, mice lymph node tissues were washed 5 times for 3 to 5 minutes each wash with permeabilizing and blocking solution. Lymph nodes were then incubated in a 1:200 solution of secondary antibody (Catalog # 406416;

BioLegend) diluted using the permeabilizing and blocking solution for a duration of one hour. After secondary antibody incubation, mice lymph node tissues were washed 5 times for 3 to 5 minutes each wash with permeabilizing and blocking solution. TrueVIEW Autofluorescence Quenching Kit reagent (Catalog # SP-8400; Vector Laboratories) to reduce autofluorescence background was then created by creating a 1:1 solution of TrueVIEW reagent A and B and mixing for 10 seconds. Following the mix of TrueVIEW reagents A and B, TrueVIEW reagent C was then added to the mixed solution to create a 1:1:1 solution of TrueVIEW reagents A, B, and C with 10 seconds of mixing. Following washing, mice lymph node was incubated in TrueVIEW solution covered for five minutes at room temperature. Mice lymph node tissues were washed by submerging the tissue slides in a plastic cartoon with 250 mL of 1x PBS for a five-minute duration. Mice lymph node tissues were then nuclear stained and mounted using a microscope plastic coverslip and a DAPI solution with mount manufactured by VectaShield for a duration of 30 minutes and covered at room temperature. Microscope plastic coverslips were cured using clearcoat nail polish. Slides were stored in a dry condition overnight at 4℃.

Microscopy Imaging. Fluorescence microscopy images were taken using the TissueFaxs SPECTRA system manufactured by TissueGnostics at the Ragon Institute of MGH, MIT, and Harvard.

Analysis and Comparison of Germinal Center T_{fr} and T_{fh} cell counts. To determine and measure T_{fr} and T_{fh} cell counts, images were analyzed using the StrataQuest analysis software. T_{fr} cells were identified by visual identification of CD4+ (AF750), GFP+ (AF488), and nuclear (DAPI) stained cells.

Primary Antibody				
Target	**Dilution**	**Reactivity**	**Host**	**Conjugated Fluorophore**
CD4	1:100	Mu	Rat	AF750
GFP	1:1000	Mu	Goat	-
Gl7	1:100	Mu	Rat	AF594
IgD	1:100	Mu	Rat	AF647
Secondary Antibody				
Target	**Dilution**	**Reactivity**	**Host**	**Conjugated Fluorophore**
-	-	-	-	-
Goat Fc	1:200	Goat	Rat	AF-488
-	-	-	-	-
-	-	-	-	-

Table 2.1: IF Experimental Panel Setup. Fluorophore Conjugated primary antibodies were designated to target CD4, Gl7, and IgD. Unconjugated anti-GFP was incubated with fluorophore conjugated secondary targeting the Fc region of the primary anti-GFP.

Chapter Three: Results

Section 3.1: Elevated germinal center levels in PTEN-ΔT_{Reg} mice compared to Foxp3 Cre mice

In order to assess and compare the germinal center response following conditional knockout of PTEN in Foxp3+ cells, lymph nodes were harvested from immunized Foxp3 Cre and PTEN-ΔT_{Reg} mice and processed for fixation and eventual frozen sectioning. After performing our optimized immunofluorescence staining protocol, we observed greater germinal center formation ($\geq$ 2-fold increase), denoted by the orange coloration (Gl7-AF647), in the PTEN-ΔT_{Reg} mice which was in-line with what has been reported in the literature.

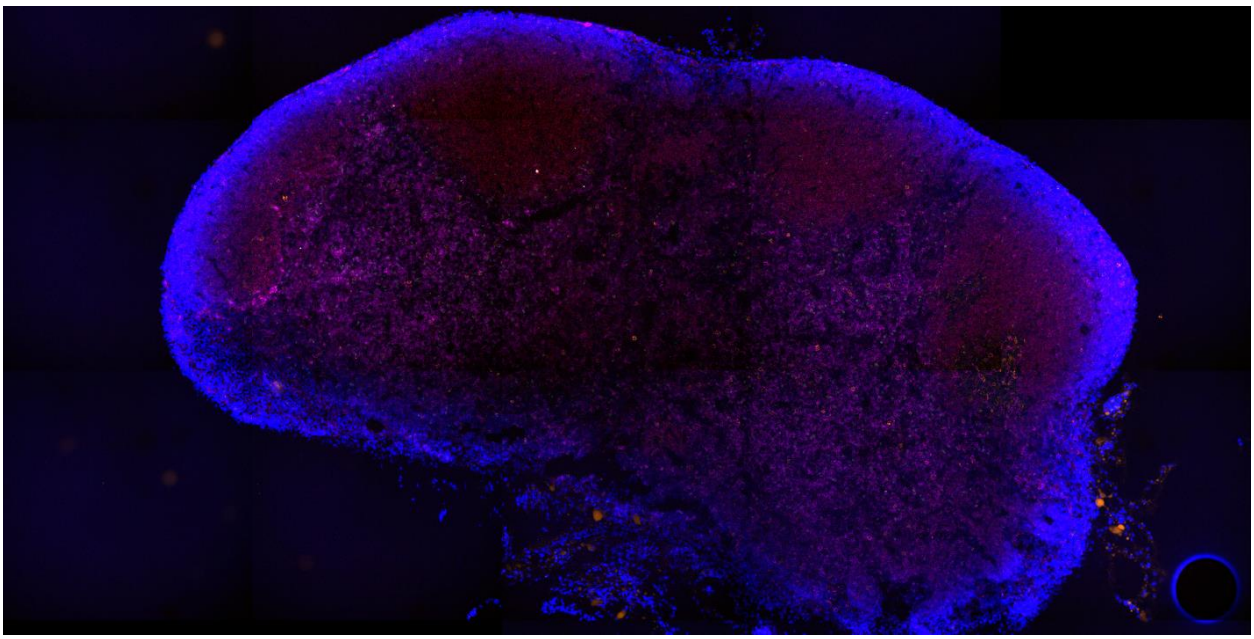

Figure 3.1: Foxp3 Cre Mice Draining Lymph Node. Frozen lymph node was obtained from Foxp3 Cre mice immunized with NP-OVA. Tissue was processed, frozen, and sectioned through use of Cryostat. Sectioned tissues were stained using our optimized IF protocol. Cells were stained for CD4 (pink), DAPI (blue), GFP (green), Gl7 (orange), and IgD (red).

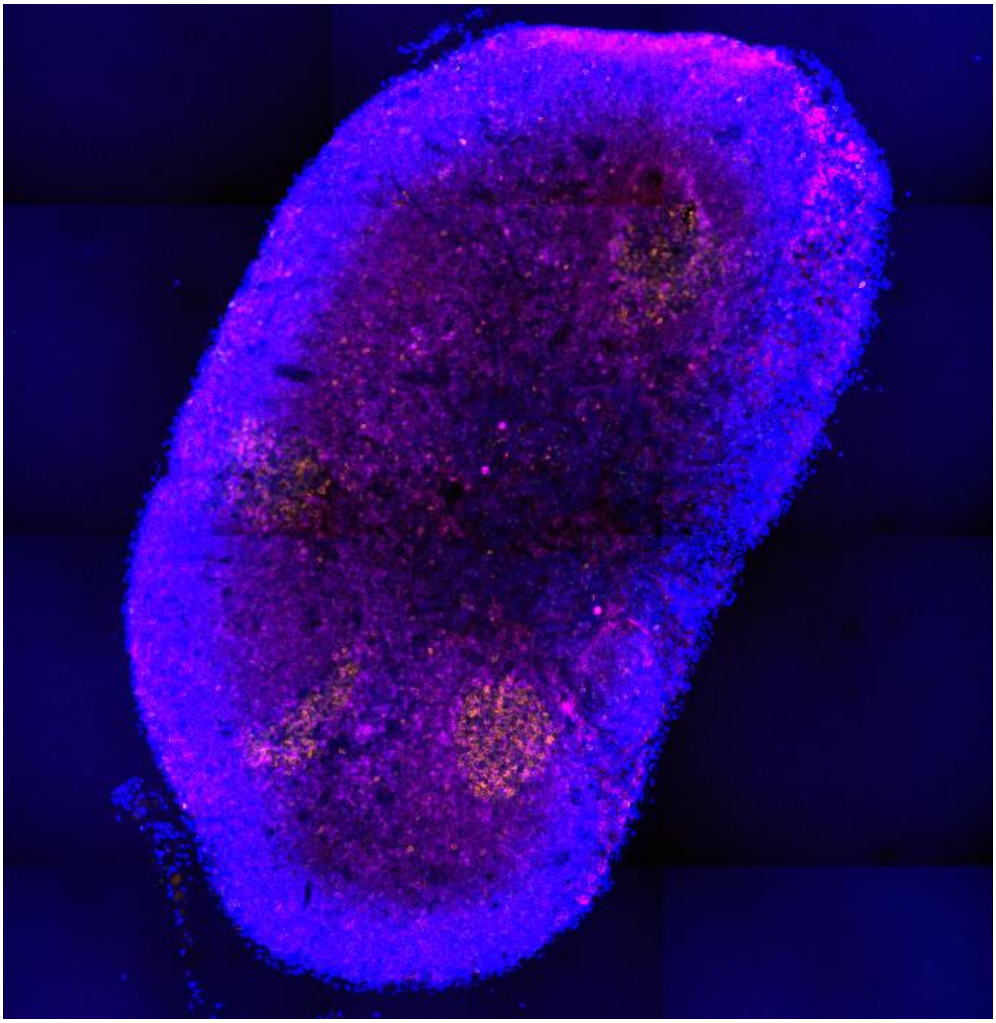

Figure 3.2: PTEN-ΔT_{Reg} Mice Draining Lymph Node. Frozen lymph node was obtained from PTEN-ΔT_{Reg} mice immunized with NP-OVA. Tissue was processed, frozen, and sectioned through use of Cryostat. Sectioned tissues were stained using our optimized IF protocol. Cells were stained for CD4 (pink), DAPI (blue), GFP (green), Gl7 (orange), and IgD (red).

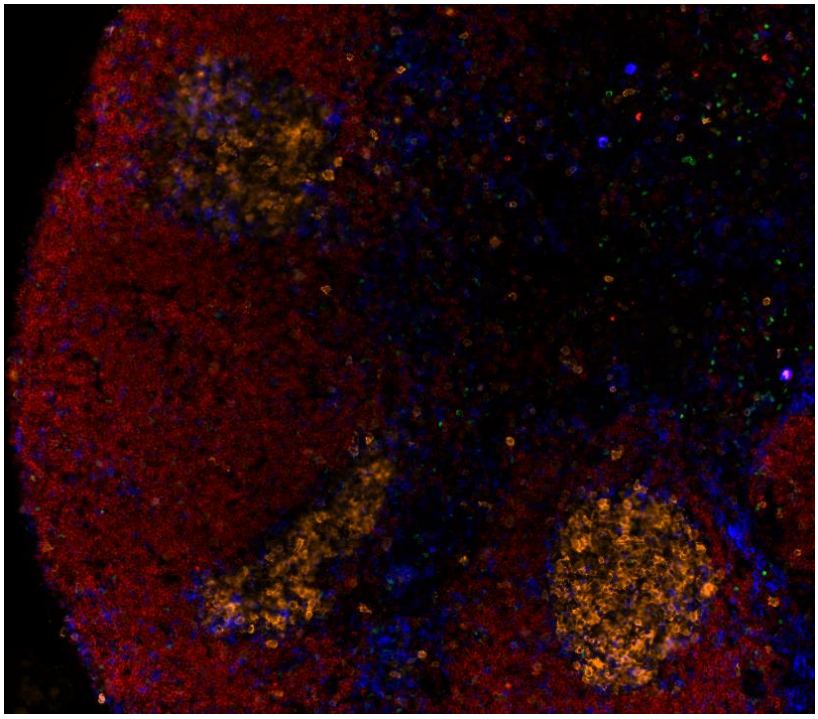

Figure 3.3: PTEN-ΔT_{Reg} Mice Draining Lymph Node Germinal Center, B Cell Follicle, and T Cell Zone. Frozen lymph node was obtained from PTEN-ΔT_{Reg} mice immunized with NP-OVA. Tissue was processed, frozen, and sectioned through use of Cryostat. Sectioned tissues were stained using our optimized IF protocol. Cells were stained for CD4 (blue), GFP (green), Gl7 (orange), and IgD (red). DAPI nuclear stain was excluded from this image but validated separately (not shown) for of image clarity.

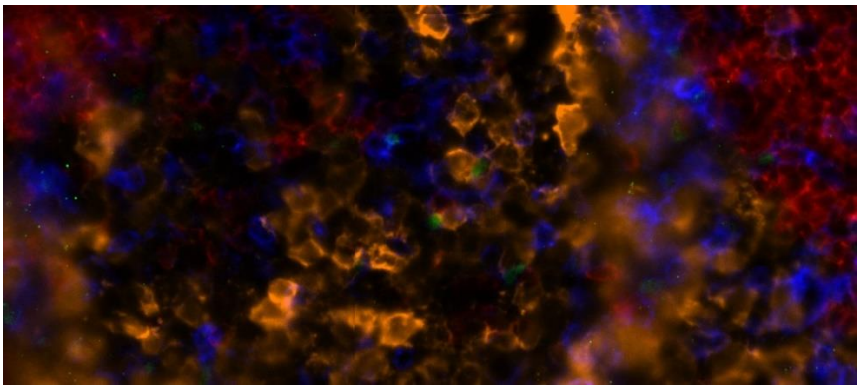

Figure 3.4: PTEN-ΔT_{Reg} mice Germinal Center Cell-to-Cell Contact. Frozen lymph node was obtained from PTEN-ΔT_{Reg} mice immunized with NP-OVA. Tissue was processed, frozen, and sectioned through use of Cryostat. Sectioned tissues were stained using our optimized IF protocol. Cells were stained for CD4 (blue), GFP (green), Gl7 (orange), and IgD (red). DAPI nuclear stain was excluded from this image but validated separately (not shown) for image clarity.

Section 3.2: Greater germinal center T_{fh} cell levels in PTEN-ΔT_{Reg} mice

In order to analyze and compare T_{fr} and T_{fh} cell levels in 2 PTEN-ΔT_{Reg} mice and 1 Foxp3 Cre mice, TissueQuest software by TissueGnostics was utilized for visualization and cell counts. To our surprise, we observed similar absolute T_{fr} cell counts in both the PTEN-ΔT_{Reg} and Foxp3 Cre mice populations. Strikingly, we observed a 59% average increase in absolute T_{fh} cell counts in the PTEN-ΔTReg compared to the Foxp3 Cre mice.

	Germinal Center		
Mouse	**T_{fr} Count**	**T_{fh} Count**	**T_{fr} + T_{fh}**
Foxp3 Cre 2-1 Male (Imm)	33	90	123
Average	*33*	*90*	*123*
PTEN-ΔT_{Reg} 1 Female (Immunized)	28	147	175
PTEN-ΔT_{Reg} 2 Female (Immunized)	30	140	170
Average	*29*	*144*	*173*

Table 3.1: Germinal Center T_{fr} and T_{fh} Cell Comparison in PTEN-ΔT_{Reg} and Foxp3 Cre Mice. T_{fr} and T_{fh} cell levels were measured from a single germinal center from each mouse type lymph node: 1 germinal center from a Foxp3 Cre mouse and 2 germinal centers from PTEN-ΔT_{Reg} mice (3 germinal centers in total) were examined. TissueQuest software by TissueGnostics was utilized to visualize tissues and conduct cell count analysis.

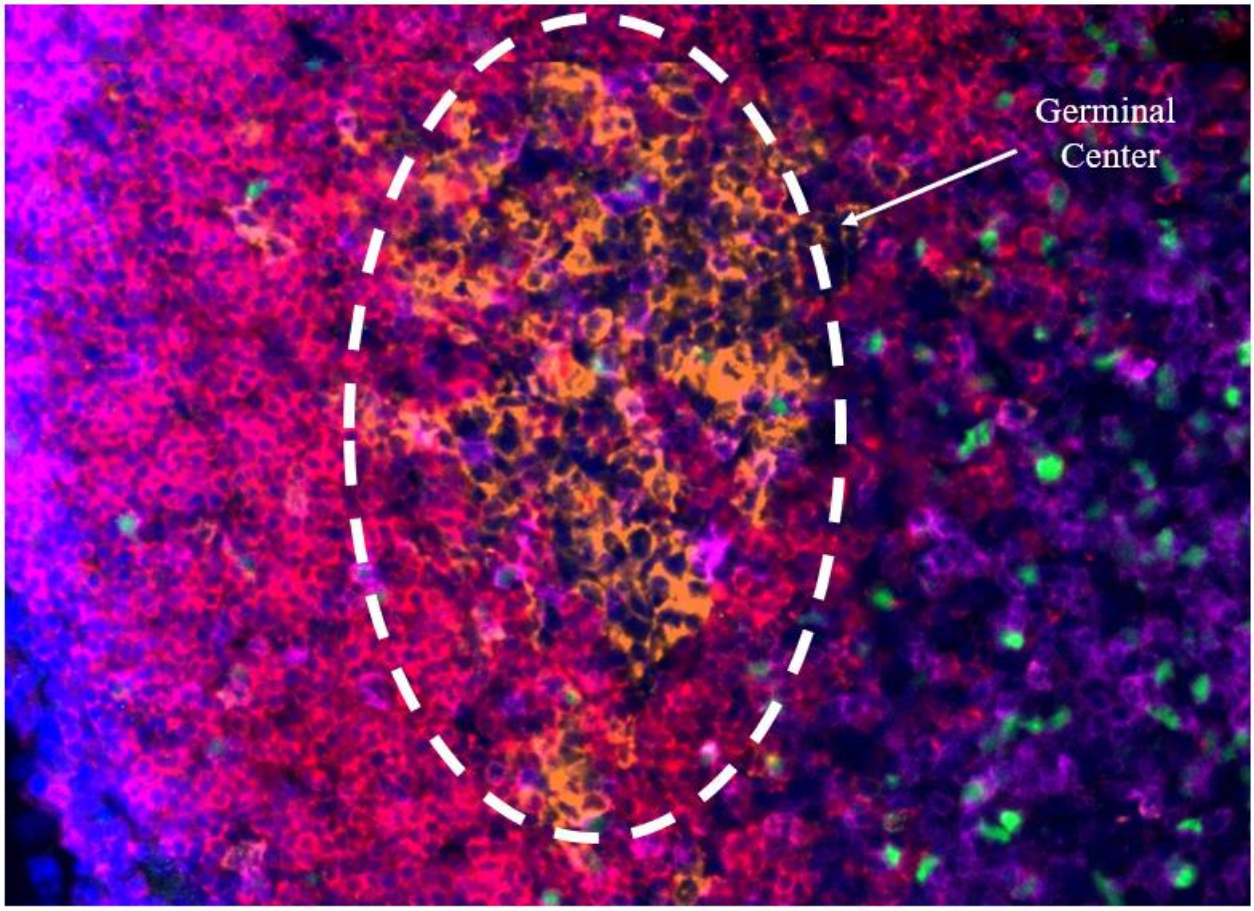

Figure 3.5: Foxp3 Cre Mice Germinal Center from Draining Lymph Node 5 Color Image. Frozen lymph node was obtained from Foxp3 Cre mice immunized with NP-OVA. Tissue was processed, frozen, and sectioned through use of Cryostat. Sectioned tissues were stained using our optimized IF protocol. Cells were stained for CD4 (blue), DAPI (Blue), GFP (green), Gl7 (orange), and IgD (red).

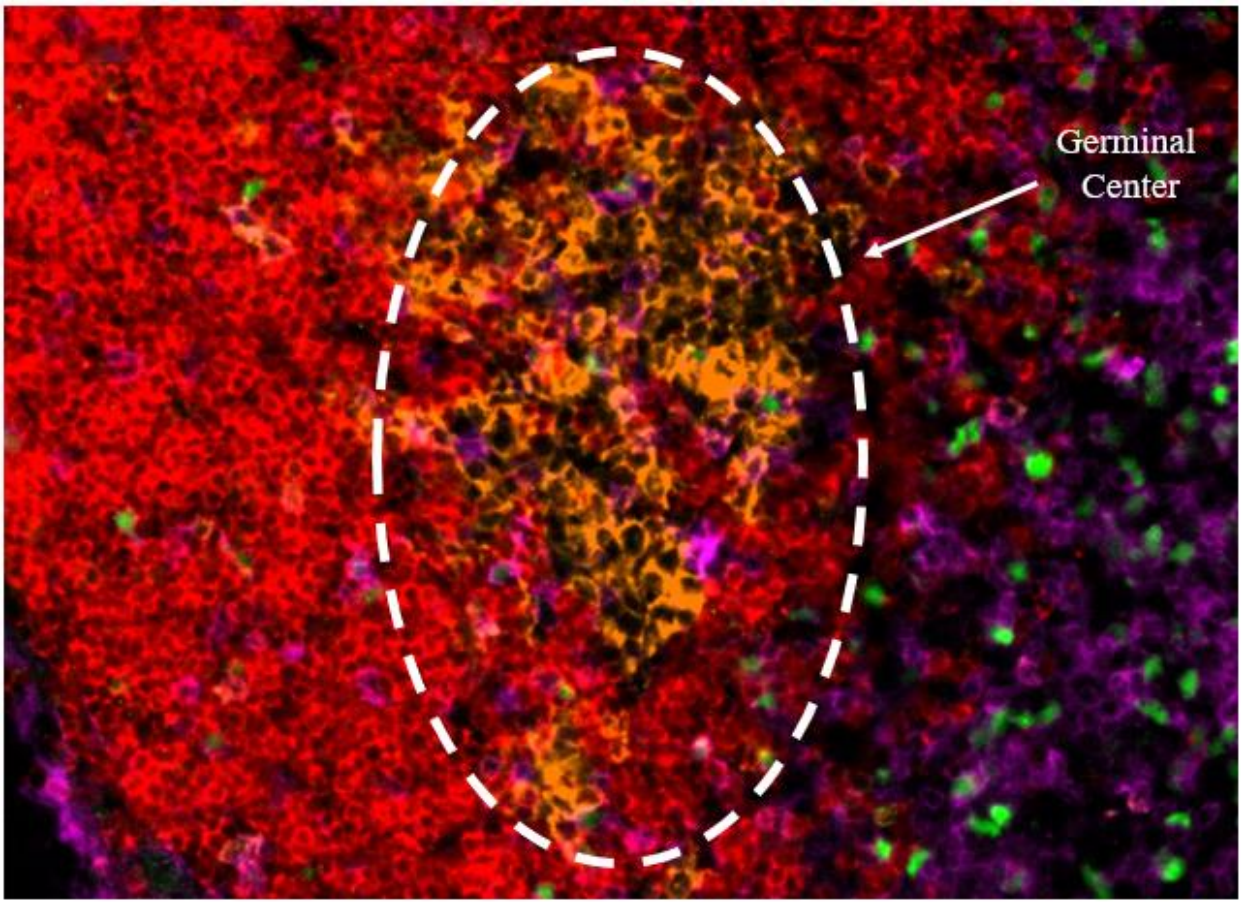

Figure 3.6: Foxp3 Cre Mice Germinal Center from Draining Lymph Node 4 Color Image. Frozen lymph node was obtained from Foxp3 Cre mice immunized with NP-OVA. Tissue was processed, frozen, and sectioned through use of Cryostat. Sectioned tissues were stained using our optimized IF protocol. Cells were stained for CD4 (blue), GFP (green), Gl7 (orange), and IgD (red). DAPI nuclear stain was excluded from this image.

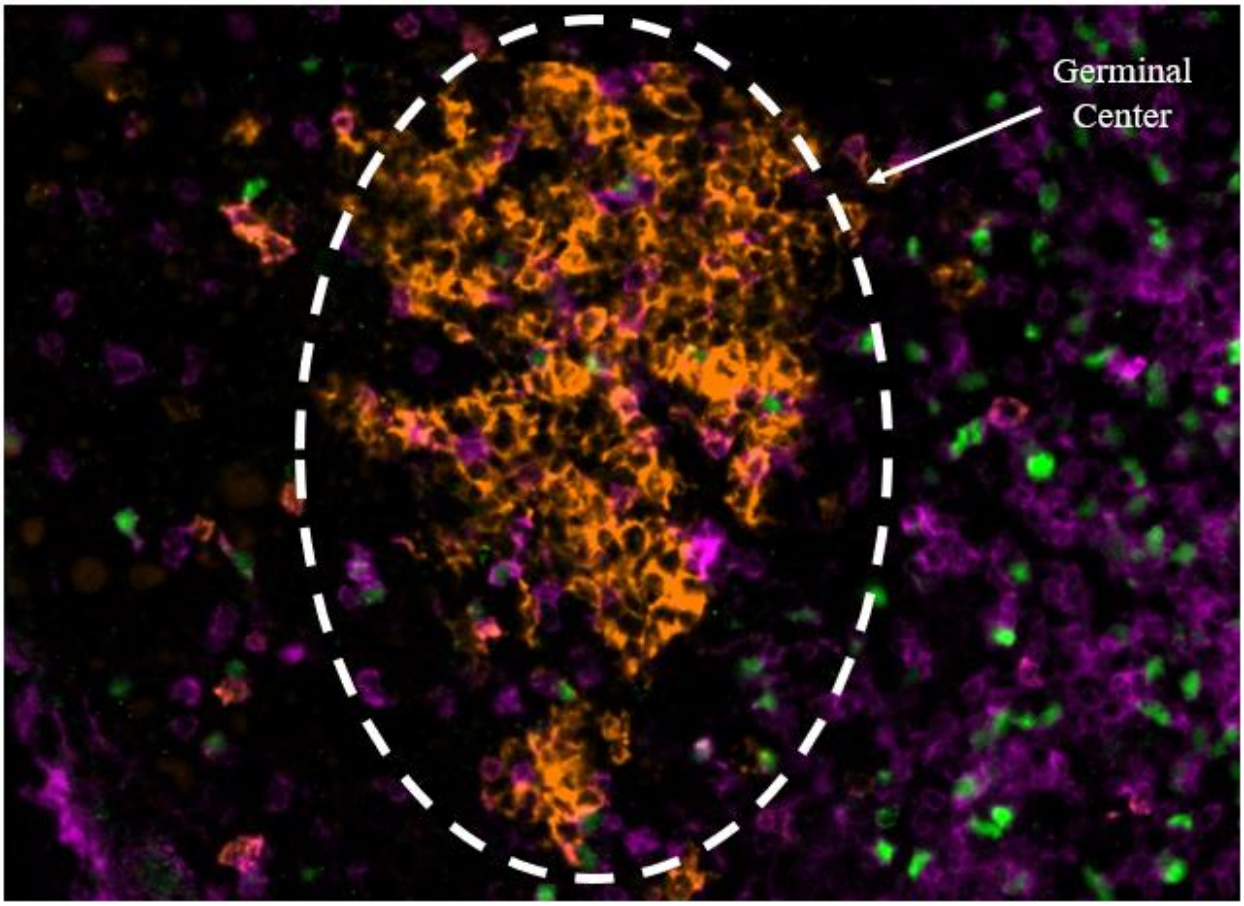

Figure 3.7: Foxp3 Cre Mice Germinal Center from Draining Lymph Node 3 Color Image. Frozen lymph node was obtained from Foxp3 Cre mice immunized with NP-OVA. Tissue was processed, frozen, and sectioned through use of Cryostat. Sectioned tissues were stained using our optimized IF protocol. Cells were stained for CD4 (blue), GFP (green), and Gl7 (orange). DAPI nuclear stain and IgD were excluded from this image.

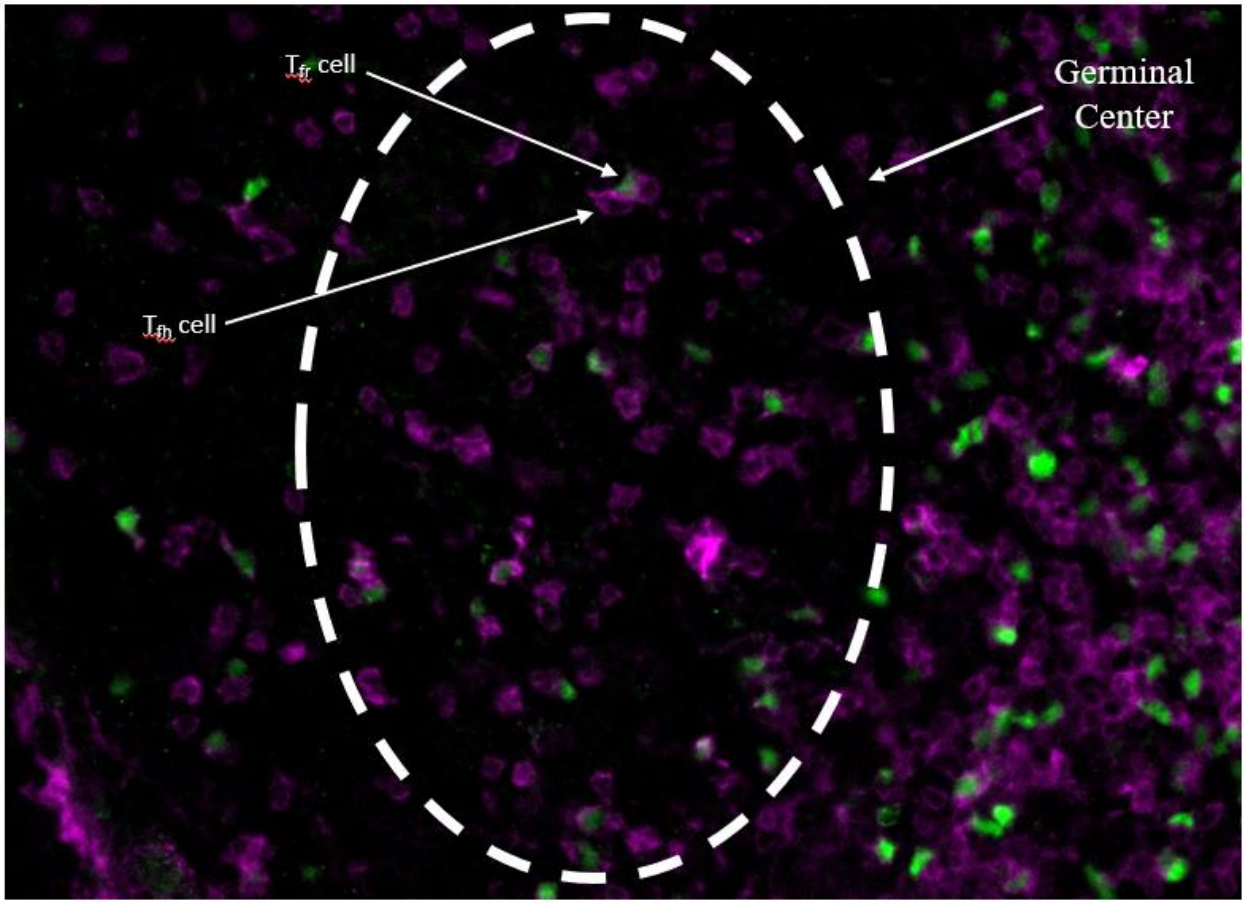

Figure 3.8: Foxp3 Cre Mice Germinal Center from Draining Lymph Node 2 Color Image. Frozen lymph node was obtained from Foxp3 Cre mice immunized with NP-OVA. Tissue was processed, frozen, and sectioned through use of Cryostat. Sectioned tissues were stained using our optimized IF protocol. Cells were stained for CD4 (blue) and GFP (green). DAPI nuclear stain, Gl7, and IgD were excluded from this image.

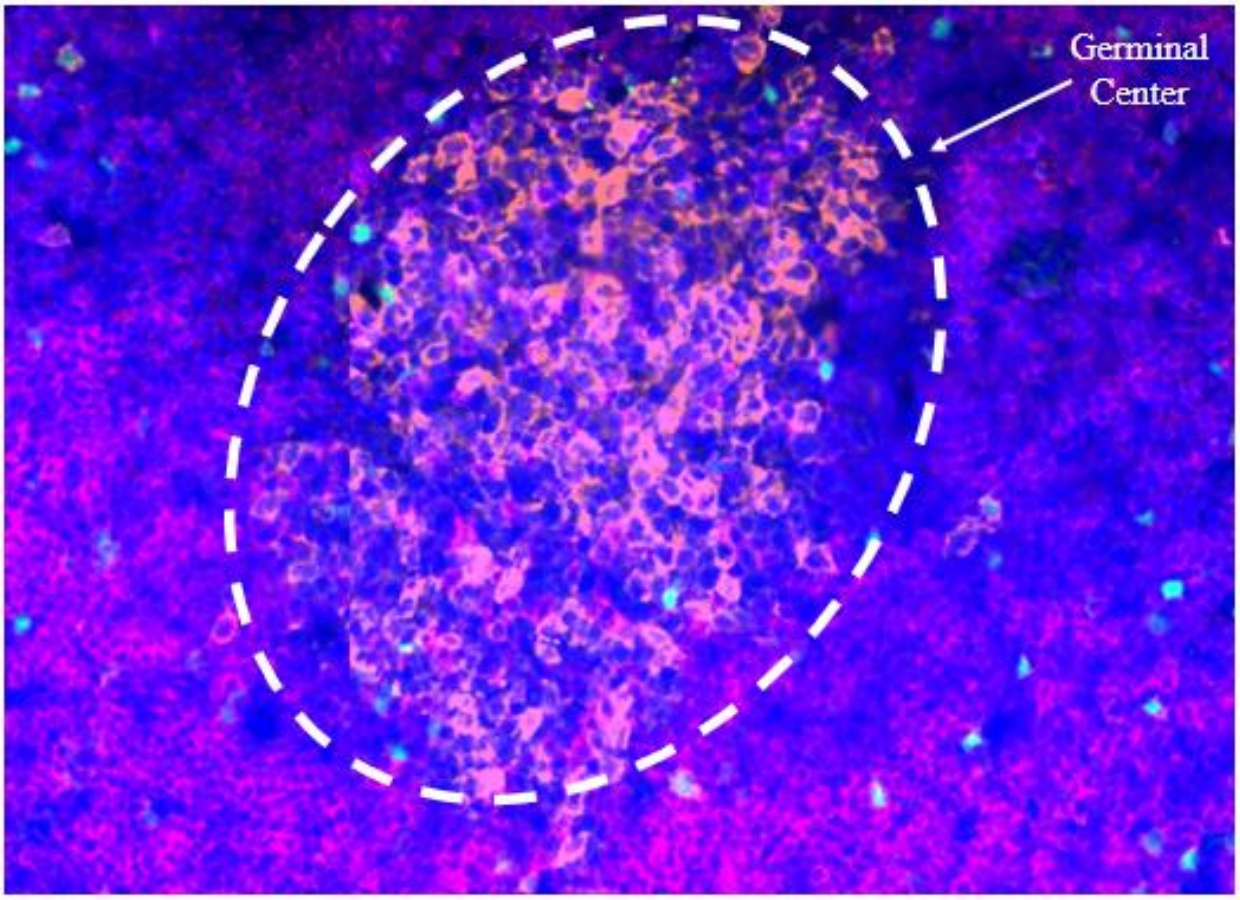

Figure 3.9: PTEN-ΔT$_{Reg}$ Mice Germinal Center from Draining Lymph Node 5 Color Image. Frozen lymph node was obtained from Foxp3 Cre mice immunized with NP-OVA. Tissue was processed, frozen, and sectioned through use of Cryostat. Sectioned tissues were stained using our optimized IF protocol. Cells were stained for CD4 (blue), DAPI (Blue), GFP (green), Gl7 (orange), and IgD (red).

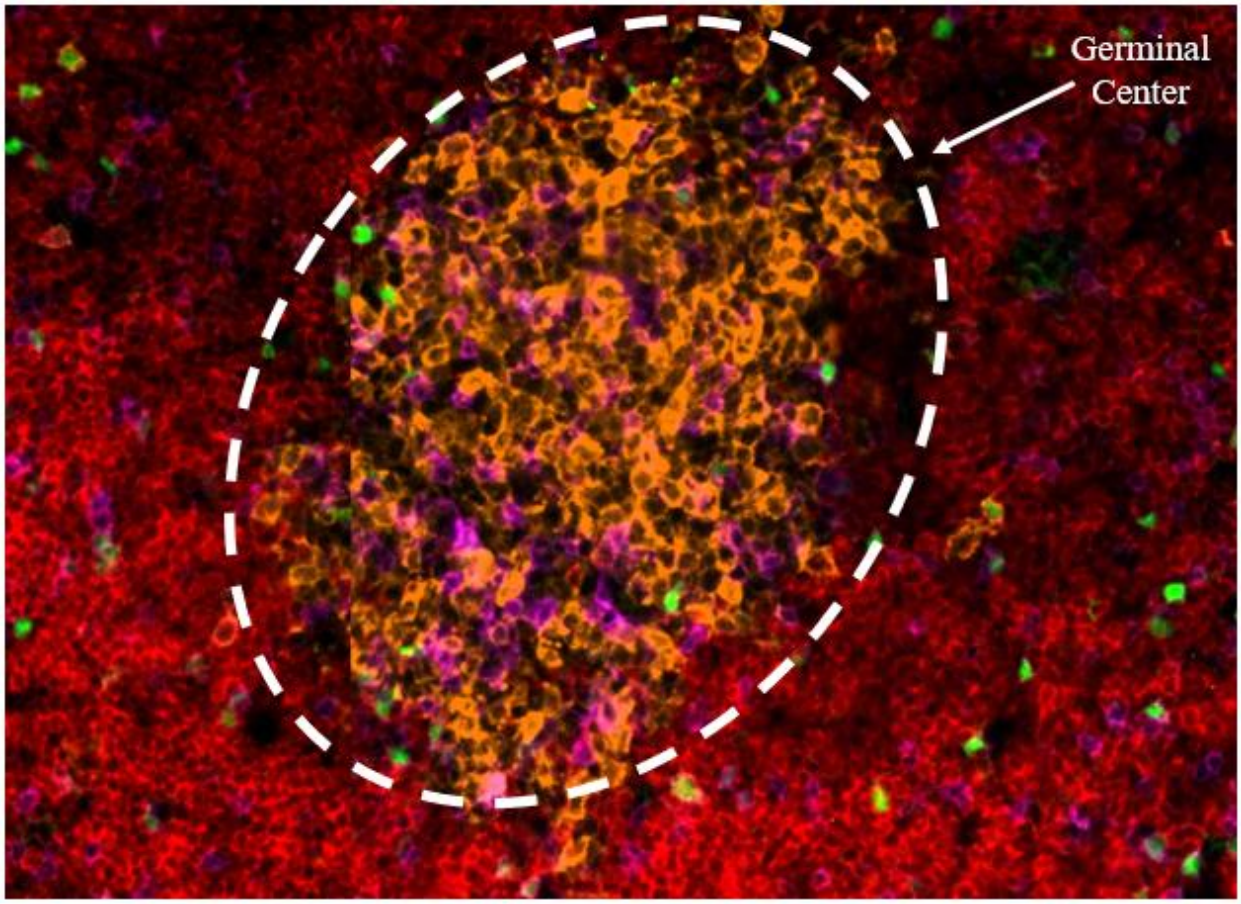

Figure 3.10: PTEN-ΔT_{Reg} Mice Germinal Center from Draining Lymph Node 4 Color Image. Frozen lymph node was obtained from Foxp3 Cre mice immunized with NP-OVA. Tissue was processed, frozen, and sectioned through use of Cryostat. Sectioned tissues were stained using our optimized IF protocol. Cells were stained for CD4 (blue), GFP (green), Gl7 (orange), and IgD (red). DAPI nuclear stain was excluded from this image.

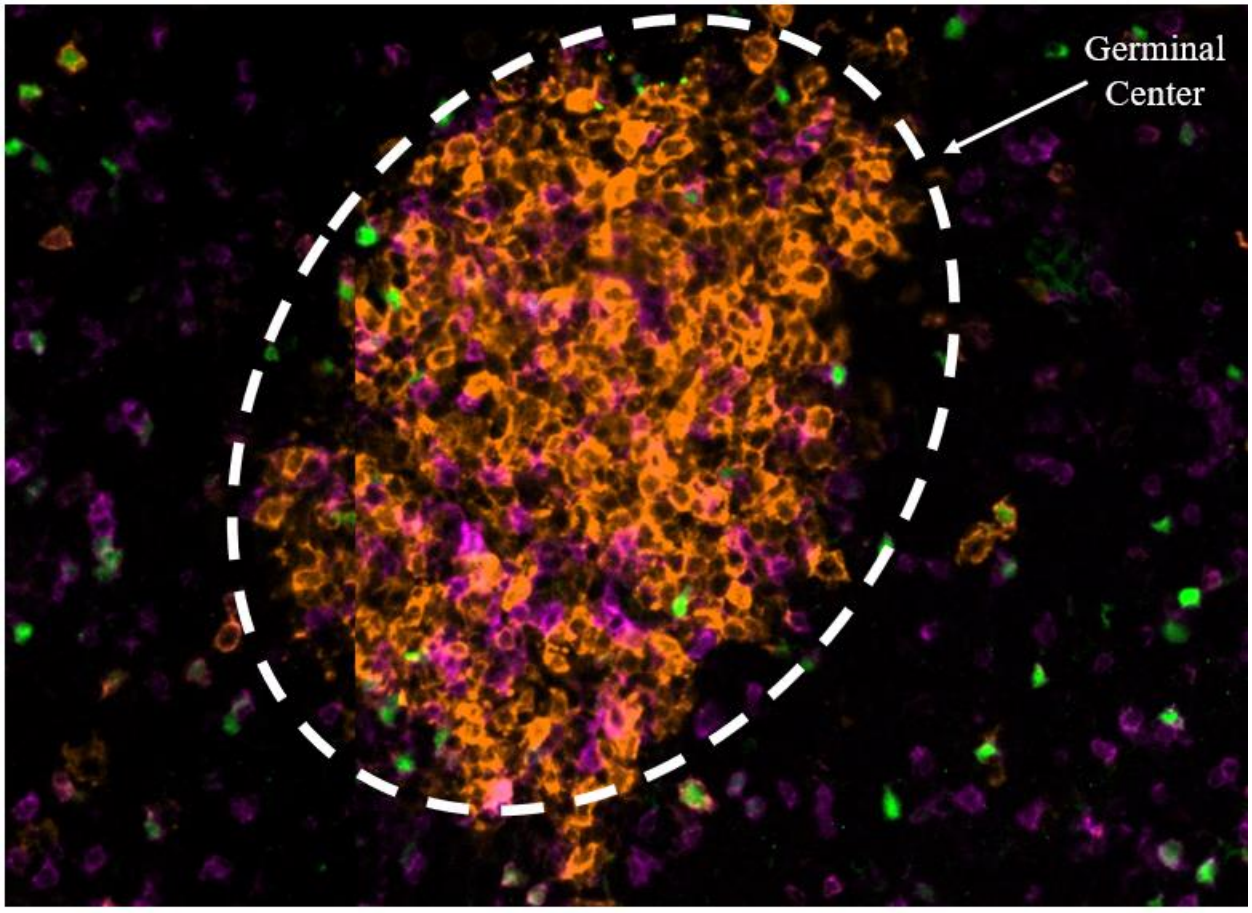

Figure 3.11: PTEN-ΔT$_{Reg}$ Mice Germinal Center from Draining Lymph Node 3 Color Image. Frozen lymph node was obtained from Foxp3 Cre mice immunized with NP-OVA. Tissue was processed, frozen, and sectioned through use of Cryostat. Sectioned tissues were stained using our optimized IF protocol. Cells were stained for CD4 (blue), GFP (green), and Gl7 (orange). DAPI nuclear stain and IgD were excluded from this image.

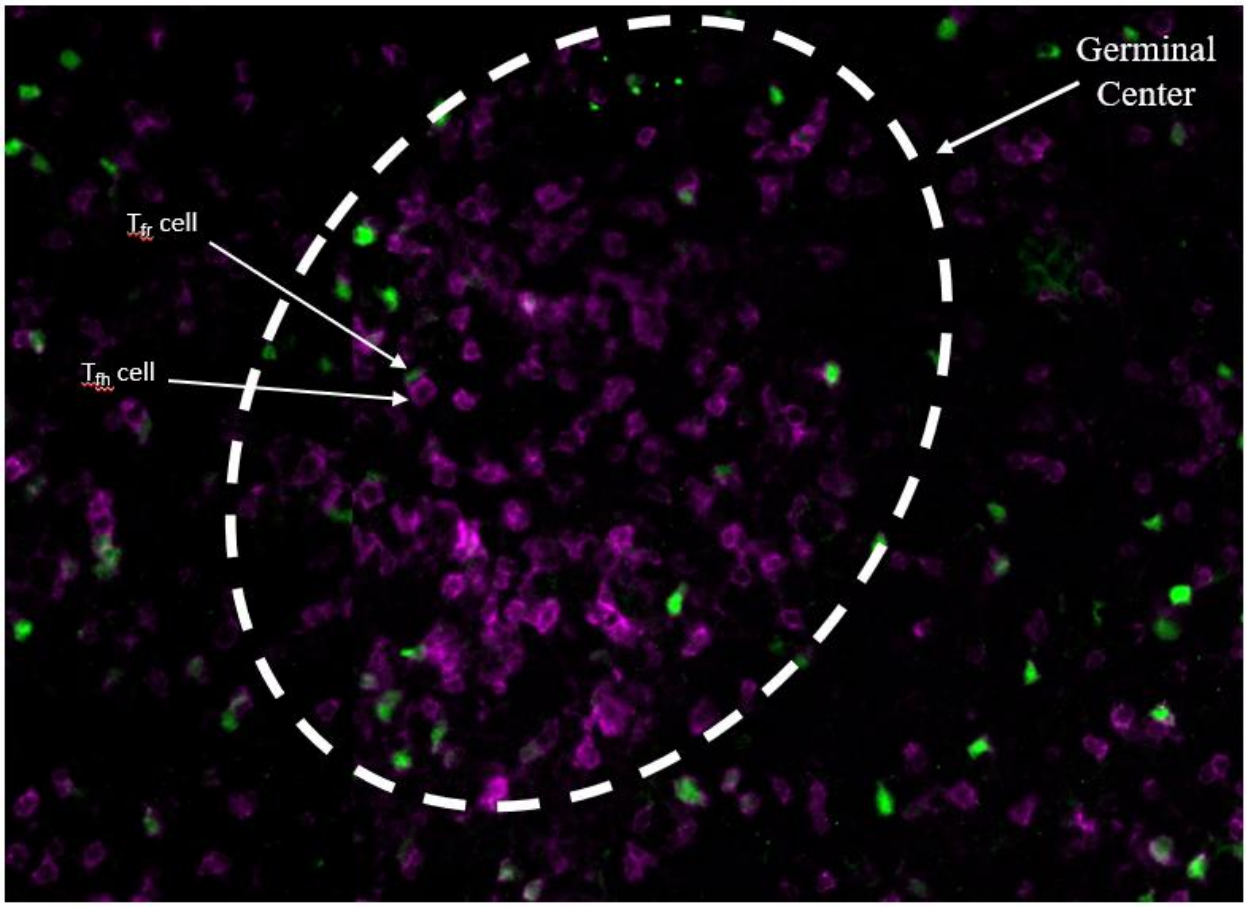

Figure 3.12: PTEN-ΔT_{Reg} Mice Germinal Center from Draining Lymph Node 2 Color Image. Frozen lymph node was obtained from Foxp3 Cre mice immunized with NP-OVA. Tissue was processed, frozen, and sectioned through use of Cryostat. Sectioned tissues were stained using our optimized IF protocol. Cells were stained for CD4 (blue) and GFP (green). DAPI nuclear stain, Gl7, and IgD were excluded from this image.

Section 3.3: Decreased T_{fr} to T_{fh} cell ratio in PTEN-ΔT_{Reg} mice compared to Foxp3 Cre mice

Following analysis of T_{fr} and T_{fh} cell levels in PTEN-ΔT_{Reg} and Foxp3 Cre mice, we compared the ratio of T_{fr} to T_{fh} cell counts within the germinal center to determine if there is perhaps a defect in T_{fr} cell germinal center localization in PTEN-ΔT_{Reg} mice. Interestingly, we observed a ~15% decrease in germinal center average T_{fr} to T_{fh} cell ratio in PTEN-ΔT_{Reg} mice compared to what was observed in the Foxp3 Cre mice. Additionally, T_{fh} cells encompassed an average of 83% of CD4+ T cells within the germinal center, a 10% increase compared to Foxp3 Cre mice.

	Germinal Center				
Mouse	**T_{fr} Count**	**T_{fh} Count**	**% T_{fr}**	**% T_{fh}**	**T_{fr} : T_{fh} Ratio**
Foxp3 Cre 2-1 Male (Immunized)	33	90	27%	73%	0.37
Average	*33*	*90*	*27%*	*73%*	*0.37*
PTEN-ΔT_{Reg} 1 Female (Immunized)	28	147	16%	84%	0.19
PTEN-ΔT_{Reg} 2 Female (Immunized)	30	140	18%	82%	0.21
Average	*29*	*144*	*17%*	*83%*	*0.20*

Table 3.2: Comparison of Germinal Center T_{fr} and T_{fh} Cell Levels and Relative Ratio. Germinal center % T_{fr} and T_{fh} cell levels were calculated with reference to total germinal center CD4+ (T_{fr} + T_{fh} cells) cell count. Germinal center T_{fr} : T_{fh} ratio was calculated by dividing T_{fr} by T_{fh} cell counts.

Section 3.4: Brief Discussion

Our research collectively suggests that the germinal center dysfunctions observed in PTEN-ΔT_{Reg} mice may be due to dysfunctional T_{fr} cells. Importantly, the ~15% decrease in T_{fr} to T_{fh} ratio in PTEN-ΔT_{Reg} mice compared to the Foxp3 Cre mice would suggest that T_{fr} cells are dysfunctional with PTEN deletion, in-line with the reported decrease in high affinity antibody levels and humoral driven defects observed in PTEN-ΔT_{Reg} mice.

Chapter Four: Discussion and Perspectives

Section 4.1: Discussion

In this study, we investigated and compared differences in T_{fr} and T_{fh} cells within the germinal center. In accordance with prior studies, we observed increased germinal center formation in lymph nodes from PTEN-ΔT_{Reg} mice compared to control mice (Foxp3 Cre). Based on our findings of similar germinal center T_{fr} cell counts, increased T_{fh} cell counts, and a decreased T_{fr} to T_{fh} cell ratio in PTEN-ΔT_{Reg} mice, we believe these results support a view that T_{fr} cells are dysfunctional with PTEN deletion. It is likely that the dysfunctional T_{fr} cells are unable to perform their effector function of T_{fh} cell suppression leading to elevated T_{fh} cell counts in PTEN-ΔT_{Reg} mice compared to Foxp3 Cre mice. These findings likely validate the findings of a decrease in high affinity antibody production capability in PTEN-ΔT_{Reg} mice compared to Foxp3 Cre mice (unpublished data adapted from Kelsey Finn, PhD Candidate).

Based on T_{fr} cell expression of CTLA-4, it is likely that the T_{fr} cells prevent an autoreactive adaptive response by regulating B and T cell collaboration through trans endocytosis of CD80/CD86, preventing the ability for B cells to provide signal 2 to activated T cells.[26] Additionally, a recent study demonstrated an ability for T_{Reg} cells to physically remove pMHCII from dendritic cells.[27] Due to the nature of T_{Reg} cells having TCR specificity that are skewed towards self-reactive peptides, T_{fr} cells may be removing pMHCII complexes presenting peptides that are self-reactive in nature.[28] Further supporting this claim, a recent study found that T_{fr} cell TCRs resembled that of T_{Reg} TCRs.[29] Considering the literature regarding the function and specificity of T_{Reg} and T_{fr} cell TCR, it is possible that T_{fr} cells are essentially reducing the presence of antigen presenting B cells presenting self-reactive peptides to T cells at the B and T cell border and germinal center.

Collectively these findings advance our understanding of the regulatory elements of the robust and targeted adaptive immune system. Knowledge of a role of T_{fr} cells during the creation of high affinity antibodies could potentially be exploited in designing and advancing therapies focused on modulation of the germinal center response through the inhibition of PTEN in T_{fr} cells.

Section 4.2: Experimental Perspectives

In order to measure T_{fr} and T_{fh} cell frequencies in PTEN-ΔT_{Reg} mice, an immunofluorescence protocol developed internally was initially chosen. This protocol was unique to traditional immunofluorescence protocols in its ability to produce robust immunofluorescence images utilizing over four different fluorophores. This was possible due the utilization of horseradish peroxidase (HRP) conjugated secondary antibodies which functioned by catalyzing the oxidation of the dye into a fluorescently active tyramide derivative. This reaction led to the covalent deposition of the fluorophore near the HRP site and more importantly the respective biological target.[30] This approach resulted in greater ratio of fluorophore to target leading to a more robust fluorophore signal and greater resolution compared to traditional immunofluorescence approaches.

This approach proved to be challenging to apply within our experimental construct that were investigated in various angles. These approaches resulted in the inability to produce/detect a positive stain in paraffin embedded mouse spleen and lymph node. This could be due to many reasons including technique errors performed by human error; however, this was accounted for by a second student performing the same protocol which led to the same negative outcome. Additionally, the negative results could be due to product malfunctions at multiple steps within the protocol including antigen retrieval, blocking, permeabilizing, antibody incubation (primary

and secondary), and fluorophore. The fact that none of the antibodies produced signals gave us confidence that the discrepancies were not due to the antibody or fluorophore products. It is possible that the setbacks were tissue processing related; however, due to the interest of time, frozen section IF was the next approach.

To continue forward with the experimental investigation, we performed IF staining on frozen lymph node tissue. Based on prior approaches, we decided that this would be the least risky approach to obtaining our scientific results. We initially set out to perform IF through use of primary antibodies conjugated to AF488, AF594, AF648, and AF750 targeting Foxp3, IgD, Gl7, and Foxp3, respectively. We initially encountered difficulty with the antibody stains as only IgD-AF594 provided positive signals verified by TissueFaxs microscopy and through tissue morphology characteristics. Additionally, the Gl7 antibody stain proved to be the most challenging as the signals continued to show no signs of positive staining. To rule out technique specific human error, we decided to repeat the experiment side-by-side with an individual with prior experience in conducting the experiment. This approach still did not yield positive result which led us to consider investigating the functioning of our experimental protocol.

To troubleshoot our protocol, we decided to perform an IF stain alongside another individual using their IF protocol, equipment, and tissue samples from their M6 mice and our PTEN-ΔT_{Reg} mice to serve as a control for positive staining specifically for Gl7. In this protocol, we stained for CDR3, Gl7, IgD, and Black6. Following the experiment, we were able to identify clearly visible positive Gl7 stains in the M6 mice through using TissueFaxs microscopy. Interestingly, in the PTEN-ΔT_{Reg} lymph node tissue did not show visible signs of positive Gl7 staining. These findings drove us to consider investigating the possibility that perhaps the discrepancies involve the method of tissue processing. As a result, we switched from overnight

fixation in 4% paraformaldehyde (PFA) to a 1-hour fixation in the respective fixative per the protocol that led to the discovery of our optimized protocol.

Within the application of the optimized protocol, we began to produce positive mice lymph node stains. Initially, these positively identified biological markers included primary antibodies targeting CD4, Gl7 and IgD conjugated to AF488, AF594, and AF647 respectively (Figure 4.1). The CD4 clone utilized worked consistently; however, we needed an AF750 conjugated CD4 because our anti-GFP (Foxp3 proxy) worked well with AF488 and it would be more efficient to alter the CD4 antibody fluorophore pairing rather than modifying the anti-GFP primary and AF488 bound secondary antibody construct. With preliminary stains, we began to already notice key differences in the resolution and quality of the stained lymph nodes.

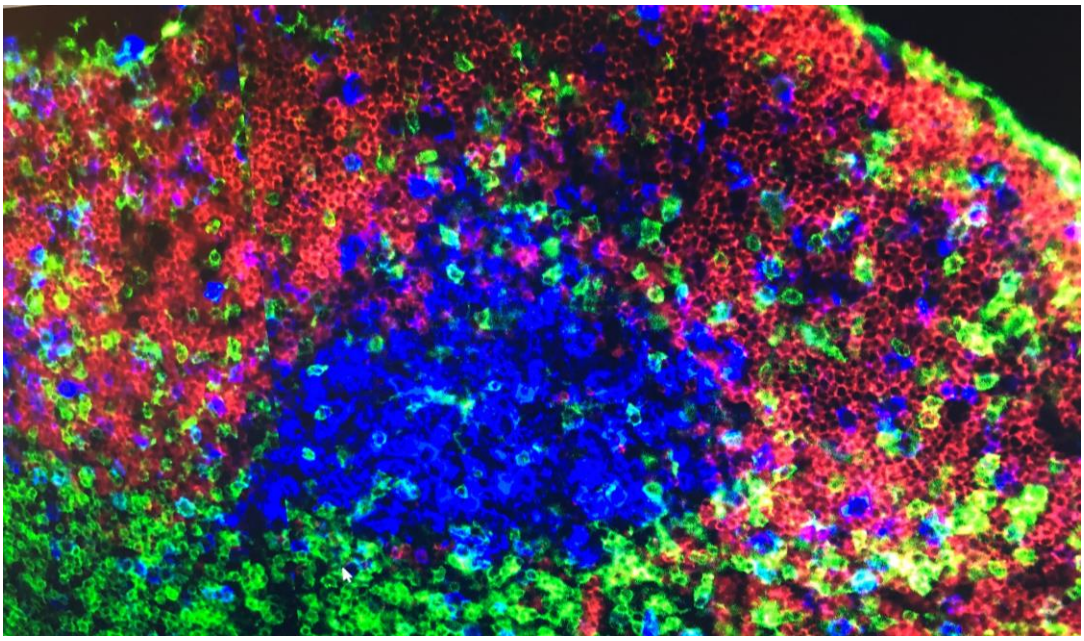

Figure 4.1: Lymph Node Morphology in wild type Mice. Frozen lymph nodes were obtained and stained using our optimized IF protocol. Lymph nodes were stained for Gl7 (blue), CD4 (green), and IgD (red).

To advance with staining, we purchased 1 mg of the same CD4 clone (GK1.5) in its unconjugated form to perform a manual conjugation experiment using ThermoFisher Scientific's SAIVI Rapid Antibody Labeling Kit using the Alexa Fluor 750 fluorophore. This resulted in the manually AF750 labeled CD4 antibodies. Following CD4-AF750 conjugation, we then designed and proceeded with our experiment using the designated panel (Table 2.1).

Section 4.3: Experimental Limitations

Although these experiments provide evidence of the effector function of T_{fr} cells during the germinal center reaction, further studies are needed to further determine any changes in T_{fr} to T_{fh} ratios in both types of mice. These later studies would require analysis of T_{fr} cell levels within the B cell follicle in addition to the germinal center. Additionally, it is currently challenging to achieve PTEN deletion in specifically T_{fr} cells. Our mice model does not isolate the biological affects to the T_{fr} cell population as the T_{Reg} population were also PTEN deficient inherently leading to the possibility that physiological changes were in-part due to the dysfunctional T_{Reg} population. For efficient isolation of T_{fr} cells effect in vivo, further experiments could be performed including adoptive transfer of PTEN-ΔT_{Reg} T_{fr} cells into a Foxp3 Cre $Bcl6^{fl/fl}$ mouse model.

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