DISSERTATION

T ZONE LYMPHOMA: CELLULAR ORIGIN AND FUNCTION

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ABSTRACT

T ZONE LYMPHOMA: CELLULAR ORIGIN AND FUNCTION

The lymphoid system is exceedingly complex with specialized subsets of lymphocytes involved in both the innate and adaptive immune response. Lymphocytes are subdivided into T cells and B cells with lymphoproliferative disorders comprising a heterogeneous group of diseases arising from various lymphocyte subsets. Dogs are a natural model for studying cancer in humans with overlapping subtypes of lymphoproliferative disease seen in both species. We were particularly interested in characterizing lymphoma subtypes in dogs as a model for studying human disease progression. We believe characterization of lymphoma subtypes in dogs will contribute to enhanced understanding of the pathogenic mechanisms leading to the development of malignancy in both humans and dogs.

T zone lymphoma (TZL) is a subtype of peripheral T cell lymphoma (PTCL) occurring in both humans and dogs. Determination of the cell-of-origin of PTCLs in humans has pointed to a subset of the larger disease, but up to half of these lymphomas cannot be further classified and are referred to as PTCL – not otherwise specified (PTCL-NOS). TZL has unique phenotypic characteristics including absence of the pan-leukocyte transmembrane protein, CD45, allowing for reliable identification by flow cytometry. CD45 has been found to be critical for T cell signaling through the T cell receptor (TCR).

We hypothesized TZL has been derived from an activated, mature T cell. We used gene expression in an attempt to classify the cell-of-origin in TZL. Then we used *in vitro* systems to identify proliferative mechanisms, cytokine production, and immunosuppression at play in this

disease. We determined TZL cells express genes associated with T helper 2 (Th2) and T regulatory (Treg) cells. We further confirmed that TZL cells do not proliferate through the TCR, likely due to their lack of CD45. We identified IL-2 may be involved in TZL signaling mechanisms.

There are reports of TZL occurring with an opportunistic follicular mite infection, suggesting a component of immunosuppression with this disease. We thus anticipated TZL cells would have an immunosuppressive phenotype and inhibit normal T cells. Using *in vitro* methods, we were unable to confirm immunosuppression through production of TGF-β, inhibition of proliferation and inhibition of IFN-γ production. Finally, we observed older Golden retrievers having a higher frequency of cells with the same aberrant phenotype as TZL, but without evidence or suspicion of lymphoproliferative disease. We believe this finding resembles clonopathies of unknown significance in older human individuals, also with rare progression to disease.

We now believe TZL is derived from a Th2 or Treg-like mature T cell which may be responsible for the indolent nature of this disease due to the tolerant behavior of those T cell subsets. Furthermore, inducing proliferation in this disease was challenging, which is consistent with slowly progressive biologic behavior. Expression of immunosuppressive molecules may also contribute to the indolent nature of this disease. While we were not able to observe immunosuppression *in vitro*, it is reasonable to assume there may be *in vivo* mechanisms of immunosuppression resulting in clinical manifestations seen in TZL. Characterization of cell-of-origin of this disease, neoplastic cell function, along with recognition of a pre-neoplastic state, contributes to enhanced understanding of the pathogenesis of lymphoproliferative diseases in both humans and dogs.

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DEDICATION

I dedicate this dissertation to Dr. Anne Avery, my mentor and advisor. This work would not have been possible without Dr. Avery's guidance, patience and forethought. I am a better scientist and person for having worked with you.

TABLE OF CONTENTS

INTRODUCTION

Cancer hallmarks

Cancers are an extremely heterogeneous group of diseases but fundamentally share the feature of uncontrolled cell growth by a number of mechanisms.[1,2] This common growth advantage shared by all cancers and its recurring features has become the focus of study for many involved in the discipline of oncology research.[2] Uncontrolled proliferative signaling from growth factors leads to a loss of homeostasis and disruption of tissue architecture.[1] For example, CD28, a prominent co-stimulatory receptor on T cells involved in T cell proliferation, was found to have a recurrent mutation in 11.3% of angioimmunoblastic T cell lymphomas sampled and 1 case of peripheral T cell lymphoma not otherwise specified.[3] Another important mechanism, programmed cell death has been well established as a natural protection from cancer development,[4] yet cancer cells are able to evade internal apoptotic signals generated by cellular stress.[1] BCL2 has a prominent role in blocking apoptosis and has a well-known gene translocation within the immunoglobulin loci in follicular lymphoma.[5,6] Similarly, this translocation can also be found in 20 - 30% of diffuse large B cell lymphomas.[7] For transformation to occur, there must be multiple 'hits' or mutations effecting regulation of the cell cycle. Likewise, tumors do not develop in isolation and also depend on the microenvironment for pathogenesis.[1] Inflammation within tumors can have opposing effects, either tumor eradication or tumor promotion. Inflammation may promote production of growth factors leading to cell proliferation, enhanced survival factors, or enzymes which can facilitate invasion.[8,9] Immunomodulation can also have a growth promoting effect. The immune

response may be altered to release cytokines which support tumor-promoting macrophages and neutrophils while suppressing the inflammatory response.[8,10] Our work was focused on the cell-of-origin and cellular functions related to mechanisms of proliferation, evasion of apoptosis, and shifting of the microenvironment as means to cancer development in a subtype of peripheral T cell lymphoma**.**

Peripheral T cell lymphoma

Lymphomas are a broad group of diseases encompassing a number of different subtypes with highly variable prognoses. Categorization of the specific subtype entails a number of diagnostic tests in order to accurately predict survival and identify the best treatment options. The most often employed classification system is the World Health Organization (WHO) classification of lymphoid neoplasms. Classification relies on using all available information including cellular morphology, immunophenotype, genetic features, and clinical presentation.[11] This classification system has been adapted to canine lymphomas with an overall accuracy of 83% between 17 international veterinary pathologists.[12] The first step in classification requires determining if the neoplasm is derived from an immature or mature cell and then determining B or T; or non-B, non-T cell lineage. T cell lymphomas are a diverse group of non-Hodgkin lymphomas (NHL) accounting for the minority of hematopoietic neoplasms compared to B cell lymphomas. Peripheral T cell lymphomas (PTCLs) are composed of a variety of subtypes of mature T cell lymphomas accounting for 15% of all NHL in humans[11] and 15% - 30% of all lymphomas in dogs.[12-16] Up to 50% of PTCLs cannot be further characterized in humans and are referred to as PTCL – not otherwise specified (PTCL-NOS).[11] Heterogeneity of PTCLs hinders identification of molecular mechanisms of disease. Furthermore, their relative

rarity in humans limits pharmaceutical interest from a cost benefit standpoint resulting in an absence of tumor models.[17] T cell signaling through the T cell receptor leads to proliferation and a functional immune response through production of cytokines, cell receptor upregulation, and transcription of genes involved in survival, death, and signaling. It is not surprising that in humans many T cell lymphoma subtypes are associated with signaling molecules that interact with these imperative TCR pathways.[18-20] The pathogenesis of PTCL in humans is slowly becoming more well-defined, but the cell-of-origin for several PTCLs remains to be identified.[17] An in depth understanding of T cell biology is needed to determine the cell-of-origin and possible underlying mechanisms of pathogenesis in these diseases.

T cell Development

T cells develop in the thymus where positive and negative selection occurs to prevent autoreactive T cell responses. From the bone marrow, thymic seeding progenitors arrive in the thymus as the earliest stage of the T cell.[21] T cells begin their development without expression of either subset marker, CD4-CD8- T cells (double negative T cells; DN), or the T cell receptor (TCR).[22,23] TCR rearrangement begins in the DN2 stage with gene rearrangement occurring in the TCR γ, δ, and β loci.[24] Rearrangement continues and if the cell ultimately has a γδ arrangement, T cell fate commitment occurs at this point, whereas, αβ T cells continue development through the β-selection check point.[25] A pre-TCR is formed with a rearranged TCRβ chain and an invariant preTα chain.[26] After pre-TCR signaling, thymocytes upregulate both subset markers, CD4 and CD8, to the double positive (DP) stage.[27] The *TCRA* recombination originates with re-expression of the *RAG* gene resulting in an αβ TCR in which specificity and binding strength for MHC determines positive and negative selection thymocyte fate.[25] Self-

peptide-MHC complexes are presented by thymic epithelial cells, dendritic cells, and fibroblasts to be positively selected for commitment to the single positive (SP) stage of either CD4+ or CD8+ cells.[28] Negative selection follows in the medulla and thymocytes continue on as MHC class I or MHC class II restricted T cells.[29] During selection, SP cells are negatively selected if their TCR shows high-affinity for self-antigens,[28] however, this process is imperfect and there are mechanisms to lessen the degree of auto-reactivity in the periphery.[30] Many transcription factors and signaling molecules are responsible for T cell development involving different stages and compartments of the thymus including Notch-signaling in T cell lineage commitment and development,[31,32] IL-7 in growth and development,[33,34] and GATA3 expression is required for T cell development.[35] The transcription factors: ThPOK, TOX, and GATA3 are involved in CD4 lineage fate, whereas RUNX3 is required for the CD8 lineage commitment.[36] After successful TCR recombination and progression through positive and negative selection, CD4+ T helper (Th) or CD8+ cytotoxic T lymphocytes (CTLs) leave the thymus in a resting, naïve state to circulate through the periphery.[36]

T cell Activation

T cell activation occurs when an antigen presenting cell (APC) containing the antigen bound to major histocompatibility complex (MHC) class I or class II is recognized by the TCR.[37] APCs include macrophages, dendritic cells, and B cells. In addition to the interaction between the MHC and TCR, there are a number of co-receptors involved. Activation only occurs when there is both antigen recognition and a second signal.[38] The costimulatory signal is transmitted through distinct receptors on the cell surface, CD28 being a well-recognized co-receptor. CD28 binds to receptors on antigen presenting cells via CD80 and CD86.[39] Engagement of CD28

generates phosphorylation of tyrosine motifs of the cytoplasmic tail of CD28 through Src-family kinases. Downstream proteins are recruited and activated including PI3K.[40] This signaling cascade is similar to TCR activation which suggests synergy with TCR signaling, rather than being a distinct pathway.[41]

Peptide-MHC recognition by the TCR facilitates phosphorylation of CD3 chains.[42] The Src Kinase leukocyte-specific kinase (Lck) is associated with CD4 or CD8 and phosphorylates immunoreceptor tyrosine-based activation motifs (ITAMs) contained in the CD3 chains.[43] Phosphorylation of ITAMs enables sites for the Syk family kinase, Zeta-activated protein 70 kDa (ZAP-70); recruitment is also initiated by Lck-mediated phosphorylation. The membrane anchored protein, linker for the activation of T cells (LAT) and cytosolic adapter protein Src homology (SH2) domain are targets of ZAP-70 and once phosphorylated function as protein docking sites.[44,45] Effector molecules, such as phospholipase Cy1 (PLCy1) can be incorporated into these docking sites stabilizing communication between the APC and the T cell.[46] Activated PLCy1 has the ability to hydrolyze phosphatidylinositol 4,5–bisphosphate (PIP2), a membrane phospholipid, leading to the production of second messengers: inositol trisphosphate (IP3) and diacylglycerol (DAG).[47] There are a number of transcription factors activated through the TCR including the nuclear factor of activated T cells (NFAT), nuclear factor of kappa-light-chain enhancer of activated B cells (NF-kB) and activator protein-1 (AP-1).[48] The NFAT pathway requires Ca^{2+} which comes from PLCy1 mediated IP3 generation leading to Ca^{2+} influx when Ca^{2+} channel receptors increase permeability in the endoplasmic reticulum.[49] Ca^{2+} is a universal second messenger and increased intracellular Ca^{2+} levels lead to activation of Ca^{2+} and calmodulin-dependent transcription factors through the phosphatase, calcineurin.[50] Calcineurin

can dephosphorylate proteins of the NFAT family, allowing for access to the nucleus and gene expression.[49] AP-1 proteins can partner with NFAT, which can result in particular gene expression patterns.[51] The production of DAG leads to activation of the rat sarcoma (RAS) and protein kinase C theta (PKCθ) pathway. Activated RAS induces the mitogen-activated protein kinase (MAPK) cascade, which can result in the activation of the AP-1 complex.[52] DAG production can also lead to the activation of NF-kB signaling through PKCθ.[52] NF-kB can regulate a wide variety of genes involved in the immune response and T cell growth, survival and differentiation.[53] Normally NF-kB is resting in the cytoplasm associated with (inhibitor of kB) IkB. Phosphorylation of IkB leads to degradation of IkB and release of NF-kB to allow for nuclear localization and gene transcription.[54] AP-1, NFAT, and NF-kB behave in concert, resulting in the expression of interleukin-2 (IL-2).[55] TCR signaling resulting in IL-2 production provides both autocrine and paracrine signals for T cell proliferation through the IL-2 receptor (IL-2R) and JAK/STAT pathways.[56,57]

During the proximal TCR signal, the process is regulated via inhibitory phosphates. CD3 is not directly involved in antigen recognition, but is involved in transmitting the TCR signal through ITAM phosphorylation by the Src-family protein tyrosine kinases such as Lck, Fyn and Zap-70. Phosphorylation of Y505 by C-terminal Src kinase (Csk) maintains Lck in an inactive conformation.[58] Dephosphorylation of this inhibitory Y505 residue of Lck by CD45 tyrosine phosphatase yields auto-phosphorylation at Y394, resulting in the activation of Lck.[58] In some circumstances CD45 can inhibit Lck and other molecules though dephosphorylation of activating residues.[59] The fine tuning of T cell response by CD45 has yet to be completely elucidated,[55] but CD45 does appear to be essential for the TCR response.[60-62] Proliferation and

cytokine production has been shown to be impaired in CD45-deficient T cell clones; however proliferation could be rescued through TCR independent mechanisms such as IL-2 signaling or through exogenous mitogens.[60,63] Additional impairment in CD45-deficient clones included their inability to produce cytokines, and in CD8 T cells, cytolysis has also been shown to be hindered.[60,64]

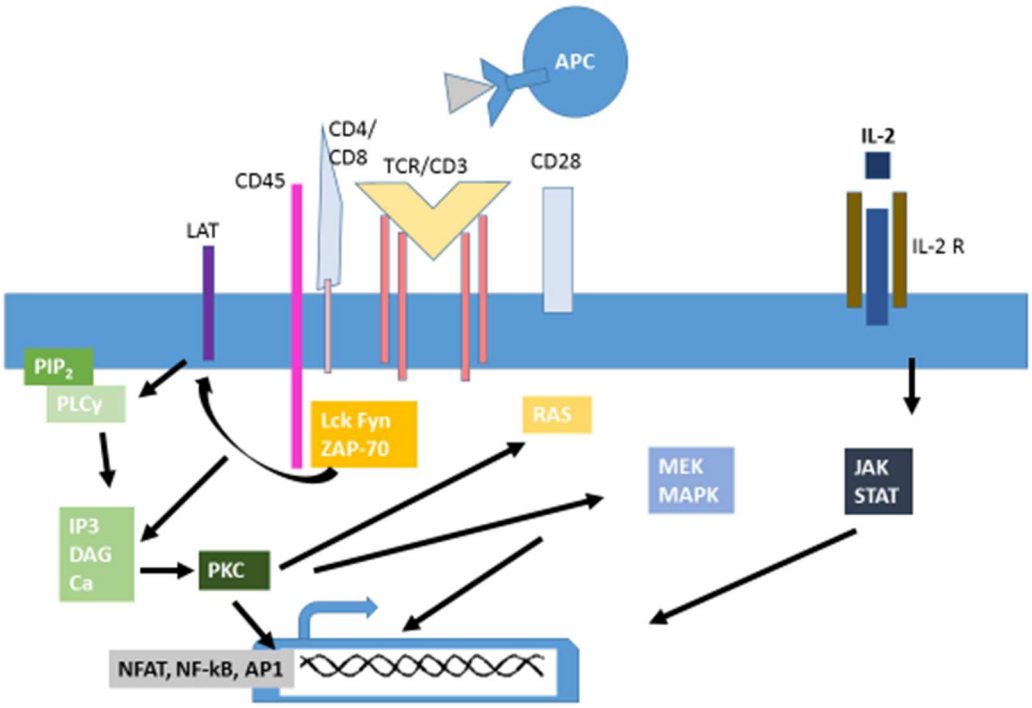

Figure 1. Schematic of T cell activation. T cell activation begins with antigen recognition. The TCR has affinity for antigen presented by the antigen-presenting cell (APC). Costimulation through CD28, along with sustained interaction between the T cell and APC, is facilitated by other connections including CD4 or CD8. These interactions form an immune synapse promoting activation of phosphorylation cascades and docking sites involving Lck, Fyn, ZAP-70 and LAT. PLCγ produces secondary messengers including IP3, DAG, and Ca^{2+} with subsequent activation of multiple pathways such as RAS and MAPK. Ultimately there is release or activation of transcription factors (NFAT, NF-kB and AP-1) in the nucleus for transcription of genes involved in proliferation and effector functions. Upregulation of IL-2 and the high affinity IL-2 receptor propagates the response. IL-2 can act upon the IL-2R and through JAK/STAT proteins to upregulate genes involved in this pathway.

T cell differentiation

Naïve T cells in the peripheral lymphoid tissue undergo differentiation in order to produce an appropriate immune response. The two main branches of T cells are CD4+ Th cells and CD8+ CTLs, which interact with MHC II and MHC I, respectively.[65] The T helper subsets include Th1 and Th2, which were the first discovered T helper subsets,[66] Th17 and Th9 which produce the cytokines IL-17 and IL-9,[67-69] follicular helper (Tfh) with functions in the lymph node follicle[70] and regulatory (Treg) T cells regulate the immune response and control self-tolerance.[71] CTLs are activated by cells that are infected with intracellular pathogens.[72]

Virus or intracellular bacteria can promote Th1 and CTLs through type I IFNs and IL-12 produced in response to these pathogens.[73,74] The expression of the transcription factor, T-bet, which is the master transcription factor for Th1 cells,[75,76] is elicited in these responses. Mice deficient in the IL-12 or the IL-12 receptor have a poor Th1 response and reduced ability to produce the classic Th1 cytokine, IFN-γ.[77,78] T-bet/TBX21 controls expression of IFN-γ and can repress production of the Th2 cytokines, IL-4 and IL-5.[76,77] Similarly Th2 responses are more prominent in mice lacking IL-12,[77] which illustrates the reciprocal nature of promotion and inhibition of Th1 and Th2 cells. Similarly, IFN-γ has been shown to directly inhibit Th2 cell proliferation,[79] as well as to boost Th1 programming through up regulation of IFN-γ receptor mediated STAT1 generated T-bet expression.[80]

Th2 cells are elicited in response to parasites and allergic disease, and are induced by IL-4.[81-83] Th2 cells also produce IL-4, IL-5, and IL-13, inducing alternatively activated macrophages (IL-4, IL-13)[84] recruitment of eosinophils (IL-5),[85] and initiating B cell production of IgG and IgE (IL-4).[86] GATA3 is the major transcription factor of Th2 cells and is upregulated by IL-4 though

STAT6 mediation.[87,88] STAT6 phosphorylation results in gene induction of *GATA3*.[89] STAT6 has been identified as a requirement for activation of 77% of enhancers specific for Th2.[90] However, GATA3 expression can direct Th2 differentiation independently of IL-4 and STAT6.[91] GATA3 can also inhibit Th1 differentiation; for instance, ectopic GATA3 expression in Th1 cells leads to IFN-γ repression and expression of Th2 cytokines.[92] GATA3 expression is induced by IL-2/STAT5[93] and through Notch pathways,[94] which are also involved in Th2 differentiation. Another important Th2 transcription factor is c-Maf, which contributes to peak IL-4 production and increased levels of CD25 in emerging Th2 cells.[95,96]

Th9 cells develop in conditions characterized by TGF-β and IL-4 and they produce IL-9,[97-99] which is a common γ-chain cytokine involved in parasitic infection[100,101] and allergy.[102] Th9 responses are often in accordance with Th2 cellular reactions delaying identification of this subset. There are several transcription factors that have been identified to be involved in Th9 cells, however, PU.1 appears to be required.[67] GATA3 involvement in Th9 differentiation has shown variable results. *GATA3* mRNA is increased during differentiation, but retroviral expression of GATA3 repressed IL-9 production in Th9 cells;[103] additionally, GATA3 expression was absent in Th9 cells in some studies[98,104] but was identified with intracellular staining in others.[103]

Th17 cells are involved in the clearance of organisms which Th1/Th2 cannot combat successfully. For instance, fungal and extracellular pathogens have been shown to elicit a Th17 response.[105-107] TGF-β and IL-6 have been found to induce naïve T cells to produce IL-17.[108,109] In humans, TGF-β is necessary to induce RORγt which is the major transcription factor for this subset. IL-6 and IL-23 or IL-21 are necessary to allow RORγt to upregulate the transcription of

IL-17.[110] RORγt is necessary for Th17 differentiation and transduction of T cells with this transcription factor induces production of IL-17 in naïve T cells.[111] STAT3 has also been found to be important in Th17 differentation.[112,113] IL-17A and IL-17F produced by Th17 cells leads to activation and recruitment of neutrophils to areas of infection.[114,115] Although TGF-β is necessary for Th17 cell differentiation, high concentrations of TGF-β will shift the environment away from Th17 cells and induce T regulatory (Treg) cell differentiation through the transcription factor, Forkhead box P3 (FoxP3).[116]

Treg versus Th17 cell development has been found to be mutually dependent on communication from the innate immune system.[109] With TCR stimulation in the presence of TGF-β, a naïve cell can express Foxp3 leading to Treg differentiation; however if IL-6 or IL-21 is also present, Th17 cells develop instead.[116] Th17 cells have been implicated in autoimmune disease,[68,69,117] whereas Treg cells inhibit autoimmunity.[71] Treg cells actively downregulate the activation and proliferation of self-reactive T cells; Treg cells are also involved in suppressing excessive inflammation in other immune responses, including T cells recognizing infectious antigens.[71,118-120] Treg cells develop both in the thymus and in the periphery; naïve T cells can differentiate into Treg cells in the presence of IL-2 and TGF-β.[121] Thymus and peripheral derived Treg cells express the master transcription factor, Foxp3,[122-124] which is induced in naïve T cells by high levels of TGF-β.[125] TGF-β has also been shown to inhibit Th1 and Th2 differentiation through down regulation of the differentiation transcription factors, T-bet and GATA3, respectively.[126,127]

Follicular helper T cells (Tfh) are identified as a T cell subset, independent of Th1, Th2, and Th17 with reliance on the master transcription factor, Bcl6.[128-131] The other T helper cell

subsets express high levels of Blimp-1,[128,132,133] which is an antagonist of Bcl6 and vice versa.[133,134] After antigen recognition, T and B cells migrate to the T cell zone-B cell zone border where their interactions increase.[135-138] T cells express high levels of CXCR5 in order to maintain follicular center localization and continued contact with B cells.[128,139,140] Tfh cells are involved in development and maintenance of the germinal center (GC), along with the production of plasma cells and memory B cells.[70,141] This process involves a number of signaling pathways, including survival signals necessary for generating GC B cells dependent on somatic hypermutation with eventual downregulation of proliferation, and replacement by signals for plasma cell differentation.[70] IL-6 and IL-21 have also been implicated in TfH cell development.[129,130] IL-21 is expressed by Tfh cells[139,142-144] but not exclusively.[142,145-147] IL-21 expression leads to plasma cell differentiation and GC B cell proliferation.[148,149] IL-4 is also produced by Tfh cells for the process of B cell help[139,144] and may be involved in B cell survival.

CD8+ T cells interact with MHC class I expressing cells which present cytosolic peptides that have been degraded by proteasomal mechanisms and transported to the endoplasmic reticulum where they are bound to MHC class I molecules and presented on the cell surface.[150] CD8 T cell response in acute infection includes clonal expansion with differentiation into effector and memory cells. CD8 T cells mediate defense against intracellular organisms through secretion of INF-γ, TNF-α[151] and cause cytolysis through perforin and granzyme delivered to target cells.[152,153] Additionally, upregulation of FasL allows for ligation via Fas-FasL causing cell death.[154] After cell expansion and clearing of the pathogen, CD8+ T cells enter a contraction phase whereby < 10% survive to become memory CD8+ T cells with phenotypically increased expression of the IL-7 receptor-α, CD27, BCL2, and decreased expression of killer cell lectin-like

receptor G1 (KLRG1).[155-158] Memory cells are further characterized as central memory CD8+ T cells, which occupy secondary lymphoid organs with proliferative capacity; and effector memory CD8+ T cells which inhabit non-lymphoid tissue and continue to have cytotoxic function.[159] T-bet and eomesodermin (EOMES) are vital transcription factors for function of CD8+ T cells [156,160-162] and are reciprocal in their expression in differentiation. T-bet expression is the highest in early effector CD8+ T cells and then declines, whereas EOMES is upregulated in early effector cells but expression increases with memory differentiation.[156,162] Additional CD8+ effector T cell subsets are non-cytotoxic and promote a Th2 response. There is evidence that IL-4 can induce CD8+ T cells with Th2-like effector functions.[163] Furthermore, CD8+ T cell subsets in human peripheral blood were identified by high expression of IL-6R, expression of GATA3, along with production of IL-5 and IL-13; in patients with asthma, there was increased prevalence of this subset.[164] Additionally, this subset also expresses low levels of terminally differentiated CD8+ T cell genes including T-bet, EOMES, and Blimp-1 with a possible Th2-like polarized state.[164]

Cell-of-origin in lymphoma

In humans, identification of the cell-of-origin for various lymphoma subtypes has shown biologic[165] and prognostic significance,[166,167] as well as elucidating potential interventions for therapy.[168,169] Peripheral T cell lymphomas (PTCLs) lack many of the definitive diagnostic and/or cytogenetic markers found in B cell lymphomas complicating the classification and subsequent investigation of this branch of lymphoid neoplasms.[167] While there are several subtypes of PTCLs including angioimmunoblastic T-cell lymphoma (AITL), anaplastic large cell lymphoma (ALCL), and adult T-cell leukemia/lymphoma identified by WHO classification, 30 - 50% of PTCLs

cannot be further defined and are categorized as PTCL-not otherwise specified (PTCL-NOS).[11] PTCL-NOS are considered a heterogeneous cohort of multiple entities with no reliable criteria for delineating these neoplasms.[16] Gene expression profiling (GEP) of T cell lymphomas has led to advances in classification[170] and identification of the cell-of-origin in some subtypes. For example, AITL and follicular T cell lymphoma (FTCL) have been identified to have a similar GEP leading to a common lineage for these two subtypes, compatible with a T follicular helper cell.[170,171] There is evidence that adult T cell leukemia/lymphoma is derived from a T regulatory cell with expression of CD25 and FoxP3,[172] the T regulatory cell transcription factor. Recently, GEP has identified two major categories of PTCL-NOS with high expression of either GATA3 or TBX21 (T-bet), [167] the transcription factors responsible for Th2 and Th1 differentiation, respectively. Expression of these transcription factors was not only useful for categorization of PTCLs-NOS, but was also prognostically important.[167] Through gene expression, most PTCLs-NOS have been more closely related to a mature CD4+ Th cells with a marginal number of cases with gene patterns more similar to CD8+ CTL.[173] Derivation from mature T cells is supported by variable function and expression of the αβ T cell receptor (TCR).[171]

In dogs, investigation into the cell-of-origin of lymphoma subtypes has been limited. Gene expression profiling of canine diffuse large B cell lymphoma (DLBCL) revealed a similar pattern to that seen in human diffuse large B cell lymphoma.[174] In humans, DLBCL is derived from germinal center B cells and activated B cells.[175,176] The gene expression pattern in canine DLBCLs was consistent with germinal center B cells and post-germinal center B cells similar to the findings with humans.[174] T cell lymphomas are more common in dogs than in humans, but

are still less common that B cell tumors, accounting for nearly 30% of all lymphomas in dogs.[12] Still, molecular investigations involving canine T cell tumors is very limited.[177,178]

Dogs as a natural model for cancer

While mice are a common model for studying human disease genetics because of the ease and degree to which these animals can be modified; for cancer, this model is hindered by the lack of spontaneous disease development compared to humans.[**179**] Dogs, on the other hand, live in the same environment as their humans with > 40% of households in the US having at least one dog.[180] Further, dogs receive a high level of health care. In dogs over 10 years old, cancer is the leading cause of death.[181,182] Dogs provide an obvious system for studying spontaneous disease development and cancer growth within an intact immune system.[183] Many types of naturally occurring cancers in dogs overlap with human disease in histomorphology, genetics, biologic behavior, and therapeutic response.[184] There are nearly 400 dog breeds with variable affinities for specific diseases and their associated mutations.[179,185] In fact, 41 naturally occurring dog diseases have homologous human genes associated with similar disease phenotypes in humans.[186] Canine genome mapping has also identified marked genetic similarities between dogs and humans.[187,188] Breed associated diseases suggests there is a limited number of loci involved in each disease, which presents an advantage for determining underlying genetic susceptibility, when compared to humans.[189,190] Lymphomas are one of the more common tumors in dogs[191] and several breeds have been identified to have a higher relative risk for developing various lymphoma subtypes.[192] For instance, Boxers have a predisposition to the developing an aggressive CD4 T cell lymphomas,[193-196] while American Golden Retrievers have a high incidence of T zone

lymphomas.[197] The incidence of canine non-Hodgkin lymphoma is similar to that seen in humans.[198,199] Canine lymphomas have similar morphology to their human counterparts with high reproducibility and adaptability of the human WHO classification scheme for lymphomas in dogs.[12-14]

T zone lymphoma in dogs

While PTCL in dogs is likely a heterogeneous groups of diseases, specific characterization of several T cell lymphomas in dogs has aided in the identification of distinct subsets of T cell lymphomas in dogs.[15,194,196,200,201] The most common types of T cell lymphomas in dogs are PTCL-NOS and T zone lymphoma (TZL).[12,16] TZL occurs rarely in humans, but shares a similar histomorphology with the canine disease.[202,203] In dogs, TZL accounts for 4-13% of all lymphomas.[12,15] TZL has a unique histologic pattern with compression of follicles by the expanded paracortex (T zone) and infiltration of the medullary cords by neoplastic cells with maintenance of nodal architecture.[203] The cells are small to intermediate in size with folded or indented nuclei and indistinct nucleoli; mitoses are rare.[12,203] Because the histologic evolution associated with this disease retains follicles and the pattern is nodular,[204] accurate diagnosis may be challenging in the earlier stages and fine needle aspiration may be equivocal in distinguishing malignant TZL from benign lymphocytes.[12] Due to phenotypic abnormalities, this disease can be reliably diagnosed using flow cytometry.[197] The neoplastic cells can express either or both subset antigens, CD4+ and CD8+, or neither,[197,200,205] but consistently do not express CD45 protein which has been verified by flow cytometry[197,205] and histopathology recognizing multiple CD45 clones.[206] Additionally, CD45 gene expression[206,207] is absent in TZL, even though CD45 DNA can be found.[206] Other aberrant phenotypic characteristics include

increased expression of the activation markers: CD21,[178,197,205] CD25,[197,208] and class II MHC.[197] Clinically, systemic lymphadenopathy is reported,[208,209] even when clinical illness is absent.[12,203] The median age at diagnosis is 9 -10 years old in dogs[197,200,203,204] and peripheral blood involvement is common.[197,200,204] In a study on nodal TZL, all cases with available blood had evidence of aberrant TZL cells in the peripheral blood even when there was a normal lymphocyte count.[197] TZL is considered indolent and survival times of 622 to >1000 days have been reported.[15,197,200,204] This disease has also been reported extranodally, in the tongue, with most cases also showing indolent behavior.[210] The best treatment for this disease has not been systematically determined. There is evidence that the common multidrug chemotherapy used in many types of canine lymphoma does not improve survival.[15] In a couple of case series, 10-50% of dogs with TZL also had demodicosis[204,208] which has been associated with immunosuppressive diseases in adult dogs.[211] Additionally in 10% of cases of TZL, a separate tumor was reported,[200] suggesting there is a clinically significant degree of immunosuppression in at least a subset of TZL patients.

Although TZL appears to be relatively rare in humans,[212] the phenotypic features and indolent clinical course in dogs provide an opportunity for studying early and late phases of pathogenesis of a lymphoid neoplasia. In humans, there are multiple B and T cell clonopathies identified by their phenotypic abnormalities even though progression to disease is rare. These include monoclonal gammopathy of undetermined significance (MGUS), monoclonal B cell lymphocytosis (MBL), and T-cell dyscrasia of undetermined significance or T cell clones of undetermined significance (TCUS). MGUS is found in ~4% of Caucasian individuals over the age of 50 and there is an average risk of developing multiple myeloma of 1% per year.[213,214] In

MGUS, the phenotype includes expression of CD138+/CD38+, which is expressed on normal plasma cells but there is usually one or all of the following additional, aberrant phenotypic characteristics: CD19-, CD45-, or CD56+.[215,216] MBL is also an age related asymptomatic phenomenon characterized as an expansion of B cells with the same phenotype as CLL (CD5+, CD19+, CD23+, CD20+, and sIg dim), with an overall risk of transformation to CLL of 1 – 2% per year.[217] A possible precursor or benign counterpart to cutaneous T cell lymphoma was identified as a monoclonal expansion of CD3+CD4+CD7-CD26- lymphocytes in the peripheral blood in patients with erthroderma.[218] In another T-cell dyscrasia, clonality was defined by Vβ expression. T cell subset markers were varied, similar to canine TZL, with cases being either single positive CD4+ or CD8+, having co-expression of CD4+ and CD8+, or negative for both subset markers: CD4-CD8-; the most common feature was decreased or absent CD5.[219] These phenotypic aberrancies allow for screening for these syndromes and prompt investigation into risk factors associated with disease development. Distinct, early pathogenic events have been identified in MGUS and multiple myeloma but how these events contribute to different stages of disease is still not well understood.[220] Prediction of disease development in MBL has improved by stratifying individuals into high and low B cell count subgroups.[221] High-count MBL individuals have a higher risk of serious infection than progression to CLL,[222] highlighting the need for investigation of cellular function and pathogenesis in this syndrome in order to optimize patient management. Genetic factors in familial diseases play a role in determining relative risk for both MBL and MGUS.[223-225] TZL has been identified to be highly prevalent in American Golden retrievers;[197] this breed susceptibility suggests an inherited component for this disease. TZL may have a similar pre-neoplastic state and genetic risk as these human

entities and phenotypic abnormalities afford the prospect for early identification and investigation of pathogenic stages of this disease.

Conclusion

A fundamental principal of cancer development is uncontrolled cell growth. The pathogenesis of PTCL is still under investigation but various TCR signaling mutations have been identified. Determination of the cell-of-origin in PTCL subsets has already provided more accurate prognostic information. Identification of the cell-of-origin has led to better understanding of the mechanism of T cell activation and improved patient management through targeted therapeutic options. Dogs are an excellent natural model for studying lymphoid neoplasia and T zone lymphoma is the second most common T cell tumor in dogs. TZL is a slowly progressive disease with unique phenotypic features allowing for early identification and rich investigation into the mechanisms of pathogenesis at various stages of this disease. We intended to answer fundamental questions related to TZL cellular function as it contributes to pathogenesis. We asked:

1. What is the cell-of-origin for TZL?
2. What is the driving mechanism for proliferation in this disease?
3. Is there *in vitro* evidence for immunosuppression in this disease?
4. Is there a pre-neoplastic state in TZL and are Golden retrievers predisposed to carry a pre-neoplastic phenotype?

We were ultimately able to characterize a molecular signature for TZL which is suggestive of Th2-like cells with highly expressed regulatory molecules. Further, TZL's proliferative capacity is TCR independent, with lacking CD45. Despite expression of immunosuppressive molecules and

clinical evidence of immunosuppression in TZL, suppression of normal T cells within the tumor microenvironment was not observed *in vitro*. Lastly, while Golden retrievers do have an increased prevalence of TZL cells in their peripheral blood, there is still very low incidence of progression to disease.

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CHAPTER 1: DETERMINING THE CELL-OF-ORIGIN OF TZL

Summary

Gene expression profiling in human lymphoproliferative disorders has identified targets for therapy as well as beginning to differentiate subtypes of peripheral T cell lymphoma by cell-of-origin. T zone lymphoma (TZL) is a subtype of peripheral T cell lymphoma and is the second most common T cell lymphoma in dogs. We sought to investigate the cell-of-origin of TZL by investigating over 250 genes involved in T cell differentiation. We found increased expression of GATA3, the master Th2 transcription, factor and the Th2 specific receptors, CRTh2, CCR3, and CCR4 in TZL cases compared to sorted CD4+ and CD8+ T cell controls from healthy dogs. We validated gene expression of GATA3 and the expression of immunosuppressive molecules, galectin-1 and galectin-3 with immunohistochemistry. These findings suggest that TZL is derived from a Th2-like cell with expression of immunomodulatory molecules.

Background

Lymphoproliferative disease encompasses a large, assorted group of neoplasms derived from normal lymphocyte counterparts. In humans, in lymphoma subtypes in which the cell-of-origin has been identified, there have been improvements in prognostic information,[1,2]as well as guiding potential interventions for therapy.[3,4] Peripheral T cell lymphomas (PTCLs) have fewer cytogenetic markers than B cell lymphomas impeding differentiation of this division of lymphoid neoplasia.[2] In humans, PTCLs are much less common than B cell lymphomas, accounting for only ~12% of non-Hodgkin lymphomas internationally.[5] There are several subtypes of PTCLs within the World Health Organization (WHO) classification scheme of

lymphoid neoplasms including: angioimmunoblastic T-cell lymphoma (AITL), anaplastic large cell lymphoma (ALCL), and adult T-cell leukemia/lymphoma.[6] The majority of PTCLs are categorized as PTCL-not otherwise specified (PTCL-NOS) due to inability to further differentiate subtypes.[6] More recently, gene expression profiling (GEP) of T cell lymphomas has led to advances in classification,[7] and determination of the cell-of-origin. GEP has identified two major categories of PTCL-NOS demonstrating high expression of either GATA3, the master transcription factor for Th2 cells or T-BET, [2] the master transcription factor for Th1 cells. Further, expression of these transcription factors not only distinguished PTCLs-NOS, but also had prognostic significance.[2]

T zone lymphoma (TZL) is a neoplasm of the blood and lymph nodes that has an indolent clinical course in dogs.[8,9] In the lymph node, TZL is associated with an expansion of T cells in the paracortex or "T zone" which compresses germinal centers.[10] Normally T cells migrate through the T zone to interact with dendritic cells as part of the immune response.[11] The T cells migrating through this area can be of various subtypes. Naive T cells develop in the thymus prior to differentiation; immature T cells undergo positive and negative selection in the thymic cortex and medulla to become MHC class I or class II restricted.[12] Positively selected T cells differentiate to become CD4 T helper (Th) or CD8 cytotoxic T lymphocytes (CTLs) and single positive cells leave the thymus to circulate in the periphery.[13] In the periphery, T cells are dormant until they encounter antigens or activating signals.[13] T cell survival is dependent on activation of the TCR by co-stimulatory molecules and cytokines, including IL-2.[13]

In the case of naïve CD4+ T cells, master transcription regulators signal Th cell differentiation,[14] and cytokine signaling influences the appropriate transcription factor for the

optimal immune response. Classically Th1 cells are induced by IFN-γ and IL-12 and produce IFN-γ participating in elimination of intracellular pathogens along with activating mononuclear cells.[15] Whereas Th2 cells produce IL-4, IL-5, and IL-13 and are involved in responding to parasitic infections and forming the allergic response.[16,17] Other, well characterized Th subsets include, Th17 cells which utilize several signaling cytokines including: IL-17, IL-21, IL-23, and TGF-β; retinoic acid receptor-related orphan receptor gamma-T (RORγt) is the master transcription factor of Th17 cells which are involved in inflammation and combating extracellular bacteria and fungi.[18] TGF-β is the major initiating cytokine and FOXP3 is the major transcription factor for T regulatory (Treg) cells which help regulate the immune response.[19,20] T follicular helper (Tfh) cells signal to B cells in the germinal center and are favored by production of IL-6 and IL-21;[21,22] BCL-6 is the major transcription factor for this lineage.[23] Th17, Treg, and Th2 cell are capable of plasticity in differentiation depending on cytokine signals dictating conversion to different phenotypes.[24] For example, in the presence of TGF-β and IL-6 the response can be skewed toward Th17 proliferation, whereas TGF-β in the presence of IL-10 shifts naïve T cells toward Treg differentiation. Additionally, TGF-β is also involved in Th9 differentiation in the presence of IL-4,[25] which was originally thought to be a reprogramming of Th2 cells.[26]

In dogs, identifying the cell-of-origin responsible for various lymphoma subtypes has been limited. Gene expression profiling of canine diffuse large B cell lymphoma (DLBCL) cells has shown a pattern similar to what has been seen with human DLBCL counterparts.[27] Investigations looking at the cell-of-origin of T cell lymphomas in dogs has not been performed and there are few studies reporting molecular profiles.[28,29]

We sought to explore the cell-of-origin of TZL lymphoma through the interrogation of genes involved in T cell differentiation. Using NanoString technology, we investigated the gene expression profile of TZL in comparison to normal sorted CD4+ T cells and CD8+ T cells in dogs. We hypothesized that TZL is derived from an activated, mature T cell counterpart based on phenotypic aberrancies.

Methods

Selection of cases and controls subjected to cell sorting for gene expression analysis

To investigate the gene expression signature of TZL as it relates to T cell differentiation, we compared the expression levels of mRNA from TZL samples and controls. A diagnosis of TZL required greater than 60% of the lymphocytes in the lymph node characterized by a TZL phenotype (CD5+CD45-) with lymphadenopathy or ≥ 5000 TZL cells/μL in the peripheral blood of dogs with lymphocytosis. Case samples included peripheral blood (25 samples; 7 CD4+, 10 CD4-CD8-, 8 CD8+) and lymph nodes (11 samples; 3 CD4+, 2 CD4-CD8-, 6 CD8+) from dogs diagnosed with TZL by flow cytometry through the Colorado State University Clinical Immunology (CSU-CI) laboratory during the time period from December 2013 to March 2015 as previously described.[30] The control samples consisted of CD4+ and CD8+ sorted T cells from the lymph nodes (11 samples) and thymus (5 samples) of healthy, hound mix dogs utilized as normal controls for a series of gene expression studies aimed at profiling T cell lymphoproliferative disease.[30]

TZL cells were harvested from blood or lymph nodes of known TZL cases, as previously described.[30] TZL cells were purified from samples by magnetic depletion of CD45-expressing cells based on the Miltenyi Biotec protocol utilizing MACS buffer. The samples were loaded

onto an LD column suspended in a MidiMACS separator and unbound TZL cells (CD45- cells) were collected.[30] Anti-CD45-PE (pan-leukocyte, clone YKIX716.13, Bio-Rad [AbD Serotec], Hercules, CA) was the primary antibody and the sample was further depleted by anti-PE beads (Miltenyi Biotec, San Diego, CA). Purity of the CD45- unbound cells was then confirmed by flow cytometry. For control samples, cells were purified from the lymph nodes and thymus of healthy dogs by fluorescence-activated cell sorting on a MoFlo cell sorter (Beckman Coulter, Brea, CA) as previously described.[30] The cells were stained with the following antibodies purchased from Bio-Rad: anti-CD18-PE (Human CD18, clone YFC118.3), anti-CD5-FITC (T cells, clone YKIX322.3) and either anti-CD4-Pacific Blue (PB) (CD4 T cells, clone YKIZ302.9), or anti-CD8-FITC (CD8 T cells, clone YCATE 55.9).

Gene expression analysis

To investigate the cell-of-origin of TZL, we measured the expression of 195 genes involved in the differentiation of T cell subsets using a custom-designed codeset. An additional subset of 68 genes involved in various pathways of Th2 cells, GATA3, galectin, and T cell tumors in dogs and humans were subsequently investigated. Expression of these genes was quantified by NanoString technology (NanoString Technologies, Seattle, WA) using the nCounter Digital Analyzer (4.0), through the University of Arizona Genetic core. These genes were selected following literature review of gene expression studies characterizing T cell subsets. Genes of interest for this study were identified from investigations characterizing T regulatory cells,[31] differentiation of central and effector memory CD8+ T cells,[32] CD4+ and CD8+ T cell differentiation,[33,34] Tfh cell differentiation,[35] and Th17 cell differentiation.[36] The additional set of 68 genes was included to obtain more information about genes involved in Th2

differentiation and signaling,[37,38] GATA3 target genes in PTCL in humans,[2,39] and canine T cell lymphomas.[28] Assimilation of these references led us to categorize sets of genes into T subsets in order to look at cell type scores and gene set enrichment using nSolver advanced analysis 2.0 and gene set enrichment analysis (GSEA), respectively. Raw counts were normalized to 6 housekeeping genes: GUSB, SDHA, HPRT1, EEF1G, TBP, and POLR2A. Counts of +/- 3 standard deviations below the negative controls were excluded from heat map analysis.

Immunohistochemistry

Immunohistochemistry was performed on lymph node samples from known TZL cases. We compared these findings to cases of known lymphoid hyperplasia. We noted the expression of GATA3, galectins-1 and -3, and multiple myeloma oncogene 1/interferon regulatory factor 4 (MUM-1/IRF-4). Immunostaining was performed using the BOND-III Fully Automated IHC and ISH stainer. Immunohistochemistry for GATA3 (Invitrogen, Waltham, MA, monoclonal mouse, clone 1A12-1d9, 1:500), galectin-1 (Invitrogen, polyclonal rabbit, C-terminal region of galectin-1, 1:50), galectin-3 (Abcam, Cambridge, MA, polyclonal rabbit, clone synthetic peptide ab31706, 1:150) and MUM-1/IRF-4 (Biocare Medical, Concord, CA, monoclonal rabbit anti-human MUM-1, clone BC5, 1:75) was performed on 5 μm paraffin embedded lymph node sections.

Statistical analysis

Differential gene expression was determined using nCounter advanced analysis 2.0. The Benjamini-Yekutieli test was used to determine the False Discovery Rate. Significant genes were determined by an FDR adjusted p-value of < 0.05. The cell type scores were calculated by taking the average of log2 expression of the relative characteristic genes for each subtype in TZL cases, compared to controls. We also employed GSEA as an additional method looking for substantial

differences in our gene subsets.[40] Significant enrichment was identified by FDR < 25% with GSEA using a permutation test 1000 times. To determine the significance in Th transcription factors and Th2 genes in all samples for the first set of genes and GATA3 expression and CRTh2 expression in CD8+ cases versus CD8+ controls, the data was analyzed and R and log2 transformed; a t-test was performed with Benjamini Hockberg adjustment for multiple tests. Unsupervised hierarchical clustering was performed with nSolver 4.0 using the Euclidean distance which calculates the distance between two samples or genes as the square root of the sum of squared differences in their log count values.

Results

TZL patients have a unique immunosignature that is distinguishable from healthy dogs and independent of phenotype

TZL cases displayed a common profile that was distinguishable from normal CD4+ and CD8+ T cells when samples from the lymph node and thymus were analyzed (see first branching in the top of the dendrogram; Fig 1.1). The controls readily clustered based on phenotype (CD4+ vs. CD8+ (Fig 1.1). However, the TZL cases were not distinguishable by phenotype and were instead intermingled (CD4+ vs. CD8+ vs. CD4-CD8-) (bottom of dendrogram; Fig 1.1). Based on dissimilar branching with unsupervised hierarchical clustering of TZL cases and controls (CD4+ and CD8+) we pooled control CD4+ and CD8+ T cells compared to all TZL cases to determine differential expression. There were 127 genes differentially expressed in all TZL cases versus all controls with an adjusted P value of < 0.05 (Table 1.1).

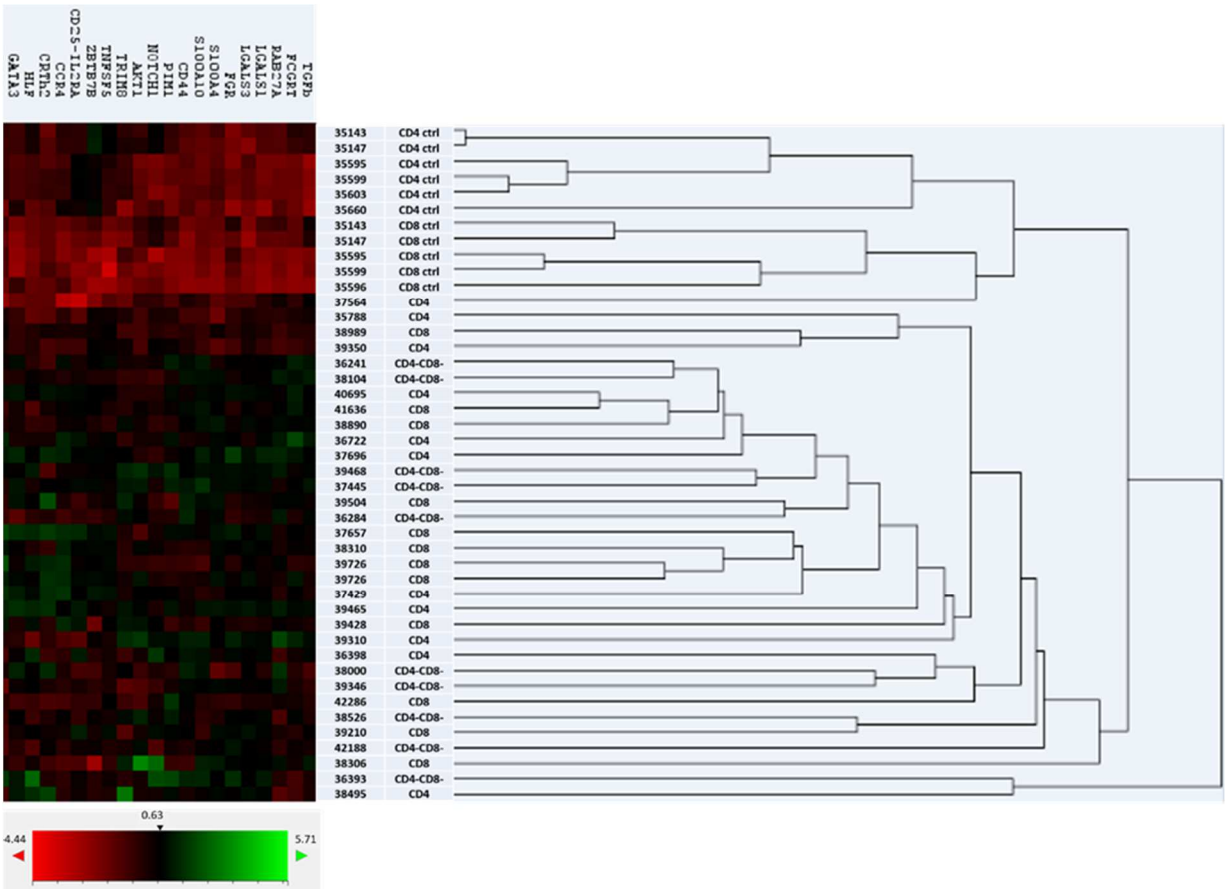

Figure 1.1. TZL patients have a unique immunosignature. Unsupervised hierarchical clustering of control CD4+ and CD8+ T cells compared to all TZL cases. Red indicates decreased expression and green indicates increased expression. The controls readily cluster based on phenotype (1st branch; CD4+ top vs. CD8+ bottom). The phenotypes of the TZL cases are intermingled, failing to cluster (2nd and 3rd branch). This heat map is showing a small portion of the genes that were expressed at high levels in cases compared to controls. The figure highlights the branching of cases and controls.

Table 1.1. Differential gene expression in TZL. The gene symbol, Log2 fold change of cases vs. controls, standard error, P-value, adjusted P-value based on the Benjamini-Yekutiedli adjustment with multiple comparisons, probe.ID for probe design of each gene.

	Log2 fold change	std error (log2)	P-value	BY.p.value	probe.ID
PECAM1	6.32	0.622	7.02E-13	1.72E-10	XM_848326.1:1355
LGALS3	5.65	0.343	5.79E-20	1.14E-16	NM_001197043.1:575
TIMP1	5.64	0.576	2.15E-12	3.52E-10	NM_001003182.1:145
S100A4	4.17	0.406	4.95E-13	1.39E-10	NM_001003161.1:247
LGALS1	3.37	0.243	2.59E-17	2.40E-14	NM_001201488.1:160
MYCT1	3.12	0.525	4.78E-07	2.19E-05	XM_849728.3:321
CRTh2	2.74	0.44	1.77E-07	8.68E-06	NM_001048107.1:459
S100A10	2.53	0.246	4.67E-13	1.39E-10	XM_003432257.2:1075

ITGAM	2.46	0.568	8.92E-05	0.00216	XM_005621235.1:3260
CCR4	2.37	0.504	2.83E-05	0.000795	NM_001003020.1:865
NFIL3	2.33	0.486	2.16E-05	0.000662	XM_003638793.3:1131
NELL2	2.24	0.478	2.83E-05	0.000795	XM_005636954.1:2175
HPGD	2.22	0.462	1.95E-05	0.000627	XM_005635643.1:2135
IL18RAP	2.16	0.456	2.55E-05	0.000748	XM_538448.2:1835
LHFPL2	2.05	0.578	0.000971	0.0169	XM_005618223.1:1475
TNF	1.91	0.487	0.00032	0.00617	NM_001003244.4:185
CCR3	1.89	0.457	0.000165	0.00364	NM_001005261.1:515
HLF	1.88	0.38	1.30E-05	0.000434	XM_005624698.1:755
SLC40A1	1.86	0.407	4.32E-05	0.00113	XM_005640439.1:605
CD44	1.74	0.162	1.49E-13	7.30E-11	XM_851912.1:453
PTAFR	1.66	0.392	0.000127	0.00288	NM_001287047.1:1371
PLD1	1.65	0.527	0.00311	0.0481	XM_005639954.1:2355
IFIT1	1.64	0.401	0.000195	0.00407	XM_843271.3:260
BHLHB2	1.56	0.303	6.77E-06	0.000237	XM_541795.4:1920
TNFRSF1B	1.5	0.346	8.90E-05	0.00216	XM_005617982.1:200
A2A	1.47	0.32	3.80E-05	0.00101	NM_001003278.1:1525
GATA3	1.43	0.235	3.06E-07	1.47E-05	XM_844060.1:1148
FCGRT	1.41	0.165	9.68E-11	1.19E-08	XM_014118885.1:1280
TNFSF5	1.36	0.392	0.00124	0.021	NM_001002981.1:1037
MYO6	1.32	0.348	0.000466	0.00866	XM_005627548.1:2255
IL10RA	1.29	0.236	2.19E-06	8.29E-05	XM_005620306.1:690
FGR	1.28	0.157	3.30E-10	3.61E-08	XM_544467.4:2847
NFATC2	1.15	0.117	2.00E-12	3.52E-10	XM_005635184.1:1315
TGFb	1.15	0.167	2.15E-08	1.51E-06	NM_001003309.1:733
TGFBR3	1.12	0.318	0.00104	0.018	XM_005621935.1:4115
RAB27A	1.1	0.136	4.72E-10	4.57E-08	NM_001048130.1:640
PPARG	1.06	0.325	0.00224	0.0361	NM_001024632.2:1005
FASLG	1.06	0.341	0.00322	0.0494	XM_848916.2:1315
PIM1	1.04	0.145	7.68E-09	6.28E-07	NM_001146177.1:710
ZC2HC1A	0.998	0.173	8.82E-07	3.85E-05	XM_005638063.1:525
UBXN2B	0.992	0.265	0.000547	0.00995	XM_535083.5:2410
DBF4	0.876	0.279	0.00307	0.0478	XM_532451.5:1020
PENK	0.867	0.204	0.00012	0.00273	XM_003640011.3:861
IFNGR1	0.86	0.202	0.000113	0.00268	XM_003638758.2:1290
NOTCH1	0.839	0.221	0.00046	0.00866	XM_005625433.1:3623
XBP1	0.828	0.204	0.000209	0.00432	XM_849540.1:1565
TRAF1	0.798	0.212	0.000528	0.00969	XM_850435.1:795
IL2	0.791	0.209	0.000467	0.00866	NM_001003305.1:70
MAP2K1	0.776	0.167	3.37E-05	0.000908	NM_001048094.1:333

TMEM243	0.747	0.178	0.000133	0.00298	XM_014118895.1:479
SND1	0.712	0.125	1.11E-06	4.66E-05	XM_532436.5:989
IMPDH1	0.615	0.108	1.10E-06	4.66E-05	XM_014119169.1:1448
ITPKB	0.574	0.135	0.000119	0.00273	XM_547504.3:5265
SRI	0.539	0.149	0.000813	0.0144	XM_005628477.2:2419
MYO5A	0.513	0.125	0.000188	0.00397	XM_005638688.1:3485
PSMA1	0.509	0.156	0.00221	0.0358	XM_005633695.2:381
TRIM8	0.507	0.12	0.000118	0.00273	XM_543993.4:933
AKT1	0.421	0.0875	2.00E-05	0.000632	XM_548000.4:1579
COX8A	0.411	0.0935	7.50E-05	0.00186	NM_001252370.1:350
CROT	-0.405	0.0984	0.000174	0.00376	XM_014119108.1:1082
LEF1	-0.482	0.145	0.00181	0.0299	XM_858284.3:955
CNOT2	-0.482	0.149	0.00245	0.0392	XM_005625628.1:785
TCF7	-0.492	0.143	0.00132	0.0222	XM_003639372.2:1860
PTEN	-0.501	0.108	3.25E-05	0.000899	NM_001003192.1:721
CD3e	-0.528	0.112	2.52E-05	0.000748	NM_001003379.1:445
CRLF3	-0.529	0.117	5.18E-05	0.00132	XM_005624833.1:785
GCC1	-0.538	0.142	0.000458	0.00866	XM_539388.5:2646
PITPNC1	-0.557	0.152	0.000708	0.0128	XM_005624306.1:2255
NFIA	-0.57	0.158	8.00E-04	0.0143	XM_005620235.1:1970
SLC25A40	-0.573	0.16	0.000902	0.0158	XM_014118965.1:308
NFATC3	-0.58	0.113	6.65E-06	0.000237	XM_536809.2:2630
Canis_TRBC	-0.581	0.129	5.17E-05	0.00132	Canis_TRBC.1:255
SEP6.	-0.637	0.115	1.72E-06	6.88E-05	XM_014111690.1:800
WASL	-0.641	0.123	5.47E-06	0.000199	XM_532445.5:4165
TGFBR2	-0.645	0.134	1.90E-05	0.000623	XM_005634331.1:2775
DGKA	-0.694	0.136	8.03E-06	0.000272	XM_531626.2:1042
IL6ST	-0.727	0.22	0.00199	0.0326	NM_001287021.1:4745
PRKCA	-0.795	0.203	0.000317	0.00617	XM_005624271.1:3050
DMTF1	-0.796	0.145	2.09E-06	8.16E-05	XM_005628451.2:1009
FBXO22	-0.892	0.141	1.39E-07	7.16E-06	XM_544796.5:1496
STAT4	-0.898	0.228	0.000301	0.00592	XM_005640471.1:1410
MSH6	-0.901	0.192	2.81E-05	0.000795	XM_531814.5:1695
FKBP5	-0.959	0.234	0.000186	0.00397	XM_005627260.1:1085
ASB15	-0.961	0.301	0.00265	0.042	XM_014119083.1:1464
ASPH	-0.989	0.31	0.00267	0.042	NM_001003196.1:606
SATB1	-1.01	0.158	9.99E-08	5.30E-06	XM_005634642.1:1455
LAG3	-1.07	0.223	2.19E-05	0.000662	XM_005637438.1:130
HPS3	-1.09	0.15	6.38E-09	5.70E-07	XM_005634557.1:2220
CCR7	-1.16	0.293	0.000299	0.00592	XM_548131.2:335
IL17F	-1.21	0.307	0.000294	0.00591	XM_538959.1:120

RORC	-1.38	0.271	7.88E-06	0.000272	XM_005630826.1:1840
CDK5R1	-1.43	0.213	3.74E-08	2.29E-06	XM_005624820.1:1700
PRF1	-1.49	0.217	2.28E-08	1.55E-06	XM_005618879.1:1396
TRPS1	-1.61	0.39	0.00017	0.00371	XM_539139.5:3750
CXCR7	-1.68	0.306	2.12E-06	8.16E-05	NM_001003281.2:1312
GCNT1	-1.7	0.32	3.90E-06	0.000145	XM_005615841.1:1346
PTPN22	-1.7	0.366	3.33E-05	0.000908	XM_005630623.2:1535
CCR5	-1.76	0.527	0.00174	0.029	NM_001012342.2:1060
ABCB1	-1.81	0.449	0.000228	0.00466	NM_001003215.1:1425
ICOS	-1.89	0.283	4.00E-08	2.35E-06	NM_001002972.1:635
CHN1	-1.91	0.319	4.06E-07	1.90E-05	XM_845334.3:570
IL10	-2.22	0.324	2.38E-08	1.56E-06	NM_001003077.1:555
ITGAD	-2.24	0.502	5.87E-05	0.00148	NM_001110802.1:2061
CXCR5	-2.32	0.321	6.93E-09	5.92E-07	XM_546496.3:1426
AKAP2	-2.4	0.605	0.000277	0.00562	XM_005626415.1:3520
ITGAE	-2.57	0.734	0.00113	0.0193	XM_005624962.1:2375
TIMP4	-2.6	0.251	4.09E-13	1.39E-10	XM_003432743.2:3130
FOXP3	-2.63	0.263	1.10E-12	2.17E-10	NM_001168461.1:629
ADAM12	-2.66	0.393	3.25E-08	2.06E-06	XM_005637842.1:845
TOX	-2.78	0.475	6.50E-07	2.90E-05	XM_005638006.1:1300
EGR1	-2.8	0.583	2.03E-05	0.000634	XM_846145.3:1787
IFNG	-2.96	0.471	1.60E-07	8.04E-06	NM_001003174.1:430
CD160	-3	0.701	0.000106	0.00253	XM_005630717.1:220
IL21	-3.01	0.374	4.89E-10	4.57E-08	NM_001003347.1:2360
PDCD1	-3.17	0.315	8.91E-13	1.95E-10	XM_543338.2:601
GZMA	-3.28	0.496	5.28E-08	2.97E-06	XM_544335.2:140
PTPRC a	-3.37	0.518	7.39E-08	4.04E-06	XM_005622278.1:278
TNFSF13B	-3.55	0.365	2.56E-12	3.86E-10	NM_001161710.2:865
EOMES	-3.59	0.537	4.07E-08	2.35E-06	XM_845645.3:2095
GZMB	-3.7	0.652	1.17E-06	4.79E-05	XM_547752.2:495
KLRB1	-3.79	0.537	1.19E-08	8.69E-07	XM_005637170.1:325
CTLA4	-4.19	0.521	4.77E-10	4.57E-08	NM_001003106.1:1370
CCL4	-4.46	0.463	3.43E-12	4.81E-10	NM_001005250.1:15
KLRD1	-4.74	0.523	1.98E-11	2.59E-09	NM_001048035.1:169
TRGC2	-4.85	0.352	3.66E-17	2.40E-14	Canis_TRGC2.1:218
PTPRC b	-5.08	0.709	8.63E-09	6.79E-07	XM_005622278.1:1133
CCL5	-5.53	0.674	2.91E-10	3.37E-08	NM_001003010.1:69
GZMK	-6.51	0.913	9.43E-09	7.13E-07	XM_546318.2:195

GATA3, the transcription factor responsible for Th2 differentiation; and CRTh2, a Th2 cell specific receptor were significantly upregulated in cases compared to controls (GATA3: median cases = 2869, median controls = 1223; CRTh2: median cases = 70, median controls = 12; normalized mRNA counts) (Fig 1.2, Table 1.2 and Table 1.3). Furthermore, transcriptions factors responsible for differentiation of other Th subsets, including: TBX21 (T-bet, Th1), RORC (RORγt, Th17), and FOXP3 (Treg), were significantly down regulated in cases versus controls (Fig 1.2, Table 1.2). Th2 specific chemokine receptors, CCR3 and CCR4, were also significantly upregulated in cases versus controls (CCR3: median cases = 20, median controls = 2, CCR4: median cases = 954, median controls = 284; normalized mRNA counts) (Table 1.3). These findings suggest TZL may be derived from a Th2-like cell. However, the cytokines released by Th2 cells, and those responsible for Th2 differentiation: IL-4; IL-5; IL-13; and STAT6, an important transcription factor downstream of IL-4 signaling,[41] were not significantly different between cases and controls (Table 1.3). Most cytokines associated with other T helper subsets were also not significantly different between cases and controls (Fig 1.3) when thymic controls were excluded since cytokine production by differentiated Th cells would occur outside the thymus. Surprisingly, TGF-β, the immunosuppressive cytokine, did show significantly increased expression in cases versus controls (Fig 1.3).

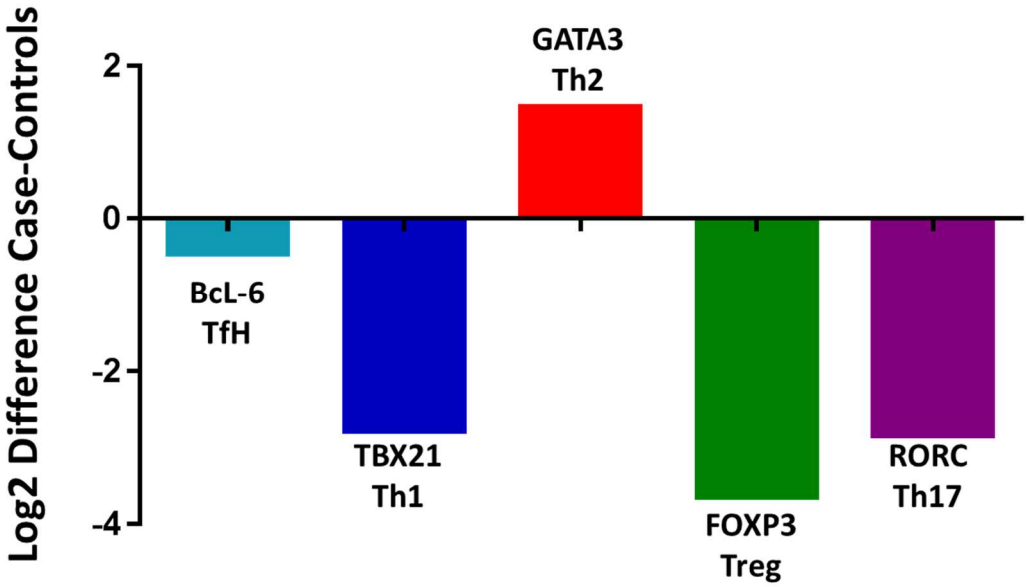

Figure 1.2. The Th2 transcription factor GATA3 is significantly upregulated in TZL. Log2 transformed gene expression difference between cases and controls for transcription factors involved in T helper cell differentiation. GATA3, the Th2 transcription factor, is significantly upregulated in TZL cases (n = 34) versus controls (n = 14).

Table 1.2. Transcription factors involved in T helper differentiation. The median normalized mRNA counts and interquartile range (IQR) for the transcription factors responsible for T helper subset differentiation. The genes with an asterisk were significantly different between cases and controls.

	Cases	IQR	Controls	IQR	P-value
GATA3 (Th2)*	2869	(2029 – 4173)	1223	(555 – 1564)	1.93E-05
TBX21 (Th1)*	27	(3 – 800)	239	(72 – 2404)	0.00367
FOXP3 (Treg)*	2	(1 – 16)	89	(2 – 138)	5.00E-08
RORC (Th17)*	1	(1 – 39)	18	(2 – 52)	0.00014
BCL-6 (TfH)	552	(219 – 5158)	974	(675 – 1610)	0.826

Table 1.3. Expression of additional Th2 genes. The median normalized mRNA expression values for cases and controls and interquartile range (IQR) for the most Th2 specific genes investigated in cases versus controls. CRTh2 is a Th2 cell specific receptor. CCR3 and CCR4 and chemokine receptors involved in Th2 cell signaling. STAT6 is a transcription factor downstream of IL-4

signaling. The genes with an asterisk were significantly different between cases and controls.

	Cases	IQR	Controls	IQR
CRTh2*	70	(36 – 246)	12	(9 – 15)
CCR3*	20	(4 – 51)	2	(1 – 7)
CCR4*	954	(432 – 2006)	284	(75 – 491)
IL4	1	(1 – 4)	2	(1 – 17)
IL5	18	(10 – 22)	9	(5 – 18)
IL13	3	(1 – 4)	1	(1 – 3)
STAT6	2056	(1853 – 22896)	2111	(2009 – 2222)

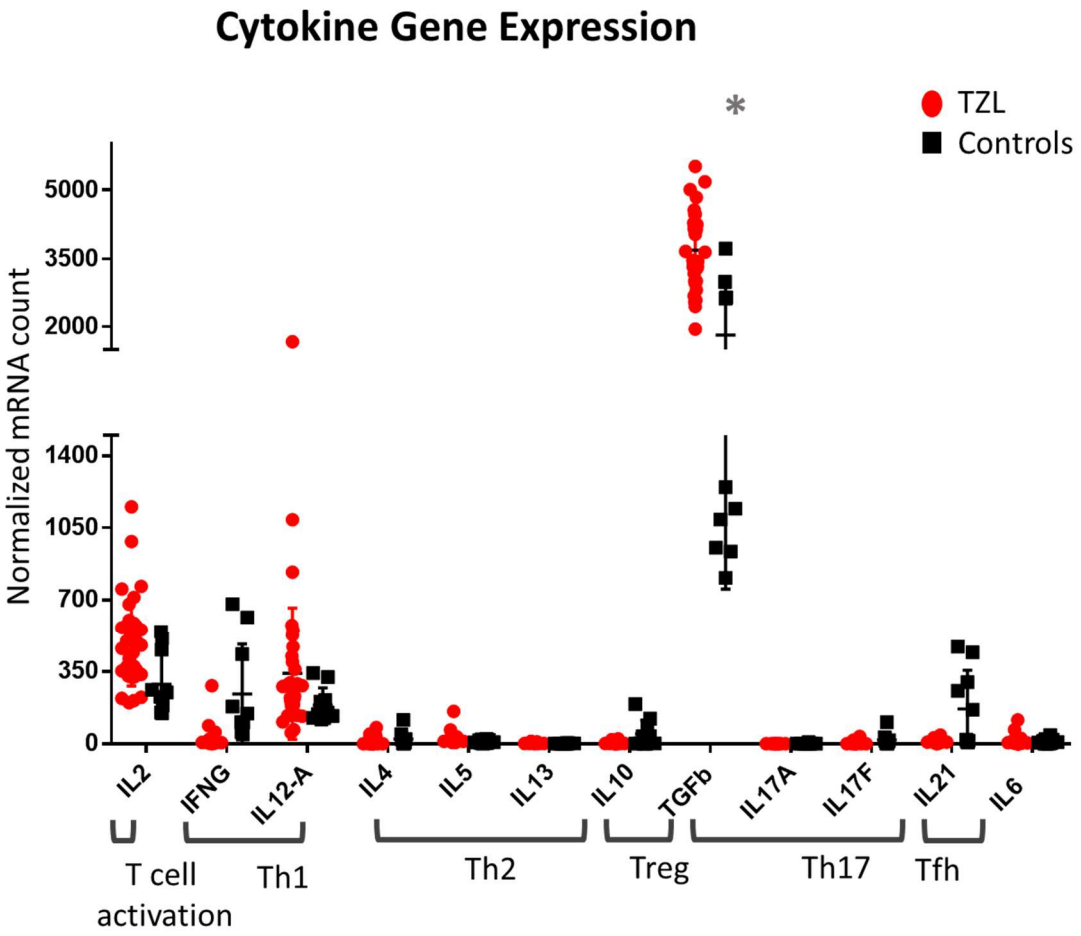

Figure 1.3. The immunosuppressive cytokine, TGF-β, showed increased expression in TZL cases versus controls. mRNA expression values of cytokines involved in T helper cell differentiation and activation for TZL cases (red) and controls (black). The cytokines associated with T helper subsets were not significantly different in TZL cases vs. controls. There was, however, a significant difference in the expression of TGF-β in TZL cases vs. controls. Controls in this comparison consisted only of lymph node tissue because we were focused on cytokines involved in peripheral differentiation.

When comparing only CD8+ TZL cases, we also saw significantly increased expression of GATA3 and CRTh2 compared to CD8+ controls (GATA3 $p = 0.0015$, CRTh2 $p = 3.956 \times 10^{-5}$) with a higher median gene expression than CD4+ controls (Table 1.4). Cases with a CD8+ phenotype did show expression of both CD8a and CD8b genes (alpha and beta chains, respectively), but did not show expression of CD8 effector granzyme molecules: GMZA, GMZB, GMZK, or other effector molecules including perforin and IFN-γ (Fig 1.4). These findings provide evidence that expression of CD8 in TZL cases does not correlate with CD8+ T cell function. Furthermore, the interspersion of various phenotypes across cases suggests CD4+ and CD8+ subtype molecules are not essential to the function of TZL cells.

Table 1.4. Median mRNA normalized count by phenotype. The median normalized mRNA counts for the transcription factor responsible for Th2 differentiation, GATA3 and CRTh2, the Th2 specific receptor, delineated by phenotype

	CD4+ cases	CD8+ cases	CD4-CD8- cases	CD4+ controls	CD8+ controls
GATA3	2989.67	2232.77	4102.7	1534.23	500.3
CRTh2	58.4	61.8	100.3	13.5	9.4

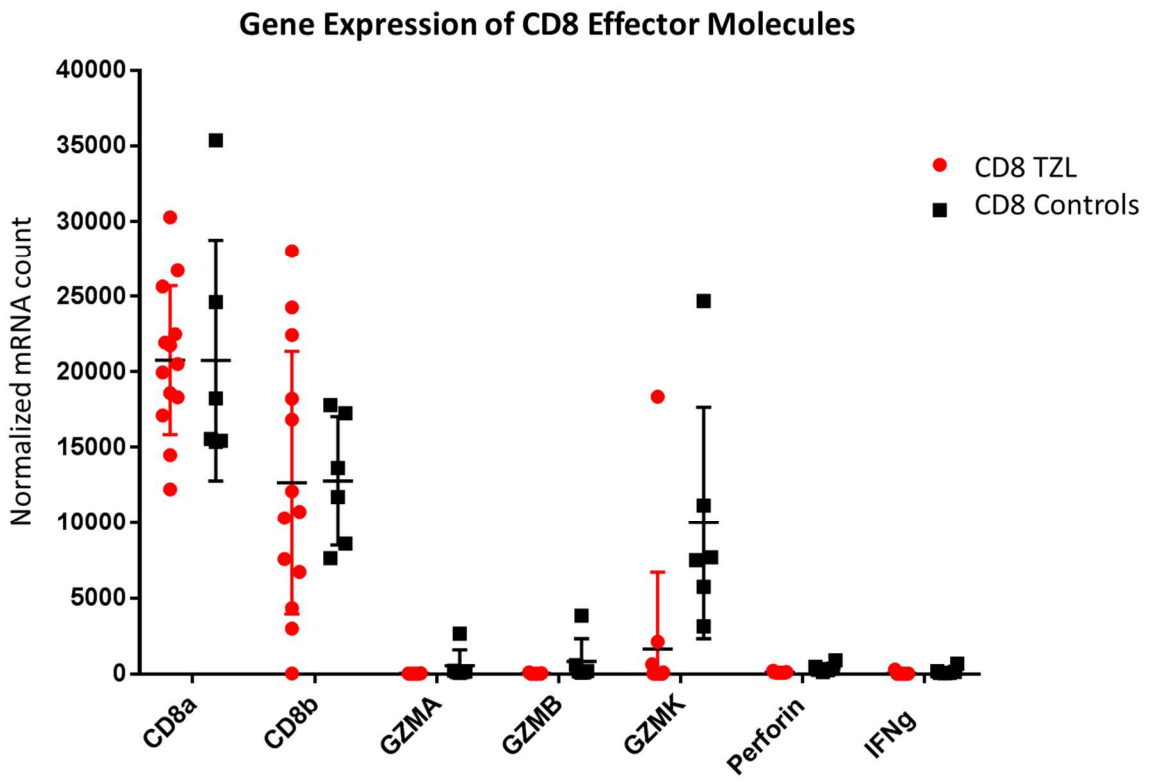

Figure 1.4. TZL cases can show expression of CD8 but do not show expression of effector molecules. mRNA expression of CD8a; CD8b (CD8 alpha and beta chains, respectively); and CD8+ effector granzyme A, B, and K molecules (GZMA, GZMB, GZMK); perforin; and INF-γ (IFNg). CD8+ TZL cases (red) and CD8+ T cell controls (black).

TZL cells express high levels of immunosuppressive molecules

There were three immunosuppressive molecules that were highly expressed in TZL cases: TGF-β, galectin-1 and galectin-3 (Table 1.5). TGF-β is produced by a number of leukocytes and stromal cells,[42] but this cytokine has been found to inhibit Th1,[43] Th2,[44,45] and effector functions of CD8+ cytotoxic lymphocytes.[46] In tumor bearing mice, TGF-β production was associated with Th2 skewing and inhibition of a Th1 response.[47] Galectins are produced by a wide variety of cells and upregulated in activated lymphocytes.[48,49] LGALS1 and LGALS3 (galectin-1 and galectin-3) are highly expressed in FOXP3+ T regulatory cells suggesting these

molecules are markers of Treg cells.[31,50] Galectins are also involved in apoptosis of activated T cells, suppressing Th1 and Th17 cells along with preferential sparing of Th2 cells promoting a Th2 cellular response.[51,52] Although there was high expression of these immunosuppressive molecules in TZL cases versus controls (Fig 1.5, Table 1.5), FOXP3 (Treg master transcription factor) was not identified in our TZL cases (Table 1.2, median = 2). The expression of these molecules may be involved in pathogenesis of this disease either creating an immunosuppressive environment, or influencing the microenvironment towards a Th2-like or Treg-like environment.

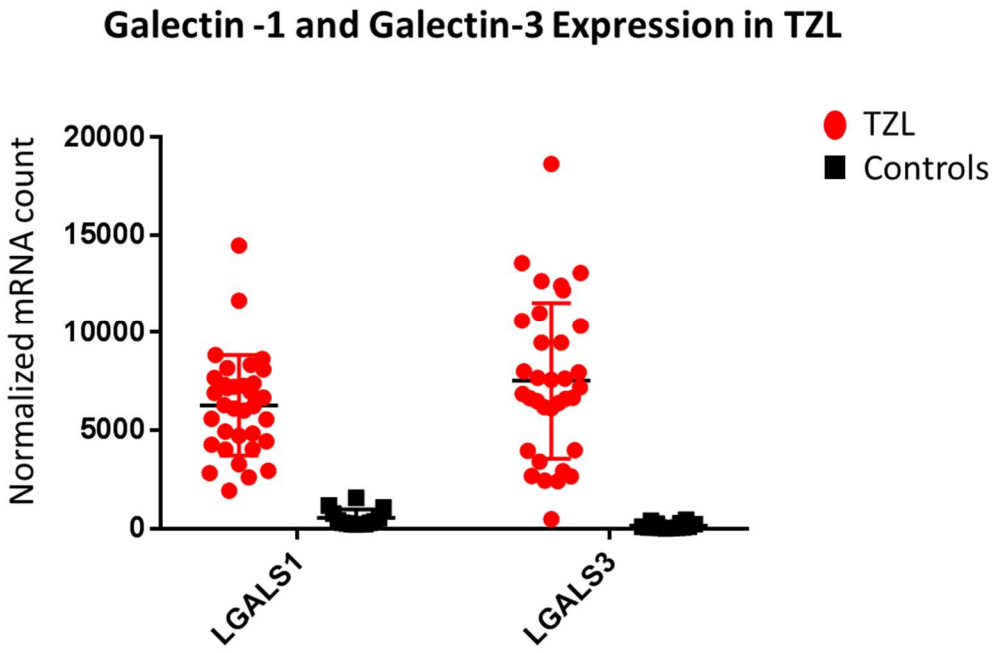

Figure 1.5.TZL cells express significantly higher levels of galectin-1 and galectin-3 which may contribute to an immunosuppressive environment. Normalized mRNA expression levels of galectins and TGF-β in TZL cases (red) and controls (black).

Table 1.5. Median normalized mRNA of immunosuppressive molecules. The median normalized mRNA expression values for cases and controls and interquartile range (IQR) for the immunosuppressive molecules, TGF-β, galectin-1 and galectin-3. All of these genes were significantly upregulated (*) in TZL cases versus controls.

	Cases	IQR	**Controls**	IQR	P-value
TGF-β*	3664	(3067 – 4229)	1081	(894 - 2206)	4.14E-10
Galectin-1*	6768	(4674 - 7517)	351	(236 - 677)	7.05E-18
Galectin-3*	7436	(4533 - 10430)	73	(46 - 247)	7.05E-18

TZL cells have features of Th2 cells and Treg cells

In an effort to investigate the cell-of-origin associated with TZL, immune cell type scores were evaluated by partitioning genes into characteristic T cell subsets. These subset genes were loaded into nSolver 4.0 advanced analysis and a raw cell score was obtained for the TZL cases versus controls based on the average log2 expression of the genes for a particular subset. The possible T cell subsets included: Th1, Th2, Tfh, Th17, Treg, and CD8+ effector memory cells (CD8 EM) (Fig 1.6). CD8+ central memory is not shown because the gene set was only 5 genes. The raw cells scores for Th2 and Treg subsets were higher for cases versus controls. These gene subsets were also uploaded into GSEA (Fig 1.7). The only subsets to show enrichment with a significant FDR were the Th2 subset (FDR = 3.2%, p = 0.01) and the Treg subset (FDR = 7.8 %; p = 0.04). In the Treg subset, only 6 of 27 genes were responsible for the core enrichment in this subset including: LGALS1, LGALS3, S100A4, S100A10, TGFb (TGF-β), GATA3 (Table 1.6).

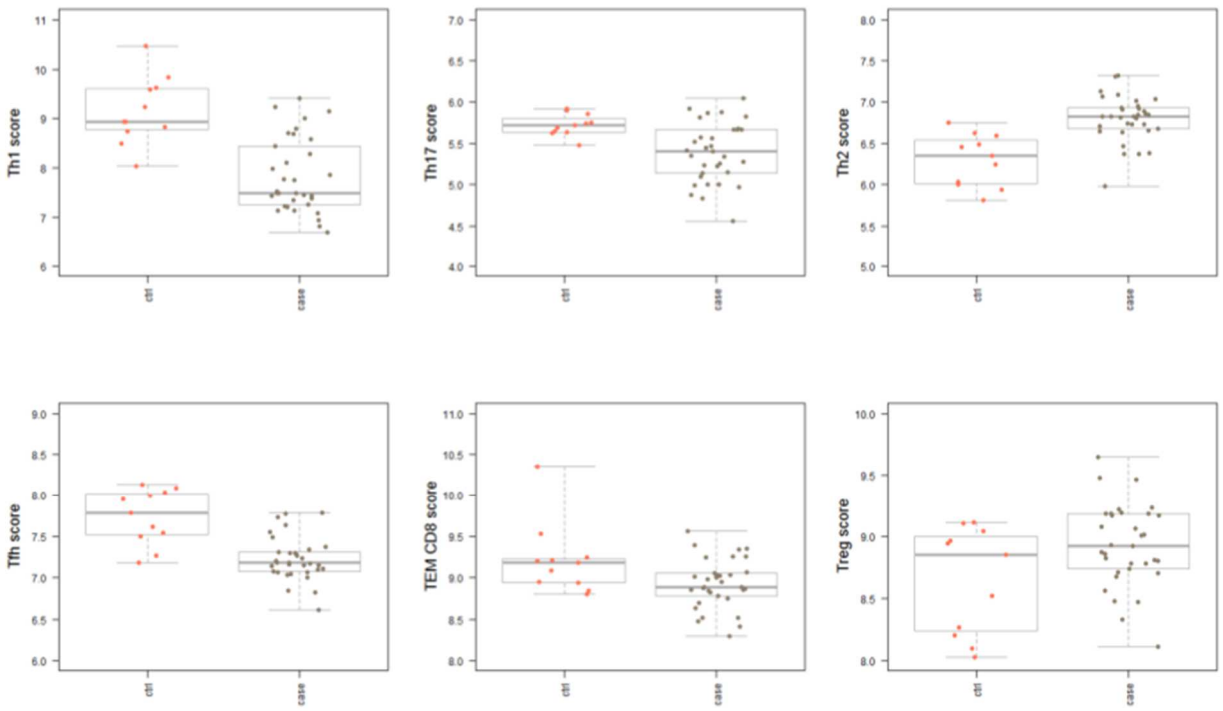

Figure 1.6. TZL cases have a higher cell score for Th2 cells and Treg cells compared to controls. Cell scores for TZL cases compared to controls (cases, grey; ctrl, salmon) derived using nSolver 4.0 advanced analysis. The cell score is based on the average log2 expression genes associated with each cell subset. TEM CD8: effector memory CD8.

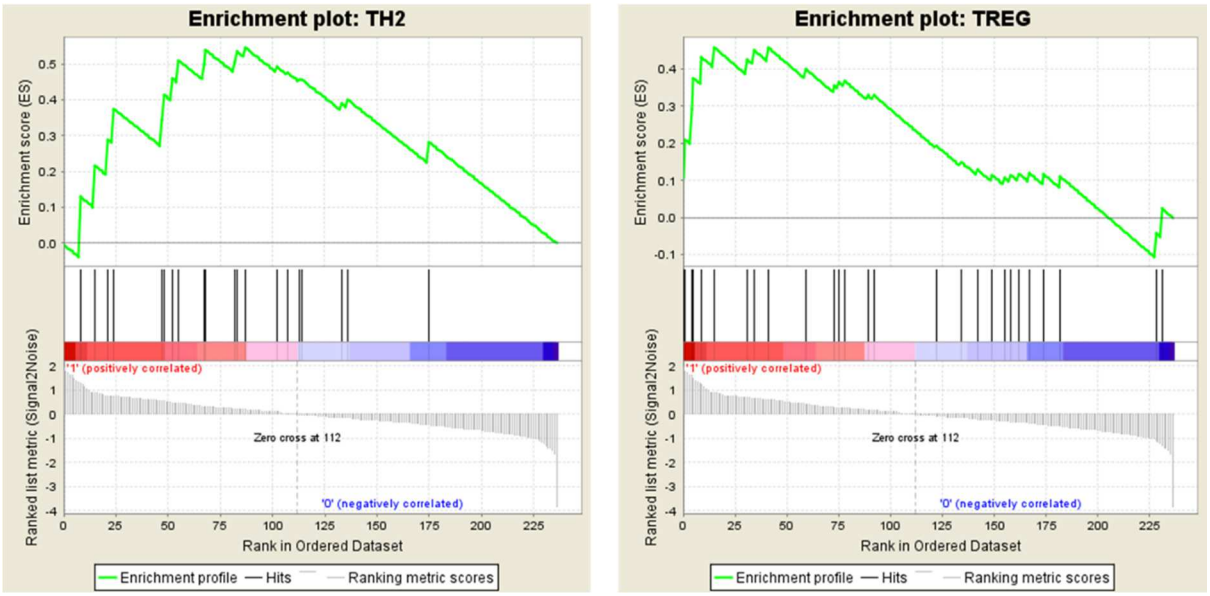

Figure 1.7. TZL cells have gene enrichment for Th2 and Treg cells. GSEA Gene set enrichment plots for Th2 (left) and Treg (right) gene sets in cases.

Table 1.6. GSEA enrichment score. Gene name, rank in gene list, running enrichment score (ES), core enrichment for Treg and Th2 gene list in TZL cases.

Treg gene subset				***Th2 gene subset***			
PROBE	RANK IN GENE LIST	RUNNING ES	CORE ENRICHMENT	PROBE	RANK IN GENE LIST	RUNNING ES	CORE ENRICHMENT
LGALS1	0	0.10842	Yes	FCGRT	8	0.12908	Yes
LGALS3	1	0.21031	Yes	GATA3	15	0.21516	Yes
S100A4	4	0.29184	Yes	MYO6	21	0.28911	Yes
S100A10	5	0.37519	Yes	CRTh2	24	0.37415	Yes
TGFb	9	0.43073	Yes	NFIL3	47	0.34346	Yes
GATA3	15	0.45761	Yes	CCR4	48	0.41356	Yes
BHLHB2	31	0.42475	No	ECM1	52	0.46006	Yes
TRAF1	34	0.45247	No	CCR3	55	0.50958	Yes
EPSTI1	41	0.45753	No	NLRP3	67	0.49885	Yes
CD25-IL2RA	59	0.40056	No	IL5	68	0.53836	Yes
FLT1	73	0.35434	No	ATF5	82	0.50639	Yes
TIAF1	75	0.36392	No	BATF	83	0.53425	Yes
HLA-DRB1	78	0.36790	No	IL3	87	0.54518	Yes
SHMT2	89	0.32996	No	IL4R	102	0.49219	No
GBP5	92	0.32931	No	EPAS1	107	0.47634	No
NINJ2	122	0.19462	No	IL24	113	0.45447	No
TRIB1	134	0.15025	No	STAT6	114	0.45634	No
SDC4	142	0.12849	No	MAF	133	0.39086	No
TFRC	149	0.11374	No	IL4	136	0.39970	No
SLAMF1	155	0.10632	No	JUN	175	0.28111	No
IL2RB	158	0.11396	No				
CEBPA	162	0.11769	No				
CCR5	167	0.11880	No				
TP53INP1	174	0.11542	No				
AKAP2	182	0.10983	No				
FOXP3	228	-0.04153	No				
CTLA4	231	0.02381	No				

Our lab has previously observed that the percentage of CD25 positive cells was higher in TZL cells than normal T cells.[53] CD25 is the IL-2-receptor-α subunit (IL-2Rα, high affinity IL-2R), prompting us to determine if any growth factor receptors were significantly upregulated in TZL.

IL-2, IL-4, IL-7, and IL-15 are all considered T cell growth factors.[54-58] We ultimately did not see a higher expression of any of the growth factor receptors in TZL cases versus controls (Fig 1.8). CD25 mRNA expression was also not significantly different between cases and controls, but there was a range of expression (TZL cases: median = 1123 range: 21 – 3666.5). IL-7R also had a higher median count in cases versus controls, but this was not statistically significant.

Figure 1.8. T cell growth factor receptor expression is not significantly different in TZL cells. Plot of normalized mRNA expression (y-axis) of receptors for T cell growth factors, IL-2, IL-4, and IL-7. IL-2 receptors included expression of the subunits α, β, and γ (CD25-IL-2RA, IL-2RB, IL-2RG).

GATA3 is diffusely expressed in the nucleus of neoplastic TZL cells

Protein expression of GATA3 was confirmed by immunohistochemistry (Fig 1.9). In TZL lymph nodes, there was compression of follicles to the capsule and intramedullary connective tissue by the expanded paracortex (Fig 1.9A). The paracortex was expanded by intermediate sized lymphocytes (nuclear size was 1 – 1.5 x RBC); nuclei were round and indented with heterochromatin and indistinct nucleoli; there was a moderate amount of homogenous

amphophilic cytoplasm and the cells expressed CD3 (not shown). In the expanded paracortex there was diffuse nuclear expression of GATA3 in neoplastic TZL cells (Fig 1.9B) Normal lymph node tissue displayed multiple primary and secondary follicles (Fig 1.9C) and there was nuclear immunoreactivity to GATA3 in cells scattered throughout the paracortex (Fig 1.9D). The diffuse nuclear expression pattern of GATA3 was confirmed in three cases of TZL.

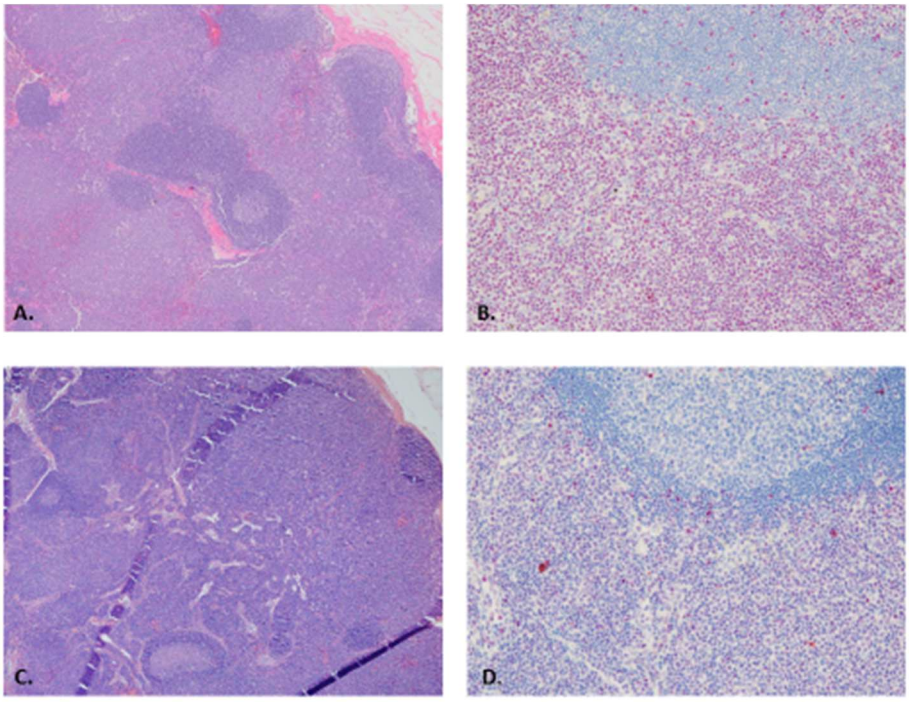

Figure 1.9. GATA3 is diffusely expressed in the nucleus of TZL cells. A., B. Lymph node tissue from a dog with TZL. A. The paracortex has been expanded by intermediate-sized neoplastic lymphocytes that compress follicles (H&E, 100X). B. Within the expanded paracortex, the neoplastic lymphocytes have diffuse nuclear immunoreactivity for GATA3. The adjacent lymphocytes in the compressed follicle are diffusely negative for GATA3 (IHC, GATA3, 200X). C., D. Reactive lymph node tissue from a 2 year-old dog. C. The lymph node is hyperplastic with multiple primary and secondary follicles. D. Within the paracortex there is scattered variable nuclear immunoreactivity for GATA3 in lymphocytes. The adjacent secondary follicle is diffusely negative for GATA3 (IHC, GATA3, 200X).

Galectin-1 and Galectin-3 expression

We anticipated the concordance of galectin protein expression with mRNA expression results. Galectins have versatile functions and galectin-3 has been found to be expressed on

many inflammatory cells including monocytes and macrophages.[59] Normal lymph node immunoreactivity toward galectin-3 was identified in the cytoplasm of dendritic cells scattered throughout the lymph nodes surrounding follicles (Fig 1.10E and F). There was also occasional scattered dim galectin-3 expression in the nucleus of cells found in the paracortex, as well as in follicles of cells with lymphocyte morphology. Galectin-1 immunoreactivity in the normal lymph node was almost identical to the galectin-3 expression pattern (not shown). In contrast, in TZL cases, there was diffuse strong immunoreactivity in the nucleus and cytoplasm of neoplastic lymphocytes in the expanded paracortex for both galectin-3 and galectin-1 (Fig 1.10A and B (galectin-3) and C and D (galectin-1)).

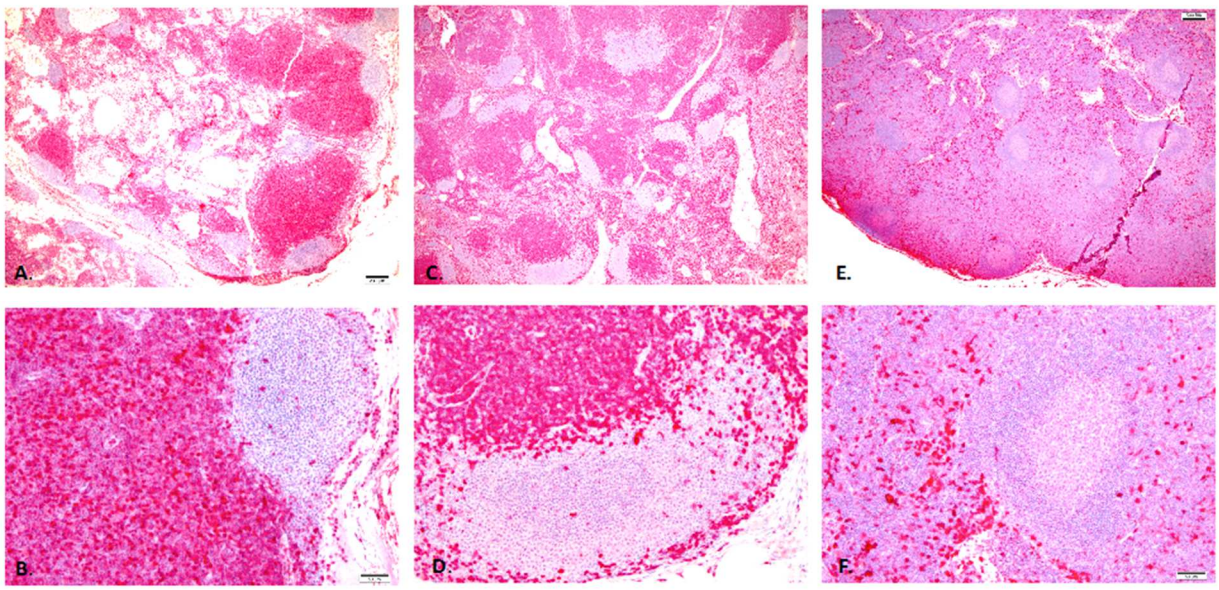

Figure 1.10. Galectin expression is abundant in the nucleus and cytoplasm of TZL cells. A., B., C., D., Lymph node tissue from a dog with TZL. A. and C. The paracortex is expanded by intermediate-sized neoplastic lymphocytes that compress follicles. In the neoplastic cells there is diffuse, strong immunoreactivity for galectin-3 (A.) and galectin-1 (C.) (H&E, 100X). B. and D. Close-up of a follicle compressed by the expanded TZL population. Within the expanded paracortex, the neoplastic lymphocytes have strong diffuse nuclear and cytoplasmic immunoreactivity for galectin-3 (B.) and galectin-1 (D.); (IHC, galectin-3 (A. and B.) and galectin-1 (C. and D.) 40x and 200X). E., F. Reactive lymph node from a 2 year-old dog. E. The lymph node is hyperplastic with multiple primary and secondary follicles. There is strong scattered cytoplasmic and dim nuclear immunoreactivity for galectin-3 in both the

follicles and paracortex. F. Within the paracortex, there is scattered strong cytoplasmic immunoreactivity for galectin-3 within dendritic cells with abundant irregular stellate cytoplasm. There are few scattered lymphocytes with dim nuclear immunoreactivity for galectin-3 (IHC, galectin-3, 200X).

TZL cells to not express IRF-4/MUM-1 when analyzed with immunohistochemistry

IL-9 was originally thought to be produced by Th2 cells but further investigation revealed a distinct subset of Th cells now called Th9 cells was ultimately responsible for IL-9 production.[26] In the presence of IL-4 and TGF-β, naïve cells differentiate into Th9 cells and produce IL-9.[60] This process requires several transcription factors including STAT6, IRF-4, and GATA3.[26,60,61] TGF-β was highly expressed in TZL cases along with GATA3 but our work did not investigate gene expression of IL-9 or IRF-4. We decided to determine if the IRF-4 protein was expressed. We preformed immunohistochemistry for IRF-4/MUM-1 on three cases of TZL to determine if this transcription factor might also be implicated in TZL. In the lymph node, the TZL cells were diffusely negative for IRF-4/MUM-1 (Fig 1.11). There were few scattered well differentiated plasma cells surrounding follicles that showed immunoreactivity for IRF-4/MUM-1. These results did not provide evidence for TZL cells being derived from a Th9 cell subset.

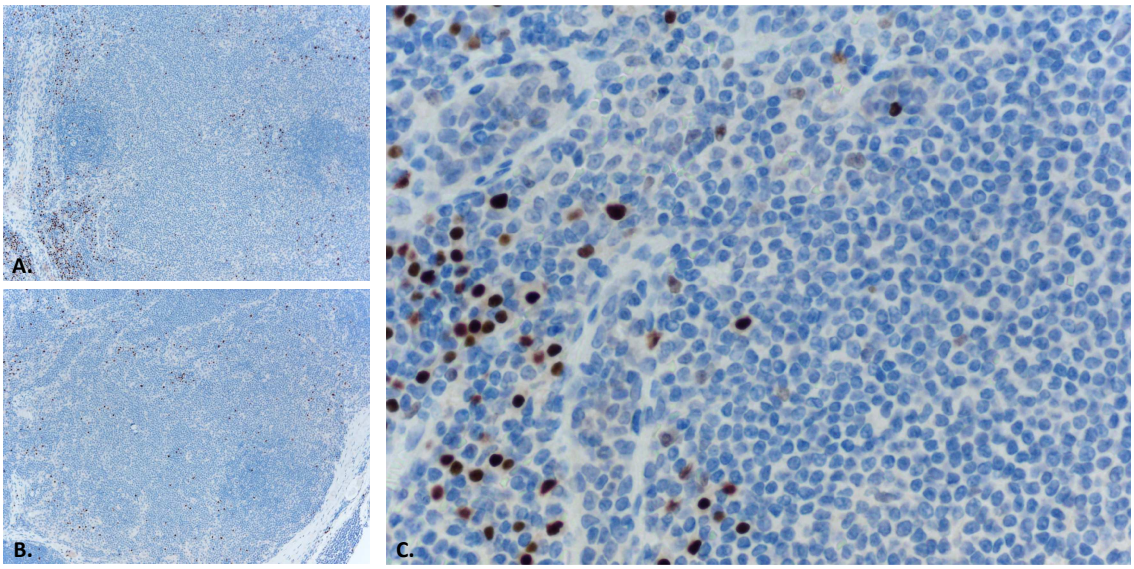

Figure 1.11. TZL cells do not express IRF-4/MUM-1. A., B. Two different TZL cases with expanded paracortex by neoplastic TZL cells and few smaller follicles on the periphery. There are scattered and peripheral clusters of cells with nuclear immunoreactivity for IRF-4/MUM-1 while the TZL cells in the expanded cortex are diffusely negative for IRF-4/MUM-1 (IHC, IRF-4/MUM-1 10X). C. Higher magnification of A. showing the neoplastic TZL cells with condensed chromatin and frequently indented nuclei do not express IRF-4/MUM-1. On the periphery, well differentiated plasma cells with round nuclei show strong nuclear immunoreactivity for IRF-4/MUM-1.

Discussion

Our investigation sought to identify the cell-of-origin in T zone lymphoma. TZL can present as a CD4+, CD4-CD8-, or CD8+ T cell phenotype.[53] The TZL cases in our study did not cluster by gene expression based on phenotype (Fig 1.1) despite protein and mRNA expression of CD4 and CD8 molecules. PTCL-NOS in humans with variable phenotypes showed that CD4/CD8 expression did not correspond to an operative lineage by gene expression.[62] Similarly, in our study, CD8 protein and gene expression did not correspond with expression of CD8 effector molecules (granzymes, perforin or IFN-γ) (Fig 1.4).

Regardless of the TZL phenotype, we were able to identify a unique immunosignature for TZL. There was significant upregulation of GATA3, the master transcription factor for Th2 cell differentiation, as well as upregulation of the Th2 specific receptors: CCR3, CCR4, and CRTh2 (Fig 1.2, Table 1.2, and Table 1.3). CRTh2 is a G-protein-coupled receptor, specifically expressed by Th2 cells and considered to be the most dependable marker for memory Th2 cells.[63] This receptor is involved in GATA3 upregulation, Th2 cell migration and cytokine production.[64] CCR4 is the dominate chemokine receptor for Th2 cells, Treg cells, and skin-homing T cells.[65] In polarized T cells CCR3 and CCR4 were found to be expressed in Th2 cells.[66,67] Eotaxin binds to CCR3 and CCR3 upregulation was found to be acquired on a subset of Th2 cells.[68]

Increased gene expression of some Th2 specific genes in TZL is suggestive of a Th2-like cell-of-origin but we did not find a difference in expression of Th2 cytokines including: IL-4, IL-13, IL-5, or the transcription factor, STAT6 (Table 1.3). Although TZL may be derived from a Th2 cell, the mechanisms involved in oncogenesis appear to have diverted from normal Th2 cellular response since they do not express increased levels of these cytokines. Furthermore, clinically dogs with TZL are not reported to present with an eosinophilia.[8] Eosinophils are associated with a Th2 response and IL-5 is involved in the production of eosinophils.[69,70] A subset of human PTCL-NOS has been identified to express GATA3 and while these patients predominantly had a normal eosinophil count even with increased expression of IL-5, there was a relationship between the degree of GATA3 expression and the development of eosinophilia.[71] In a separate study, the incidence of eosinophilia was not more prominent in patients with GATA3 positive PTCL compared to GATA3 negative patients.[72] Neither of these studies demonstrated induced secretion of Th2 cytokines in neoplastic cells.[71,72] It is possible the degree of GATA3 expression in TZL cases is not sufficient for cytokine production or eosinophil recruitment.

Even in the absence of STAT6 with Th1 primed conditions, GATA3 has been shown to induce Th2 differentiation.[73] GATA3 has been identified to bind to a large number of genes in various T cell subsets leading to activation or repression depending on the cellular context.[74] The number of binding sites for GATA3 in various T cell subsets appears to depend on the degree of expression.[74] GATA3 has shown variable levels of expression in Th1 (low expression) and Th2 (high expression) T cell clones, suggesting that the level of GATA3 expression could be linked to Th2 cytokine production.[75] This theory was further validated by generating antisense GATA3 lines from the Th2 cell clones in which all Th2 cytokines were inhibited with decreased

GATA3 expression.[75] GATA3 was unable to activate the IL-4 promoter alone, needing other activation-induced transcription factors such as NFAT or AP-1, for IL-4 promoter activation.[75] Additionally, the level of expression of GATA3 differed depending on the cytokine.[75] c-Maf has been identified to be a transcription factor necessary for the optimal expression of IL-4 in CD4+ T cells and acts synergistically with NFAT.[76,77] In our study, c-Maf was not highly expressed in TZL cases (TZL median = 246; controls median = 552; data not shown) and there could be other genes involved in cytokine regulation that not upregulated TZL cells disrupting cytokine production.

GATA3 expression could also reflect other signaling pathways and transcription factors which regulate GATA3 production being disrupted in TZL, rather than representing Th2 cell origin. GATA3 expression occurs through different mechanisms including: IL-4 signaling and downstream STAT6 activation,[78] TCR signaling occurring through PI3K-mTOR,[79] IL-33 and STAT5 activators,[80] Notch signaling,[81] and Wnt/β-catenin signaling through SATB1.[82] However, we did not find a significant difference in the expression of STAT6, mTOR, or SATB1 in cases compared to controls. The TZL cases did show significantly higher expression of NOTCH. Notch is involved in differentiation of many of the T cell subsets,[81] but GATA3 has been specifically identified to be a transcriptional target of Notch.[83] This pathway, along with others involved in GATA3 regulation, could be involved in the oncogenesis of TZL and the expression of GATA3 in this tumor. It would be useful to compare the phosphorylation of these proteins between cases and controls to determine if there is a difference in activation. Furthermore, GATA3 has also been identified to be critical for thymocyte development,[84,85] involved in differentiation and function of multiple CD4+ T cell subsets,[86] CD8+ T cell maintenance,[87] and development of innate like

lymphoid cells.[88] GATA3 has many functions in T cells and its expression in TZL may not be specific for Th2 differentiation.

Th9 cells also express GATA3 but at lower levels than Th2 cells.[89-91] The function of GATA3 in Th9 cells is still under investigation[25] as some studies have found low levels of GATA3 present in Th9 cells.[89] Th9 cells are characterized by secretion of IL-9 under the influence of TGF-β and IL-4.[92] STAT6 is an important factor in the downstream pathway of this subset.[91] TZL cells did show high expression of TGF-β, but did not show an increased expression of IL-4 or STAT6. We did not measure the expression of IL-9 in our study. BATF is another transcription factor required for Th9 cellular development[93] and expression of this transcription factor was very low in TZL cases (median = 1.23, data not shown). Through regulation of expression of IL-9, IRF-4 has been found to be essential to Th9 development.[61] We completed immunohistochemistry on two TZL cases to determine if there was protein expression of IRF-4/MUM-1 but no expression was found in TZL cells (Fig 1.11). Additionally, in cases of TZL of the tongue, MUM-1 was not expressed (single case evaluated).[94] These findings do not support TZL cells stemming from a Th9 T cell subset.

There were some features of TZL that were suggestive of a regulatory T cell progenitor, but we did not find expression of the master transcription factor of Treg cells, FOXP3. GATA3 has been identified to be important in the function of FOXP3+ Treg cells with co-expression of the transcription factors GATA3 and FOXP3.[86] Still, we were unable to detect any evidence of co-expression of GATA3 and FOXP3 in TZL cases; the median absolute count of FOXP3 expression in TZL was 2. In TZL cells, there also was not a significant difference in the expression of CD25 mRNA (Fig 1.8) when compared with controls or in expression of the Treg cytokine, IL-

10. There was increased expression of TGF-β, the immunosuppressive cytokine, in TZL cases versus controls (Fig 1.3, Table 1.5). TGF-β is an important cytokine for development of Th17 cells,[95] induced Treg cells,[19] and it is required for Th9 cell differentiation.[96] The involvement of TGF-β in various T helper subsets highlights the inherent plasticity of T cells and the myriad of complex signaling molecules involved in T cell differentiation. TGF-β expression has also been implicated in a variety of cancers associated with tumor immune evasion, metastasis, cellular proliferation, and survival.[97] TGF-β expression in TZL may be involved in the pathogenesis of this disease rather than with the cellular derivation of neoplastic cells.

Many of the cytokines investigated in this study were expressed at low levels (Fig 1.3). Cytokine production primarily occurs through stimulation of the TCR with the help of CD45. It is possible the lack of CD45 could be hindering signaling through the TCR and subsequent cytokine production then persists at low levels. In other studies, CD45 deficiency was not only associated with proliferation defects, but also diminished cytokine production.[98 99] CD45 deficient cells have been shown to still have the ability to proliferate with IL-2.[99] We did find that IL-2 was significantly higher in TZL cases (IL-2: TZL median = 470, controls median = 218; p value = 0.00866) and CD25 (IL-2R) was expressed at higher levels in cases compared to controls although this difference was not statistically significant (Fig 1.8). It is possible that TZL cell activation/proliferation occurs though the IL-2R since IL-2 is a known T cell growth factor and can be produced from many cells besides activated CD4+ Th cells including CD8+ T cells, dendritic cells and mast cells.[100-103]

Galectin-1 and galectin-3 were also both highly expressed in TZL (Fig 1.5, Table 1.5, and Fig 1.10). Galectins show variable expression by all immune cells and are upregulated in

activated T cells and FOXP3+ regulatory T cells.[104-106] High expression of galectins in TZL contributed to the increased Treg score in TZL, however galectins are not specific to Treg cells. Galectin-3 may even have an inhibitory role in expansion of the T regulatory cell population.[107,108] Galectins have been shown to mediate inflammation through apoptosis of activated leukocytes[105] and conversely amplify inflammation,[109-111] which demonstrates the multifactorial effects of galectins depending on the cellular and extracellular environment. Galectin-1 has also been identified to be activated in many tumors with methylation of the galectin-1 promoter suggested to be the primary mechanisms for expression.[112] Both galectin-1 and galectin-3 have been shown to be expressed in neoplastic tissue and in association with hypoxic conditions.[113-115] Tumor cell secretion of galectins can promote tumor growth by tolerogenic effects including cytokine skewing, promotion of T cell anergy, apoptosis of cytotoxic lymphocytes and Th1 cells, and expansion of Treg cells.[116-118] Galectin expression in TZL could have a similar function to that found in other tumors, as a mechanism to regulate the immune response; or in the case of TZL, could contribute to a Th2 or Treg-like environment.

Galectin-1 induced apoptosis in activated T cells preferentially selects Th1 and Th17 cells, sparing Th2 cells due to differential glycoproteins on the cell surface.[51] Galectin-3 has also been shown to protect cells from apoptosis when expressed intracellularly[119] within Th2 cells specifically.[120] Galectin-1 produced by Th2 cells has been shown to promote Th1 apoptosis similar to reciprocal inhibitory cytokine production in Th1/Th2 cells.[120] Furthermore, galectin-1 predominantly binds to CD45 on Th1 and Th17 cells to cause apoptosis,[121,122] and CD45 is required for galectin-3 induced cell death.[106] TZL cells could be spared from galectin-1 induced apoptosis either by lack of expression of CD45 or Th2-type glycans which are resistant to

galectin induced cell death. A similar mechanism is described in humans with cutaneous T cell lymphoma where cells have lost expression of CD7. CD7 is another important ligand for galectin-1 mediated cell death and resistance to galectin-1 induced apoptosis has been demonstrated in CD4+CD7- leukemic T cells,[123] and is also a hypothesized mechanism for development of anaplastic large T cell lymphoma (ALCL) in which the neoplastic cells also do not express CD7.[124] CD7 expression was measured in TZL cases and was not significantly different from controls.

The results of this study propose that TZL has a unique immunosignature with increased expression of GATA3 and immunosuppressive molecules: TGF-β, galectin-1 and galectin-3. Genes that were upregulated in TZL overlapped with genes associated with both Th2 cells and Treg cells. Although there was not increased expression of Th2 type cytokines, investigation into cytokine production associated with pathogenesis may provide more insight into the cell-of-origin of this disease. Mechanisms involved in the development of TZL could include signaling through TGF-β or possibly through the IL-2 receptor. Additionally, identified galectin-1 and galectin-3 production could be involved in TZL cell resistance to galectin mediated death and/or supporting a Th2 type environment.

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CHAPTER 2: T ZONE LYMPHOMA CELLS PROLIFERATE THORUGH T CELL RECEPTOR INDEPENDENT MECHANISMS

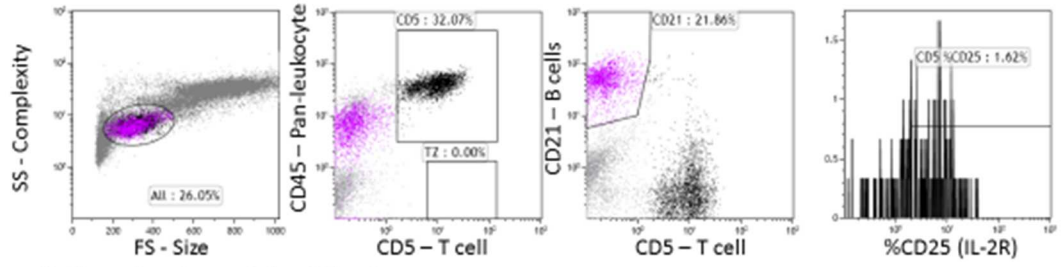
Summary

T zone lymphoma (TZL) has a unique phenotype in which the neoplastic cells lose expression of the pan-leukocyte marker, CD45. CD45 is a transmembrane receptor expressed abundantly on leukocytes and it is involved in antigen mediated T cell activation. CD45 removes inhibiting phosphates from molecules involved in T cell receptor (TCR) signaling and cell activation. Since CD45 is required for T cell signaling through antigen stimulation we hypothesized that TZL cells would proliferate through mechanisms independent of the TCR. We attempted to induce activation and proliferation through two different inducers of TCR engagement and we found TZL were unable to proliferate through these methods. TZL cells did proliferate when stimulated through mechanisms that by-pass the TCR. IL-2 was found to induce proliferation in a subset of cases suggesting IL-2 may contribute to TZL proliferative capacity. We also attempted to address resistance to apoptosis in this disease. We found that TZL cells were not immune to cell death in long-term culture even in the presence of IL-2. These findings suggest other microenvironmental factors may be involved in persistence of neoplastic TZL cells *in vivo*.

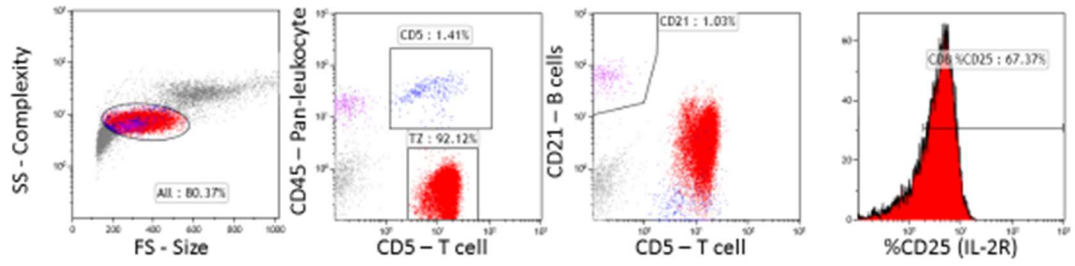
Background

T zone lymphoma (TZL) is considered a subtype of peripheral T cell lymphoma (PTCL) and, in dogs, it is an indolent disease accounting for approximately 10% of all canine lymphomas,[1,2] and up to 60% of indolent lymphomas.[3] Reliable phenotypic abnormalities aid in the identification of TZL in dogs, including loss of the pan-leukocyte marker, CD45, increased

expression of CD21, and increased expression of CD25 (IL-2 receptor-α, IL-2Rα).[4-6] CD21 or compliment receptor 2 is commonly expressed on B cells but has been known to be expressed by T cells in humans and mice, and may be involved in T cell response or activation.[7-9] CD25 is considered one of the T cell activation markers in humans.[10] These phenotypic features are suggestive of TZL cells being derived from an activated T cell (Fig 2.1). Conversely, CD45 is essential for T cell receptor (TCR) mediated activation of T cells. CD45 is the most abundant transmembrane protein on lymphocytes, and its function is best characterized in T cells.[11,12] CD45 is required for antigen stimulation with subsequent proliferation in T cells.[13] Experimental studies have found that CD45-deficient cells are unable to proliferate or produce cytokines when stimulated through the TCR.[13] Because CD45 is absent on TZL cells we questioned how these cells were being activated.



A. Normal canine peripheral blood



B. Peripheral blood from a dog with TZL

Figure 2.1. Flow cytometric features of T zone lymphoma. A. Lymphocyte characteristics of normal canine peripheral blood. The lymphocytes (circled) are small and less complex than neutrophils and monocytes (grey). The T cells (black) express both CD5+ and CD45+ (CD5 box, middle dot plot), and do not express CD21- (third dot plot). The B cells (pink) express CD21+ and do not express CD5- (middle and third dot plot). In the final histogram, 1.62% of the CD5+CD45+ T cells express CD25. B. Peripheral blood from a dog with TZL. The TZL cells (red) are intermediate in size and express CD5+ but do not express CD45- (middle dot plot). There are 1.41% of normal T cells (blue) that express both CD5+ and CD45+. The TZL cells also express higher levels of CD21 than the normal T cells (third dot plot), and 67.37% express CD25 (histogram, far right).

Prior to differentiation of Th subsets, naïve T cells are thought to remain dormant until interaction with antigen expressed by an antigen presenting cell (APC).[14] T cell activation by the TCR results in gene response leading to regulation of multiple pathways.[15] TCR engagement occurs through two signals, first through CD3, and the second signal through a co-stimulatory molecule such as CD28.[16,17] Activation through both signals is involved in TCR mediated IL-2 production,[18] which promotes differentiation and proliferation of T cells during an immune response.[18,19] Antibodies to the TCR-CD3 complex have been shown to cause an initial signal in the T cells but proliferation requires co-stimulation often through CD28.[20] Anti-CD3 and anti-CD28 coated beads have proven to provide efficient T cell expansion in humans,[21] and this has also been demonstrated in dogs.[22] Phytohemagglutinin (PHA) is a mitogen that has been utilized in many species, including dogs, to cause a rapid and marked T cell expansion,[15,23] and it has been shown to reflect the gene response of T cell activation through the TCR.[24] TCR engagement also leads to hydrolysis of membrane phospholipids and production of second messengers, resulting in elevations in intracellular Ca^{2+} concentration and protein kinase C (PKC) activation.[17] These mechanisms of T cell activation are simulated through exogenous compounds *in vitro*. Phorbol 12-myristate 13-acetate (PMA) and ionomycin are synergistic in enhancing the activation of PKC with intracellular Ca^{2+} influx.[25] IL-2 can augment T cell

expansion with other mitogens and cause preferential expansion of CD8+ T cells.[26,27] Canine T cells exhibited expansion with recombinant human IL-2 (rhIL-2) alone or in combination.[23,28]

One of the hallmarks of cancer is uncontrolled cell growth.[29] Since TZL cells do not express CD45, which impedes proliferation through the TCR receptor, we considered that resistance to cell death may be a more prominent pathway of oncogenesis in this disease. Normally, upon activation of T cells several mechanisms are in place to regulate the T cell response and maintain homeostasis. One pathway includes activation induced cell death (AICD) with upregulation of the tumor necrosis factor receptor family (TNFR) resulting in activation of the caspase cascade and cell death.[30] Fas is a TNFR leading to apoptosis when bound to Fas ligand (FasL) in T cells that have been activated.[30-32] Another mechanism of cell death in activated T cells is through galectins. Galectin-1 is a lectin that is widely expressed and causes death in activated T cells in humans.[33] Galectin-1 can control T cell fate through binding to CD45, CD43, and CD7[34] or leading to T cell sensitization of Fas mediated cell death.[35] Upon T cell activation, galectin-1 is upregulated and when bound to CD45 can lead to apoptosis of T cells.[33] Due to the lack of expression of CD45 in TZL cells, resistance to galectin mediated apoptosis may be another contributor to lymphomagenesis in this disease.

Because of the unique phenotype of TZL we wanted to investigate the mechanism of proliferation of TZL cells and determine if there was evidence of resistance to apoptosis in this disease. We used *in vitro* methods of T cell proliferation through the TCR receptor with anti-CD3/anti-CD28 coated beads and with PHA. Additionally, we investigated TZL proliferation through TCR independent pathways utilizing PMA/ionomycin and IL-2. We hypothesized that TZL cells would not proliferate when stimulated through the TCR and that IL-2 would cause

activation in TZL due to the expression of the IL-2 receptor, CD25. Furthermore, we wanted to determine if TZL cells were resistant to death in long-term culture. We hypothesized that TZL cells would remain viable for 21 days in culture and given the prolonged time, stimulation with IL-2 would induce significant proliferation in the TZL cells.

Methods

Selection of cases for in vitro proliferation assays and long-term culture

Samples that were diagnosed with TZL were obtained through the Colorado State University-Clinical Immunology (CSU-CI) laboratory and identified with the standard diagnostic flow cytometric panel 2 previous described.[4] In samples from 2017 tube 3 and 4 were adjusted and combined into tube 3d containing: Class II MHC-FITC/CD34-PE/CD21-APC. Variable phenotypes of TZL were examined including CD4+, CD8+, and CD4-CD8-. Non-leukemic canine peripheral blood was used for comparison. These 'normal' control samples consisted of peripheral blood obtained from beagle dogs being utilized for other studies at CSU and housed in the veterinary teaching hospital. These dogs were utilized while on various protocols in which peripheral blood draws did not interfere with the animal's health or current studies. These procedures were approved through the Colorado State University's Animal Care and Use Committee. Additional normal peripheral blood was obtained through the CSU-CI laboratory from samples submitted for a case-control study in Golden retrievers, samples in young dogs with heterogeneous lymphocyte expansion and not consistent with a diagnosis of lymphoma\leukemia and from samples that were serologically positive for *Ehrlichia Canis* in a study to determine the lymphocyte distribution in dogs with *E. canis.* Table 2.1 contains the

summarized patient data for the cases and controls for this study for each stimulatory method and long-term culture.

Table 2.1. Samples for in vitro cell culture methods. TZL cases (pink, left side); Clinical Immunology sample number (CI); phenotype of TZL; lymphocyte count (cells/ul); %TZL cell in the sample; breed; age; sex. Controls (Grey, Normal blood, right side); CI number, diagnosis: Heterogeneous lymphocyte expansion (hetero), expansion of CD8 T cells (CD8), T cells below normal reference range (dimished T cells), normal lymphocyte subsets (Normal), expanison of multiple T cell subsets (Hetero T cells); breed; age; sex; notes

TZL cases							Normal blood				
PMA											
CI number	**Pheno**	**Lymph count**	**%TZL cells**	**Breed**	**Age**	**Sex**	**CI number**	**Lymph count**	**Breed**	**Age**	**Sex**
73759	CD4	65247	84	LAB	11	FS	74574	6760	MIX	7	F
73775	CD8	13000	20	GLDR	9	FS	75054	2800	MIX	1	F
74234	CD4	32600	87	GLDR	8	MC	74237	4210	MIX	1	M
74721	CD8	14839	52	CORG	10	FS	84951	18600	PDL	6	FS
75048	CD4-CD8-	130200	96	MIX	11	FS					
71049	CD8	24300	75	GLDR	9	FS					
84932	CD4-CD8-	10431	57	MINPIN	5	FS					
85287	CD8	6533	82	GLDR	6	MC					
85987	CD8	25600	92	SCHN	15	MC					
Beads											
69719	CD4-CD8-	251600	93	GLDOODLE	11	MC	69718	7622	GLDR	1	F
70025	CD4-CD8-	9160	48	GLDR	12	FS	70242	1000	GLDR	11	M
70460	CD4-CD8-	22800	83		12		70364	4600	GLDR	5	MC
71049	CD8	24300	75	GLDR	9	FS	57452	1500	BGL	2	
57474	CD8	71400	93	GLDR	8	MC	48763	1750	BGL	1	M
59984	CD4	15000	61	GLDR	12	MC	48764	3780	BGL	1	M
61659	CD4-CD8-	5300	76	GLDR	11	FS					
71049	CD8	24300	75	GLDR	9	FS					
PHA											

71854	CD4-CD8-	10000	30	GLDR	12	FS	74237	4210	MIX	0	M
71856	CD4-CD8-	40800	74	GLDR	12	FS	74574	6760	MIX	7	F
73759	CD4	65247	84	LAB	11	FS	75054	2800	MIX	1	F
73775	CD8	13000	20	GLDR	9	FS	75055	5100	MIX	2	F
74234	CD4	32600	87	GLDR	8	MC	85451	2000	mix	3	M
74576	CD4-CD8-	3300	36	GLDR	11	FS	85449	4710	mix	1	F
74721	CD8	14839	52	CORG	10	FS					
75048	CD4-CD8-	130200	96	MIX	11	FS					
IL-2											
81852	CD4-CD8-	15590	54.63	GLDR	9	MC	80397	LN for IL-2 titration	MIX	1	F
81867	CD4	38800	39.43	GLDR	13	MC	80398	LN for IL-2 titration	MIX	1	F
83533	CD4-CD8-	13000	68.23	CKC	7	FS	80604	LN for IL-2 titration	BLDHD	1	M
83536	CD4-CD8-	21014	75.98	GLDR	9	FS	80605	LN for IL-2 titration	MIX	1	M
83641	CD8	6330	43.02		14	FS	82673	5340	CHI	14	MC
84158	CD4	7750	12.04		8	MC	82677	6000	MIX	3	MC
84160	CD8	18700	70.62	CHI	10	MC	83562	7400	LAB	5	M-
84187	CD4-CD8-	252588	90.05	SHTZ	9	FS	83568	10900	BRITT	2	F-
85287	CD8	6533	81.92	GLDR	6	MC	83574	3499	LAB	5	F-

85372	CD4-CD8-	78790	93.06	GLDR	9	F	85451	2000	mix	3	M
85413	CD4-CD8-	11600	71.46	GLDR	13		85449	4710	mix	1	F
85987	CD8	25600	92	SCHN	15	MC					
84932	CD4-CD8-	10431	57	MINPIN	5	FS					
Long-term culture											
86294	CD4-CD8-	162900	96.41	LAB	11	FS	87920	4500	mix	1	M
86450	CD4-CD8-	31500	88.6	BLLDFR	10	FS	88997	18300	mix	5	F
86803	CD8	57100	91.6	MALT	9	M	90120	7600	mix	5	F
87348	CD4-CD8-	16720	26.11	CCKS	12	FS					
87364	CD4-CD8-	28300	80.78	GLDR	13	FS					

Cell culture methods

Peripheral blood samples were submitted to the CSU-CI laboratory for suspicion of lymphoproliferative disease through the decision of the submitting clinician. Peripheral blood was collected in EDTA and prepared as previously described.[36] Red blood cells were lysed with filter sterilized lysis buffer (0.15M NH_4Cl, 1M KHO_3, 0.1mM Na_2EDTA, 1N HCL at a pH of 7.2-7.4) for 5 minutes at room temperature (RT). The samples were washed with HBSS (Milipore Sigma, St. Louis, MO) + 5% heat inactivated fetal bovine serum (HI-FBS) (Milipore Sigma). Lysis and wash was repeated until the cell pellet did not show any evidence of residual blood. Samples were resuspended in HBSS with no FBS. The samples were then labelled for carboxyfluorscein succinimidyl ester (CFSE, 5µM, Invitrogen, Waltham, MA) for 5 minutes and washed as described.[37] Cells were resuspended in RPMI 1640 (Milipore Sigma) media containing: HI-FBS 10%, 2-mercaptoethanol 0.05µmol/ml (Milipore Sigma), 1 x minimum Essential Medium (MEM)

non-essential amino acids (Milipore Sigma), 100U/mL penicillin and 100µg/mL streptomycin (Milipore Sigma), 1 x Glutamax (Gibco), 10mM Corning HEPES (Media Tech Inc, Manassas, VA) and 1mM sodium pyruvate (Gibco). Based on the lymphocyte count from the complete blood cell count a total of approximately 6.25 X 10^5 lymphocytes per well (62.5 X 10^5 lymphocytes/mL) were incubated in 96-well round bottom cell culture plates (M9311 Milipore Sigma) at 37 degrees Celsius (C) in a humid 5% CO_2 atmosphere. Cells were incubated for up to 7 days for proliferation assays and for 21 days for long-term culture. For long-term experiments, the cells were not stained with CFSE and the media was replaced every 7 days with fresh media. Samples were plated in duplicate wells for harvest every 7 days. For proliferation assays, cells were harvested at 24 hours, 5 days, and 7 days. All harvested cells were stained with surface antibodies by standard protocols. Briefly, the cells were harvested in a 96-well plate and washed twice with 150µL of flow buffer (PBS-2% FBS-0.1%NaAZ) and resuspended in 25µL of flow buffer and 25µL of a cocktail of antibodies in flow buffer. Surface antigens used included: CD5-APC (T cell, clone YKIX322.2, Bio-Rad, Hercules, CA), CD45-PB (pan-leukocyte, clone YKIX716.13, eBioscience, San Jose, CA), and CD8-A700 (CD8 T cell, clone YCATE 55.9, Bio-Rad). Samples were incubated for 15 minutes at RT and resuspended in 100µL of flow buffer and 100µL of 10µg/mL of propidium iodide (PI) for exclusion of dead cells. To determine the total cell count, a known amount of CountBrite beads were added prior to sample acquisition, and cells were enumerated based on bead recovery and amount of sample remaining at the time of harvest. Cell viability was determined as a percentage of the PI-CD5+CD45- events divided by the total number of PI+/-CD5+CD45- events. Samples were

acquired on a 3-laser Coulter Gallios and analyzed with Kaluza software (Beckman Coulter, Brea, CA).

Stimulatory methods

To stimulate and activate T cells, approaches included TCR-dependent and TCR-independent methods (summarized in Table 2.2). TCR-dependent methods included anti-CD3/anti-CD28 (TCR crosslinking) and PHA (lectin mitogen, non-specific TCR cross-linking). TCR-independent methods included PMA/ionomycin (bypass TCR, intracellular stimulation), and hrIL-2 (IL-2 receptor, IL-2R). For stimulation with anti-CD3/anti-CD28 beads, the manufacturer's protocol was followed. Briefly, Anti-canine CD3 (clone CA17.2A12, Bio-Rad) and mouse anti-canine CD28 (clone 5B8, eBioscience) were conjugated to magnetic Goat anti-Mouse IgG Dynabeads (Invitrogen). Primary antibody concentration was recommended at 0.1-1μg/25μl of beads. CD3 was our primary antibody and 0.1μg of CD3 and 0.5μg of CD28 were incubated with 25μL of Dynabeads (10^7 beads) for 30 min at 4 C, mixing every 5 minutes in filtered sterilized 1% bovine serum albumin (BSA) (w/v) (Milipore Sigma) in phosphate-buffered saline (PBS) (Milipore Sigma). Following incubation, the beads were washed with 3mL of 1% BSA in PBS three times using a magnet particle concentrator (MPC). The dynabeads were suspended for 1 minute with the MPC and the wash was discarded. The dynabeads were finally resuspended in the original volume of 1% BSA in PBS and stored at 4 C. Peripheral blood was cultured at a 1:1 or 1:4 ratio of beads: lymphocytes. For lectin-induced proliferation PBMCs were cultured with 2.5μg/mL of PHA (Milipore Sigma). For stimulation through the IL-2R, 1U/mL of recombinant human IL-2 (rhIL-2, Gibco) was used. Units per mL of hrIL-2 was calculated based on the reciprocal of the dilution of supernatant that resulted in half-maximal proliferation of the CTLL-

2 cells.[23,38] Finally, stimulation with PMA and ionomycin (Milipore Sigma) was performed at a final concentration of 10ng/mL and 134ng/mL, respectively.

Table 2.2 Stimulatory conditions for cell culture. The condition, final culture concentration, method of stimulation, source and location. *the source is referring to the Dynabeads; antibody sources and concentrations are listed in the text.

Condition	Final concentration	Stimulatory method	Source	Location
anti-CD3/anti-CD28 Dynabeads*	4 X 10^8 beads/mL	TCR	Invitrogen	Waltham, MA
PHA	2.5 µg/mL	TCR	Milipore Sigma	St. Louis, MO
PMA/ionomycin	10 ng/mL PMA/134 ng/mL iono	By-pass TCR	Milipore Sigma	St. Louis, MO
hrIL-2	1U/mL	IL-2R	Gibco	Waltham, MA

Intracellular Ki-67 for proliferation

TZL cases were identified through submission to CSU-CI laboratory from February 15, 2018 to April 8, 2018. The samples were handled as previously described[4] and the standard flow cytometry panel used at the CSU-CI laboratory was performed. Following diagnosis, cases with sufficient sample were processed for intracellular Ki-67 expression using Foxp3/Transcription Factor Staining buffer set (eBioscience) adapted from the previously reported protocol.[39] Zombie violet (ZV) dye (BioLegend, San Diego, CA) was used to identify dead cells. The ZV was diluted 1:1000 in PBS (0.1%) and the cell suspension was washed with 1 ml 1 x PBS. Samples were resuspended in 200µL of diluted ZV and incubated in the dark at RT for 30 minutes. Subsequently, cells were washed with flow buffer and the standard surface antibody staining protocol was performed. Surface antigens included: anti-CD5-PerCP-eFluor710 (T cell, clone YKIX322.2, Bio-Rad); anti-CD45-eFluor405 (pan-leukocyte, clone YKIX716.13, eBioscience); anti-

CD8-A700 (CD8 T cell, clone YCATE 55.9, Bio-Rad); and CD18 (human neutrophils, clone YFC118.3, Bio-Rad). Following surface staining and washing with flow buffer, the samples were resuspended in diluted fix and perm from the buffer set (1:4 dilution of eBioscience™ Fixation/Permeabilization concentrate to eBioscience™ Fixation/Perm diluent) and incubated overnight in the dark at 4 C. The next day, samples were pelleted and washed twice with diluted perm buffer (eBioscience™ Permeabilization buffer 10X in a 1:9 dilution with deionized water) and then resuspended in 200µL of diluted perm buffer. The samples were split into 2 tubes and blocked with 5µL of rat immunoglobulin and 5µL of canine Fc receptor binding inhibitor (eBioscience) for 10 min at RT. After blocking, 5µL of Isotype-FITC (Rat IgG2a, clone SeLA15, eBioscience) or 5µL of Ki-67-FITC (rat IgG2a Ki-67, cell cycle, clone SolA15, eBioscience) was added to each tube and incubated at RT for 30 minutes. Samples were washed twice with diluted perm buffer and resuspended in flow buffer and placed in cluster tubes to be analyzed. Dead cells were excluded by ZV and neutrophils were excluded by the expression of CD18. The percentage of Ki-67 positive cells was determined based on the isotype control.

Statistical analysis

Differences between non-stimulated and stimulated cells for each group, TZL, normal T cells in TZL, and normal peripheral blood were analyzed in R using a paired Wilcoxon rank sum test with significant values having a p-value of < 0.05. For Ki-67 data, a correlation matrix was computed in Prism 7.0 (Graphpad, La Jolla, CA) with nonparametric Spearman correlation. Significance was determined at a p-value of < 0.05, two-tailed. The difference in the percentage of Ki-67 positive TZL cells between blood and lymph node was analyzed using the Mann-Whitney test because the data did not pass normality. The Kruskal-Wallis test was used to

determine if there was a significant difference in the %Ki-67 TZL cells comparing TZL phenotypes, CD4+ vs. CD8+ vs. CD4-CD8-.

Results

TZL cells do not proliferate through the T cell receptor (TCR)

Canine peripheral blood mononuclear cells (PBMCs) in TZL cases and in dogs with no evidence of lymphoproliferative disease were labeled with CFSE and stimulated with either PHA (upper middle panel) or anti-CD3/anti-CD28 coated beads (Beads) (Fig 2.2, lower middle panel). Both stimuli triggered cell division by day 5 in the normal T cells (CD5+CD45+, Fig 2.2, blue) identified by loss of CFSE and increased cell size. The TZL cells (CD5+CD45-, Fig 2.2, red) did not show a decreased expression of CFSE with no stimulation or stimulation with PHA or beads and the cells remained intermediate in size (Fig 2.2, left, red). We analyzed proliferation by identifying the percentage of cells that were negative for CFSE, contained within the last decade, indicated by the orange line shown in Fig 2.2A. top panel and graphically represented in Fig 2.2B. The difference in the percentage of cells that were negative for CFSE in TZL cells with no stimulation (Fig 2.2, right side) versus PHA did reach significance but this difference was not meaningful and very low in both conditions (0.73 versus 0.27 %, Fig 2.2, right side, red). These findings support the hypothesis that because TZL cells lack CD45, they are not activated in a TCR-dependent manner.

At 5 -7 days of culture, there was a significant difference in the percentage of cells that lost expression of CFSE in TZL cells when stimulated with PMA, compared to TZL when not stimulated (Fig 2.2, right, $p = 0.004$). The TZL cells in culture with PMA also got much larger based on forward scatter (Fig 2.2, left side, bottom panel). These findings indicate that

stimulation through the TCR did not cause proliferation in TZL cells but stimulation could be provoked by bypassing the TCR in TZL cells.

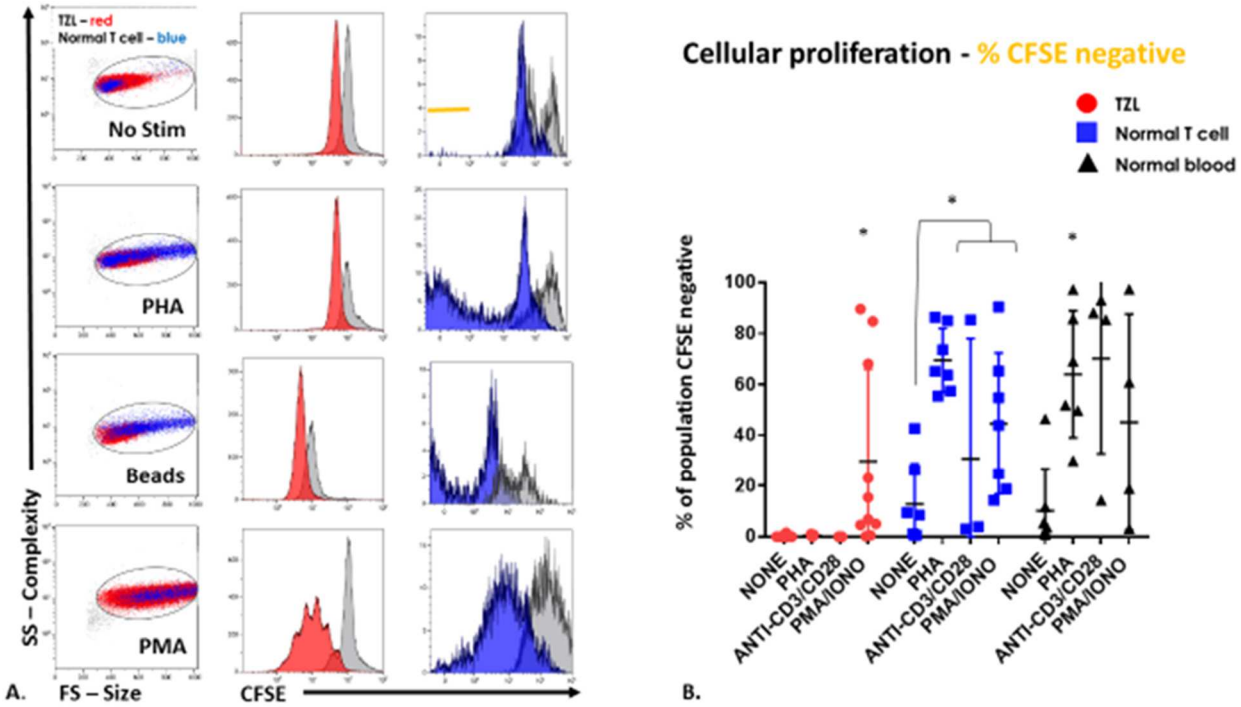


Figure 2.2. TZL cells do not proliferate when stimulated through the TCR. A.T cell proliferation in normal T cells and TZL cells in a dog after 5 days of culture measured by the loss of CFSE. Top panel: no stimulation, the normal T cells are small and the majority express high levels of CFSE after 5 days (blue). The TZL cells are intermediate in size and show consistent high expression of CFSE (red). Upper middle panel: in stimulation with PHA (stimulation through the TCR), the TZL cells show similar consistent and high expression for CFSE (red). The normal T cells are large by forward scatter and a large portion have lost expression of CFSE indicating cellular division (blue). Lower middle panel: in stimulation with anti-CD3/anti-CD28 conjugated beads (stimulation through the TCR), the TZL cells are positive for CFSE and do not show evidence of proliferation. The normal T cells (blue) have increased in size by forward scatter and a large portion have lost expression of CFSE. Bottom panel: in stimulation with PMA (bypass the TCR), the TZL cells have increased in size and have lost CFSE indicating proliferation at day 5 in culture (red). The normal T cells have also increased in size and lost expression of CFSE (blue). The area in grey on the histograms shows the starting level of CFSE in each condition at 24 hours after culture. B. The % of CFSE negative cells that are TZL cells (red) and normal T cells in the same culture (blue), and in T cells in normal blood (black) after 5 – 7 days of culture. Each point represents a different dog. There was a significant difference in the cellular proliferation of normal T cells stimulated with PHA, beads and PMA compared to no stimulation (blue, asterisk). There was only a significant difference in TZL stimulated with PMA versus no stimulation (red, asterisk). The orange bar in A. indicates the gate for determining the % for the CFSE negative cells (last decade). Red: TZL cells (CD5+CD45-); Blue: normal T cells in TZL cases (CD5+CD45+); Black: normal peripheral

blood with only CD5+CD45+ T cells. The error bars show the mean (horizontal line) and standard deviation of each set of data.

Normal T cells in a TZL environment maintain ability to activate through the TCR

There was an increase in the percentage of CFSE negative normal T cells (CD5+CD45+) in TZL cases and normal canine blood T cells between stimulated cells (beads, PHA, and PMA) compared to no stimulation and this difference was significant in all three conditions for the normal T cells in culture with TZL cells (beads $p = 0.0078$, PHA $p = 0.0078$, PMA $p = 0.0039$). The normal T cells in TZL and in normal blood also increased in size with stimulation compared to no stimulation (Fig 2.2, left, bottom three panels, blue). In the completely normal blood there was not a significant difference between no stimulation and PMA or beads (Fig 2.2, right side, black triangle); there was however a significant difference between no stimulation and PHA ($p = 0.03$). These results show that stimulation through the TCR is possible in canine T cells in normal blood and in canine T cells that express CD45 in TZL.

IL-2 signaling may be an alternative stimulatory method for TZL

IL-2, along with other mitogens, has been shown to augment T cell expansion with favored expansion of CD8+ T cells.[26,27] Canine T cells have also been shown to expand with IL-2 (rhIL-2).[23,28]TZL has previously been identified to express CD25, the IL-2α receptor (IL-2Rα).[4,6] We also found that CD25 mRNA was variably expressed in TZL cases (Chapter 1) and although not significantly different, the median count was higher in cases versus controls.

Canine peripheral blood mononuclear cells (PBMCs) in TZL cases and in dogs with no evidence of lymphoproliferative disease were labeled with CFSE and stimulated with hrIL-2 1U for 7 days. Samples were also stimulated with PHA as positive control for stimulation in the normal CD5+CD45+ T cells. hrIL-2 did cause loss of CFSE expression in both the TZL cells and the

normal T cells in many cases, but not all. The cases that often showed the most evidence for stimulation with hrIL-2 in TZL were cases that also had a higher percentage of cells expressing CD25 or showed a median fluorescence intensity of CD25 of ≥ log1.0 (Fig 2.3B, red boxes). For both TZL cells and normal T cells in culture, we analyzed the fold change of the median fluorescent intensity (MFI) of CFSE in cells with no stimulation over the MFI of CFSE in stimulated cells (either PHA or hrIL-2) (Fig 2.3B). There was not a significant difference in the MFI of CFSE fold change in TZL cells when comparing hrIL-2 versus PHA. However, there were 8 cases in which the cell count increased from day 1 to day 7 (range +297 to +473424, Table 2.3, pink), while in 3 cases the cell count decreased from day 1 to day 7 (range -39833 to -531826). In only 6 cases, the cell count increased with hrIL-2 stimulation compared to no stimulation (Table 2.3, pink).

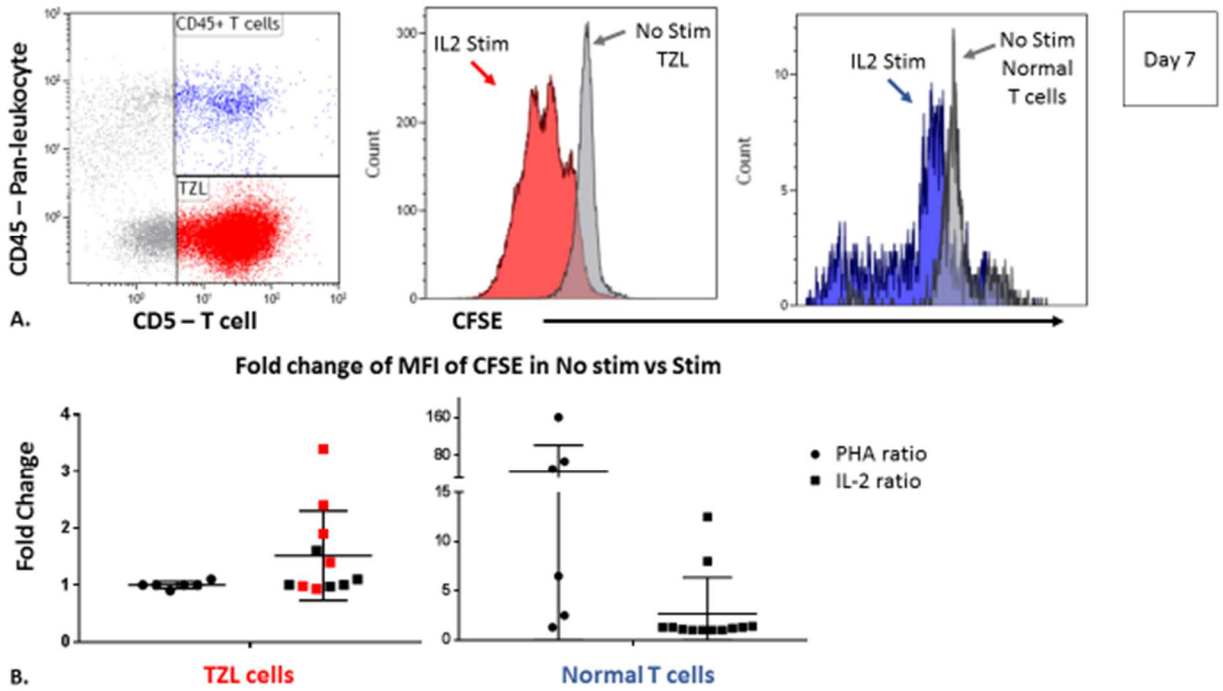


Figure 2.3. TZL cells can proliferate when stimulated with IL-2. A. Dot plot and histograms of a representative case of TZL at day 7 of culture identifying the normal T cells (CD45+ T cells) in blue and the TZL cells (TZL) in red. The TZL cells are CD5+ but do not express CD45- and the normal T cells express both CD5+ and CD45+. The histograms show the loss of expression of

CFSE, indicating proliferation, in the TZL cells in red middle panel, and the normal T cells in blue right panel. The area in grey is an overlay of the expression of CFSE at day 7 in cells that were not stimulated. There is evidence of proliferation in both the TZL cells and the normal T cells when stimulated with hrIL-2. B. The graph indicates the fold change (FC) of the median fluorescence intensity (MFI) of CFSE in non-stimulated cells/MFI of CFSE in stimulated cells at day 7 for both PHA (circles) and hrIL-2 (squares) stimulatory methods. The graph on the left is the FC of TZL cells and the red squares highlight the TZL cases with high CD25 expression (≥ log1.0). The second graph is the FC in the normal T cells in culture with TZL cases. The error bars show the mean (horizontal line) and standard deviation of each set of data.

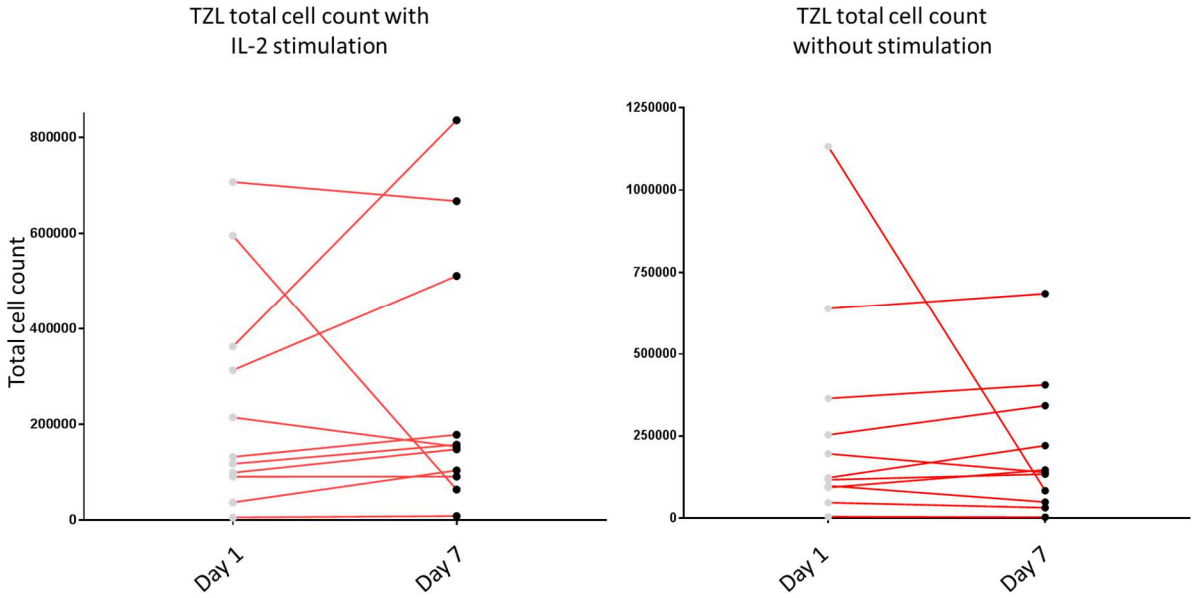


Figure 2.4 The total cell count of TZL cells in culture for 7 days with hrIL-2 (left) and with no stimulation (right). The grey dot is the total cell count 24 hours after plating and the black dot is the total cell count after 7 days in culture. The red lines connect each TZL case from day 1 to day 7.

Table 2.3. Total cell count in TZL cells stimulated with IL-2. The average total cell count and difference in duplicate wells for day 1 and day 7 of culture with no stimulation and stimulation with hrIL-2. The boxes highlighted in red identify cases with increased cell count at 7 days with IL-2 stimulation compared to no stimulation. *the cell count for day 1 with no stimulation was not obtained so the difference could not be calculated.

Case #	Avg Cell count day 1 with no stim	Avg Cell count day 7 with no stim	Difference in cell count with no stim	Avg Cell count day 1 with IL-2	Avg Cell count day 7 with IL-2	Difference in cell count with IL-2
81852*				214306	153000	-61306
81867	37307	83859	-1048315	595269	63443	-531826

83533	228502	220915	97764	131649	178041	46391
83536	401562	405780	41228	362306	835730	473424
84158	185440	147084	53346	98923	147689	48767
84160	316615	342777	88970	312835	511126	198290
84187	655909	685201	46289	706926	667094	-39833
84932	49539	49539	-49403	90359	90656	297
85287	121648	134421	17255	117093	157270	40177
85372	29801	31972	-15470	36703	103665	66962
85413	3641	3233	-1208	5245	8103	2857

TZL cells express low levels of Ki-67 in vivo

Ki-67 is a commonly used method to determine the growth fraction in neoplasia. This antigen detects a nuclear protein that is expressed in all stages of the cell cycle but is not present in resting cells (G0).[40,41] In human lymphoma Ki-67 has been employed as an independent prognostic determinant.[42-44] Recently, Ki-67 has been used in canine lymphoma to differentiate high and low-grade lymphomas by flow cytometry.[45]

The median % Ki-67 expressing TZL cells was 4.3 (interquartile range (IQR): 2.0 – 4.9). This was actually lower than the median % Ki-67 expressing CD45+ T cells in TZL cases, median = 8.3, IQR: 4.9 – 13.5 (Fig 2.5A) There was a significant difference in the %Ki-67 expressing TZL cells in lymph node versus blood samples (blood median %Ki-67 = 2.5; lymph node median %Ki-67 = 7.06; $p < 0.0001$, Fig 2.5B), but there was a not a significant difference in expression of Ki-67 based on phenotype (Fig 2.5C). There was a positive correlation between the size of the TZL cells and the %Ki-67 expressing TZL cells (Spearman $r = 0.5$, $p = 0.002$).

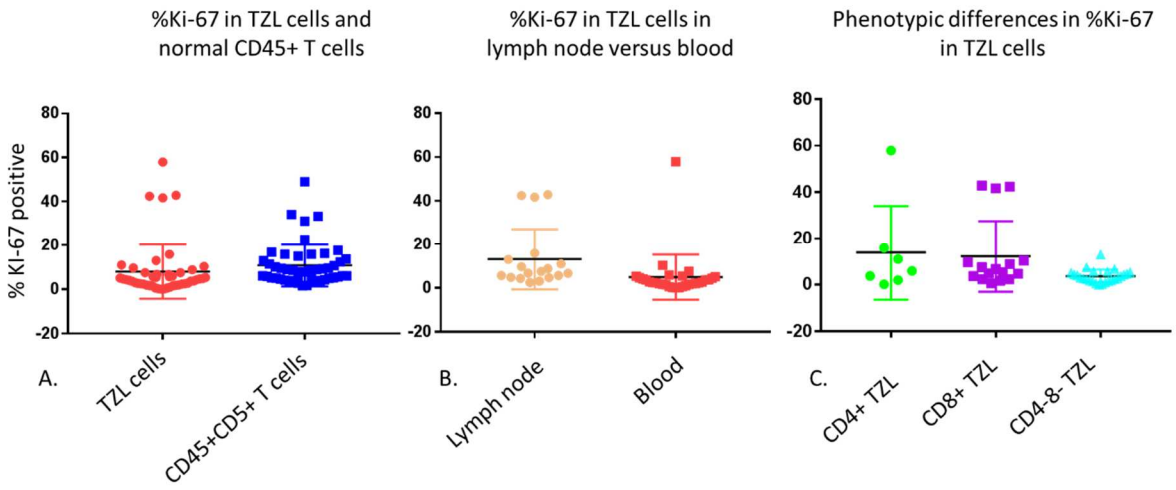


Figure 2.5. TZL cells express low percentages of Ki-67 overall. A. %Ki-67 expressing TZL cells (red) and normal T cells (CD45+CD5+ T cells, blue). B. %Ki-67 expressing TZL cells in lymph node (tan) samples versus peripheral blood (red) samples. C. %Ki-67 expressing TZL cells segregated by phenotype, CD4+(green), CD8+(purple), CD4-CD8-(aqua).

TZL cells do not appear resistant to cell death

We wanted to investigate if resistance to cell death could be a more prominent contributor to persistence of TZL cells above uncontrolled proliferative capacity. Cell viability in TZL cells remained high at 7 days of culture with no stimulation and stimulation with hrIL-2 (Day 7 median % viable: 93% with no stimulation and 93% with hrIL-2). Figure 2.6 graphically represents the % viable in TZL cells at day 7 in varying conditions. The 2 cases with the lowest viability with no stimulation were the same cases with the lowest viability when stimulated with hrIL-2. For one of these cases the viability was the lowest point for all conditions suggesting this case had an inherent cause for decreased viability. This was also the cases with the largest decrease in TZL cell number from day 1 to day 7 (Table 2.3). When reviewing this case, the sample was cultured the day after it was received but the peripheral blood appeared to be obtained at least 3 days prior and was shipped from the British Columbia. Because all of the samples were not treated exactly the same prior to arriving at our lab, there could be many

factors affecting cell viability including time in transit. However, the high percentage of viability in no stimulation and stimulation with hrIL-2 suggests the possibility for reduced susceptibility to cell death in TZL cells in these conditions.

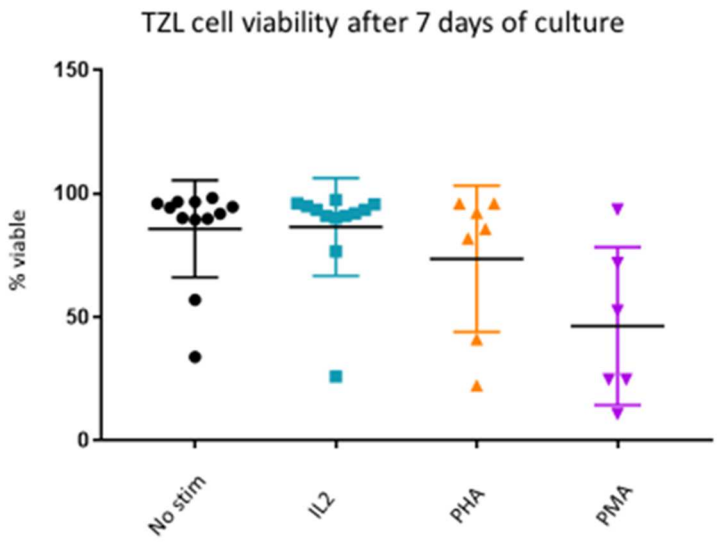


Figure 2.6. TZL cells have high viability after 7 days of culture with no stimulation and when stimulated with hrIL-2. The % viable of TZL cells (PI- TZL cells/Total TZL cells) after 7 days of culture in the following conditions: no stimulation (no stim), hrIL-2 (IL-2), PHA, and PMA/ionomycin (PMA).

In the case of IL-2 stimulation, there was proliferation with IL-2 in some cases determined by the loss of CFSE. The increase in TZL cell count was not remarkable and did not occur in every case. We theorized that the TZL cell count might increase more significantly if there were more time for a response. TZL cells were cultured for 21 days in media with no stimulation, as well as in media with hrIL-2 added every 7 days. In this system, every 7 days media was removed and replaced with fresh media with or without hrIL-2. The cells were harvested every 7 days and TZL cells were enumerated by flow cytometry. In two dogs with TZL, there was an increase in TZL cells with hrIL-2 stimulation at day 7; both of these cases were CD4-CD8- cases with the first expressing high levels of CD25, whereas the second expressed low

levels of CD25, MFI CD25= log4.3 and log0.1, respectively (Fig 2.7, magenta, 87364 and blue, 86450). In all 5 TZL cases, by day 21, the number of TZL cells had dropped significantly from the initial total cell count, and in every case there was at least one of the duplicate wells with less than 1000 total TZL cells in both non-stimulated and hrIL-2 conditions.

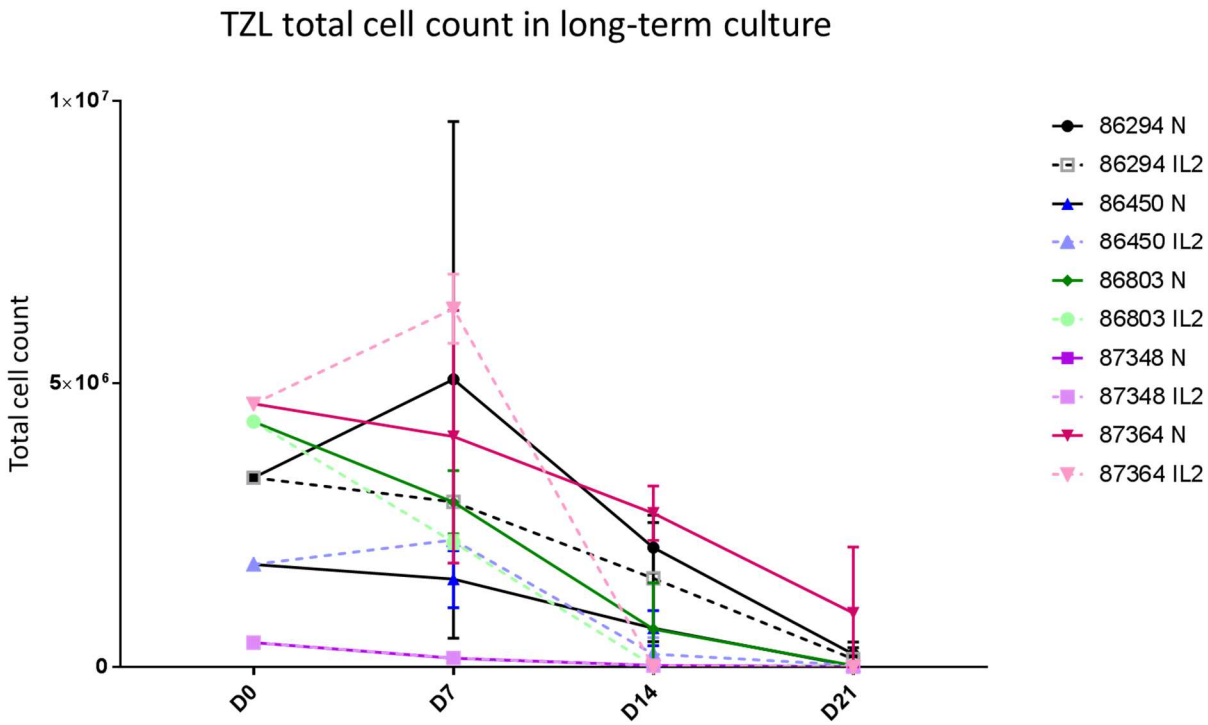


Figure 2.7. TZL cell do not persist in long-term culture. The total TZL cell count for 5 different cases. The cell count was measured in conditions of no stimulation (N, solid line) and simulation with hrIL-2 (IL-2, dotted line). All cases have decreased total cell numbers by 21 days in culture. D0 = day 0, D7 = day 7, D14 = day 14, D21 = day 21.

Discussion

In normal T cell function the TCR recognizes antigen presented by MHC class I or II[46] on the APC leading to the activation of signaling cascades, T cell differentiation and effector function, and immune response. This robust response is very specific for the inciting antigen and requires multiple checks and balances, including upregulation of numerous T cell surface receptors. These include but are not limited to CD4 and CD8 co-receptors that can initiate or

support TCR-MHC interaction,[47-49] CD2 and LFA-3 for adhesion,[50,51] and CD28 which binds to B7 molecules on APCs.[52,53] The TCR complex includes CD45, which is a transmembrane receptor that is expressed heavily on immune cells and is a necessary co-factor for T-cell activation by antigen,[54-57] and may be involved in the co-stimulating process of other co-factors.[58,59]

TZL is consistently identified by the lack of CD45 protein and gene expression.[4,5,60,61]Since CD45 has been shown to be necessary for T cell activation, we wanted to investigate mechanisms for TZL cell activation. As predicted, TZL cells did not show evidence of proliferation when stimulated through the TCR by two methods, anti-CD3/anti-CD28 beads or PHA, which causes cross-linking of the TCR (Fig 2.2). We were able to induce proliferation in TZL cells with PMA and ionomycin; this method bypasses the TCR and directly activates PKC and causes an influx of calcium.[25] However, *in vivo*, these second messengers are not a primary source of activation and require a signal from transmembrane receptors first.[62] Since this method does not normally occur *in vivo* we wanted to determine if there was a more biologically relevant method for TZL activation and proliferation. TZL cells have been described to express CD25,[4,6] the IL-2-receptor-α subunit and a marker of activation. In CD45-deficient cell lines, proliferation did not occur in response to antigen but IL-2 was still able to elicit a dampened response.[55] We hypothesized that TZL cells may be sensitive to stimulation with hrIL-2 because of increased expression of CD25. Activated T cells rapidly produce and express IL-2Rα which complexes with other IL-2R subunits to form a high affinity receptor with increased sensitivity to IL-2.[63,64] We often saw the most evidence for proliferation in TZL cases that showed increased expression of CD25, MFI ≥ log1.0 (Fig 2.3). hrIL-2 did not appear to induce a greater degree of proliferation than PMA/ionomycin based on the expression of CFSE

and did not induce proliferation in every case. Although, IL-2 may be involved in the proliferation in TZL cells, other unidentified factors are expected to be implicated in the pathogenesis of proliferation in this disease. For instance, macrophages and/or B cells may provide signals to TZL to cause proliferation. Interestingly, from the data in Chapter 1, the TZL cases had significantly increased expression levels of CD40 ligand (CD40L) compared to controls (TNFSF5; Fold change = 2.59; adj P-value = $4.6X10^{-6}$). CD40-CD40L interactions are vital for CD4 T cell effector functions including B cell differentiation and class switch, macrophage activation and cytokine production, and activation of T cells.[65] The interaction of CD40L on TZL cells and CD40 on B cells or monocytes/macrophages could be another mechanism to explore in the activation of TZL and pathogenesis of this disease.

We also measured intracellular Ki-67 in TZL cases for a better understanding of *in vivo* proliferative capacity. Previously, low Ki-67 in canine diffuse large B cell lymphoma has been defined as ≤ 20% by flow cytometry.[66] In human lymphomas a value of 45% or less was useful to distinguish indolent from aggressive lymphoma by histopathology.[44] In TZL, the Ki-67 expression was much lower with a median of 4.3 in TZL cells (CD5+CD45-) (Fig 2.5). *In vitro*, proliferation of TZL was variable and difficult to induce. The % of Ki-67 expression of TZL also does not suggest robust cell proliferation in this disease, which is similar to culture findings and may reflect the indolent nature of the disease.

Since there was some evidence for activation by hrIL-2 in TZL cells we wanted to determine if prolonged exposure to hrIL-2 would lead to more profound proliferation and increase in TZL cell number compared to what we found with 7 days of culture (Fig 2.4; Table 2.3). We also wanted to subject TZL cells to prolonged culture time to see if there was any

evidence for TZL cell resistance to apoptosis. We identified that in short term culture (7 days total), TZL cell had high viability when stimulated with hrIL-2 (Fig 2.6). The TZL cells in culture for 21 days consistently decreased in number by day 14, and by day 21 many cells were dead and there was only a small population of TZL cells remaining (Fig 2.7). IL-2 has been implicated in enhancement of AICD in T cells,[67] and has shown to activate transcription of FasL.[68] hrIL-2 may have led to enhancement of cell death in our culture system but the TZL cells also decreased in number in conditions with no stimulation. Furthermore, IL-2 priming has been show to sensitized T cells to AICD when activated with anti-CD3 antibody but not with PMA and ionomycin.[69] Since we have identified disruption of TCR signaling in TZL, IL-2 sensitization of AICD may not be a factor in these cells. Regardless, we were unable to maintain TZL cells in the presence of hrIL-2 *in vitro*. These findings further support the notion that there are likely other signaling factors that are necessary for TZL proliferation and maintenance. These factors could be produced *in vivo* within the tumor microenvironment and may involve other immune cells such as macrophages or B cells.

In conclusion, TZL cells were able to proliferate through TCR-independent mechanisms. In some cases of TZL, hrIL-2 was able to induce proliferation, however the cell count did not consistently increase in every case. With prolonged exposure to hrIL-2, we found that TZL cells did not increase in cell number after 7 days and TZL cells did not show evidence for resistance to cell death in 21 days of culture. Although IL-2 may be involved in TZL activation, it is likely that other factors are involved as well. Signaling from other cells in the lymph node could be involved in TZL proliferation. We did not investigate B cell signaling or inflammatory cell signaling in TZL cultures. Investigating TZL cell proliferative mechanisms in the lymph node may

provide more insight. Lymph node aspirates would contain cells of the microenvironment, such as B cells and dendritic cells, which might be involved in TZL cell activation. Alternatively, the use of APCs in culture might identify a possible role for microenvironmental cell signaling in TZL.

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CHAPTER 3: T ZONE LYMPHOMA CELLS DO NOT SHOW EVIDENCE FOR IMMUNOSUPPRESSION *IN VITRO*

Summary

T zone lymphoma (TZL) is not an aggressive disease but there is evidence for both genetics and environmental influence in disease development. There is also evidence for immunosuppression in at least a subset of dogs. Demodicosis is a follicular mite that can cause opportunistic infection in adult dogs and has been associated with immunosuppression. In TZL, about 10% of dogs present with demodicosis or an additional neoplastic condition. Although this disease is indolent, its influence on comorbidities has only begun to be examined, which would influence patient management. We investigated how TZL cells influence the tumor microenvironment *in vitro*. We characterize proliferation, and cytokine production in normal T cells within TZL microenvironment. We hypothesized that TZL cells would suppress normal T cells within the microenvironment. Contrary to what we expected, normal T cells in dogs with TZL proliferated at similar rates as normal T cells in dog without TZL. Additionally, IFN-γ production was not suppressed in normal T cells, and there was no difference between TGF-β production in peripheral blood TZL cases when compared with normal canine peripheral blood *in vitro*.

Background

Molecular profiling of neoplastic cells has greatly advanced the understanding of lymphomagenesis, but the interaction between the microenvironment and the tumor has also been demonstrated to be involved in pathogenesis and prognosis of cancer. Identifying functions of the neoplastic cell, as well as the interaction with the tumor stroma and non-

neoplastic cells, provides a more thorough understanding of the pathogenesis of disease in addition to more opportunities for targetable intervention. The tumor microenvironment has proven not only to be involved in preventing cancer development, but has been found to contribute to cancer progression.[1] In the tumor microenvironment, CD8+ cytotoxic T lymphocytes (CTLs) and CD4+ helper T (Th) cells combat cancer through production of IFN-γ and cytotoxic molecules such as granzymes and perforin.[2] During cancer development a state of equilibrium is reached in which there is a balance between the immune system and the cancer cells, with no appreciable growth of the cancer.[3] With progression, the tumor escapes the immune system through several mechanisms leading to clinical disease. In lymphomas several strategies of immune evasion have been identified, including loss of surface molecules that the immune system would normally use to recognize neoplastic cells such as MHC class I. For instance, in diffuse large B cell lymphoma, the loss of MHC I has been associated with fewer infiltrating CD8+ CTLs.[4] Tumor cells can also acquire surface molecules to dampen the immune response, for example, programmed cell death protein 1 (PD1) can be upregulated which inhibits T cells.[5,6] Immune evasion can also occur as a result of shifting the microenvironment toward a tolerant state, including promotion of regulatory T (Treg) cells.[7] TGF-β, produced by Treg cells or tumor cells, can lead to differentiation of naïve CD4+ cells to Treg cells, perpetuating immune suppression.[8] TGF-β inhibits proliferation of lymphocytes while IL-10 inhibits activation of macrophages and expression of co-stimulatory molecules.[9] In addition, tumors have been shown to alter the immune response from a Th1 to a Th2 reaction through TGF-β and IL-10 production.[10]

A number of small signaling molecules have also been implicated in regulation of the tumor microenviroment. Specifically, galectins have been investigated in numerous tumors due to immunosuppressive functions leading to tumor progression or immune escape. Galectin-1 and galectin-3 can bind to several T cell surface molecules including CD45, CD7, CD43, CD2, and CD3 leading to apoptosis.[14-17] Many different types of tumors have been shown to upregulate galectin expression and this is likely due to tolerogenic affects. For instance, in Hodgkin's lymphoma, neoplastic Reed-Sternberg cells have been shown to have increased galectin-1 levels resulting in an immunosuppressive Th2/Treg dominated microenvironment.[18] Additionally, in patients with leukemic cutaneous T cell lymphomas, increased galectin-1 levels have been demonstrated to promote a Th2 response leading to weakening of the anti-tumor response.[19] Th2 cytokines were found to be overproduced in patients with leukemic, cutaneous T–cell lymphoma (L-CTCL) with a strong Th2 bias.[20] The Th2 bias identified in T cells in L-CTCL was suggested as an underlying mechanism in patients' susceptibility to infection.[20] These patients not only have characteristics of a Th2-driven immune state, but they also have increased infection rates of *Staphylococcus aureus,* and increased susceptibility to cutaneous infections.[20]

Galectins promote a Th2 type environment through preferential cell death of Th1 and Th17 cells.[21] Specific modifications of oligosaccharides of CD45 are responsible for the control of T cell vulnerability to death.[22] In T cell activation, galectin-1 signaling and cell death were shown to be specific to surface glycans on Th1 and Th17 differentiated cells. In contrast, Th2 cells were protected from galectin-1 induced cell death by differential sialylation of N- and O-glycan on the cell's surface.[21] Galectin-1 has also been shown to promote production of the Th2

cytokines, IL-4, IL-5, and IL-10 with TCR induction,[23] causing suppression of chronic inflammation.[21] Furthermore, Th2 cells have been shown to promote Th1 cell apoptosis through secretion of galectin-1, thus behaving like an inhibitory cytokine.[23] Interestingly, upregulation of galectin-3 is cytokine specific and occurs with IL-2 and IL-4 but not with IFN-γ.[24] Not only does galectin-3 upregulation occur with Th2 cytokine production, but higher levels of galectin-3 were identified in Th2 cells compared to Th1 cells.[23] Galectin-3 can also bind to CD45 to modulate TCR signaling and survival.[25,26] Surprisingly, galectin-3 can act both extracellularly and intracellularly and can protect cells from death.[27,28] Intracellular galectin-3 has been shown to protect Th2 cells from apoptosis.[23] In another cancer-promoting mechanism, galectin-3 has been found to concentrate on CD45 on DLBCL cells and unexpectedly promote resistance to apoptosis.[29]

We have shown in Chapter 2 that TZL cells express high levels of galectins-1 and -3. TZL cells also express GATA3, the Th2 transcription factor and TGF-β. These features could support an immunosuppressive environment within the tumor. Interestingly, in TZL, 10-50% of dogs present with demodicosis.[30,31] Demodicosis is an opportunistic skin infection of hair follicle mites in dogs and in adult dogs the infection is often associated with an immunosuppressive state.[32] Another study reported 10% of TZL cases developing a second unrelated neoplasm.[33] These findings suggest that there is a degree of immunosuppression in a subset of dogs with TZL.

Given the unique phenotypic features of TZL, specifically the loss of CD45 in neoplastic T cells, we were able to isolate normal T cells and TZL cells in the same sample. Using these isolates we investigated whether the TZL cells could suppress CD45+ T cells. First, we wanted to

characterize cytokine production in TZL serum and TZL cell culture in order to compare *in vivo* and *in vitro* cytokine production in this disease. We also wanted to further investigate the increased expression of TGF-β identified in Chapter 2, to determine if there was increased production of TGF-β in TZL cell culture. Because of increased gene expression of TGF-β and galectins, we hypothesized that normal T cells in TZL cases would have hindered proliferation rates and IFN-γ production which we investigated through *in vitro* methods.

Methods

Cell culture methods

Peripheral blood cell culture as described in Chapter 2 was used for cytokine measurements and proliferation assays. For intracellular flow cytometry, lymph node aspirates were used with the same culture methods as described for blood in Chapter 2. The procedures for flow cytometry acquisition and analysis described in Chapter 1 were the same for initial diagnosis and case identification using the CSU-CI laboratory standard diagnostic panel. The antibodies used for surface staining of cells harvested from cell culture were the same as those used in Chapter 2. Surface staining included: CD5-APC (T cell, clone YKIX322.2, Bio-Rad, Hercules, CA), CD45-PB (pan-leukocyte, clone YKIX716.13, eBioscience, San Jose, CA), and CD8-A700 (CD8 T cell, clone YCATE 55.9, Bio-Rad). Normal T cells were identified by the expression of both CD5+ and CD45+, and TZL cells were identified by the lack of CD45 expression (CD5+CD45-). A known amount of CountBrite beads were added prior to sample acquisition, and cells were enumerated based on bead recovery.

Serum and culture cytokine measurement using canine multiplex

Serum cytokines were measured in dogs with TZL, dogs with small numbers of TZ (CD45-CD5+) cells, and control dogs with no TZ cells. Serum was provided from peripheral blood of Golden retrievers that was submitted for a case-control study of risk-factors for TZL through the CSU-CI laboratory. Dogs with small numbers of TZ cells did not have any clinical signs associated with lymphoproliferative disease such as lymphocytosis and lymphadenopathy, and these dogs are referred to as TZCUS (T zone cells of unknown significance; Labadie et al. in review JVIM) dogs. The TZCUS dogs had at least 1% TZ cells of the total lymphocyte population in their peripheral blood. If dogs had less than 1% TZ cells of the total lymphocyte population then they were considered negative for TZ cells and categorized as control dogs.

Culture supernatants were extracted from peripheral blood cell culture of both dogs with TZL and control dogs after 24 hours of culture with stimulatory methods described in Chapter 2. Using a 13-plex Milliplex MAP canine-specific assay the following cytokines and chemokines were measured in both serum and culture samples: GM-CSF, IFN-γ, KC, IP10, IL-2, IL-6, IL-7, IL-8, IL-10, IL-15, IL-18, MCP-1, and TNF-α (CCYTOMAG-90K, Millipore Sigma, Burlington, MA). Harvested serum and culture supernatants were stored at -80 C for a maximum of two years. Freeze-thaw cycles were limited to a maximum of two at the time of cytokine analysis. Analytes were measured in duplicates using the manufacturer's protocol. Briefly, 12.5µL from each duplicate sample was incubated with the panel of microspheres coupled to the capture antibodies listed above. Sample were then incubated with biotinylated secondary antibodies and streptavidin-conjugated PE. Following incubation a Bio-Plex 200 reader (Bio-Rad, Munich, Germany) with Luminex xMAP technology was used to quantify

cytokines, and calculation was performed with the Bio-Plex Manager Software 4.1.1. (Bio-Rad), which analyzes the Median Fluorescent Intensity (MFI) using a 5-parameter logistic regression curve fitting method to determine the concentration of each cytokine. The sensitivity limit for IFN-γ was 2.44pg/mL with a seven-point standard curve from 2.44 – 10,000pg/mL; for the remaining cytokines the sensitivity limit was 12.2pg/mL with a seven-point standard curve from 12.2pg/mL – 50,000pg/mL. Values below the detection limit were assigned a value equal to the minimum detectable concentration of each cytokine.

Cytokine measurement by ELISA

A DuoSet ELISA development kit (R&D systems, Minneapolis, MN) was used for quantitative analysis of IL-4 in culture supernatants following the manufacturer's protocol. Briefly, a 96-well microplate was incubated overnight with 100µL per well of diluted Capture Antibody (R&D systems). The next day, the plate was washed (5X) using an automated plate washer with wash buffer (0.05% Tween 20 in PBS, pH 7.2-7.4, R&D systems). The plate was then blocked with 300µL of Reagent Diluent (R&D systems) at room temperature (RT) for 1 hour. The plate was washed and then 100µL of sample or standards in and Reagent Diluent (1% BSA in PBS, pH 7.2-7.4, R&D systems) were added to each well in duplicates, then covered and incubated for 2 hours at RT. The plate was washed and 100µL of 200-fold dilution of Streptavidin-HRP was added to each well, then incubated for 20 minutes at RT while protected from light. After incubation, 100µL of the Substrate Solution (1:1 mixture of Color Reagent A [H_2O_2] and Color reagent B [Tetramethylbenzidine], R&D systems) were added to each well. When there was evidence of color titration in the standard curve (~20 minutes), 50µL of Stop

Solution (2 N H_2SO_4, R&D Systems) was added to each well. The optical density (OD) of each well was determined immediately using a microplate reader set to 450 nm.

Following the manufacturer's protocol, the canine IL-2 VetSet, ELISA Development Kit (Kingfisher Biotech, Inc; Saint Paul, MN) was used for determination of canine IL-2 in cell culture. The 96-well plate was coated with canine IL-2 and 50µL of standard or sample was added to each well in duplicate, covered and incubated at RT for 1 hour. The plate was washed with 0.05% Tween-20 in DPBS using an automated plate washer (5X). After washing, 50µL of Detection Antibody was added at a 1:23 dilution and incubated at RT for 1 hour, then the plate was washed. 50µL of streptavidin-HPR was added to the wells at a 1:20 dilution and incubated at RT for 30 minutes. After washing the plate, 50µL of TMB (3.3'.5.5'-tetramethylbenzidine, KingFisher Biotech, Inc.) substrate solution was added to each well and incubated in the dark at RT for 30 minutes. At this point, 50µL of the Stop solution (0.18 M Sulfuric Acid, KingFisher Biotech, Inc.) was added to each well and the absorbance was measured on a microplate reader at 450 nm.

TGF-β ELISA measurement

Following the manufacturer's protocol, the mouse/rat/porcine/canine TGF-β1 Quantikine ELISA (R&D systems) was used for measuring TGF-β in culture supernatants. Latent TGF-β1 was activated to detect immunoreactive TGF-β1 by adding 20µL of N HCl to 100µL of sample and incubated for 10 minutes at RT. The samples were then neutralized with 20µL of 1.2 N NaOH/0.5 M HEPES and assayed immediately. 50µL of Assay Diluent RD1-21 (R&D systems) was added to each sample's well, followed by 50µL of Standard, Control or activated sample. Each well was then incubated for 2 hours at RT, after which the plate was washed with an

automated washer (5X) and then blotted dry on paper towels. Following washing, 100µL of TGF-β1 Conjugate (R&D Systems) was added to each well and incubated for 2 hours at RT. The plate was then washed again and 100µL of Substrate Solution (R&D Systems) was added to each well and incubated for 30 minutes at RT while protected from light. Following this incubation 100µL of Stop Solution (R&D Systems) was added to each well with gentle mixing, after which the OD of each well was determined using a microplate reader at 450 nm. Since there was fetal bovine serum within the media, we measured the culture media by itself to determine background TGF-β1. We subtracted this background OD from the sample OD.

Intracellular flow cytometry

After lysis of lymph node aspirates the cells were resuspended in complete RPMI media as described in Chapter 2, and cells were manually counted using trypan blue to distinguish live cells. The samples were cultured at 5 x 10^5 cells/well (50 X 10^5cells/mL) in duplicate. Cells were either not stimulated or stimulated with PHA (2.5mg/mL, Millipore Sigma, St. Louis, MO) for three days. After 72 hours, PMA and ionomycin (10ng/mL and 1460ng/mL respectively; Millipore Sigma) in the presence of Brefeldin A (5µg/mL, Millipore Sigma) was added to half the stimulated wells and Brefeldin A alone was added to the other half of the stimulated wells and to the non-stimulated wells for 4 hours. After incubation, the cells were processed using the eBioscience Foxp3/Transcription Factor Staining Buffer set (eBioscience, San Jose, CA) adapted from the protocol described in Chapter 2 and previously reported[34] with the exclusion of Zombie violet dye. Briefly, the cells were first washed with flow buffer (PBS-2% FBS-0.1%NaAZ) and stained with surface antigens as previously described.[35] Cell surface antigens included: anti-CD5-PerCP-eFluor710 (T cell, clone YKIX322.2, Bio-Rad); anti-CD45-PB (pan-leukocyte, clone

YKIX716.13, eBioscience); and anti-CD8-A700 (CD8 T cell, clone YCATE 55.9, Bio-Rad). Following surface staining and washing with flow buffer, the samples were resuspended in diluted fix and perm from the buffer set (1:4 dilution of eBioscience™ Fixation/Permeabilization concentrate to eBioscience™ Fixation/Perm diluent) and incubated overnight in the dark at 4 C. The next day, samples were pelleted and washed twice with diluted perm buffer (eBioscience™ Permeabilization buffer 10X in a 1:9 dilution with deionized water) and then resuspended in 200µL of diluted perm buffer. The cells were blocked with 5% mouse serum (Jackson ImmunoResearch Laboratories, West Grove, PA) for 15 minutes at RT and subjected to intracellular staining with IFN-γ-Alexa488 (titrated to 1µL/reaction, 0.00001mg/µL, mouse anti bovine, clone CC302, Bio-Rad) for 15 minutes at RT. The cold-compete control was incubated with IFN-γ with no label, 10µL (0.0001mg/µL, mouse anti bovine IFN-γ purified, clone CC302, Bio-Rad) for 15 minutes prior to all samples receiving labelled IFN-γ. Blocking and intracellular staining were performed in 1x permeabilization buffer. After incubation the cells were washed twice with 1x permeabilization buffer and resuspended in 200µL of flow buffer. Gates were set using the cold-compete for each sample and confirmed with the fluorescence minus one control (FMO), which included all antibodies in the staining panel except IFN-γ-A88.

Statistics

For cytokine measurements, the data was analyzed by Prism 7.0 (Graphpad, La Jolla, CA). The data was assessed for normality using the Shapiro Wilk test, and was found not to be normally distributed. The non-parametric Kruskal-Wallis test was used to determine the significance across groups for each analyte and % IFN-γ production and the p-value was

adjusted for multiple comparisons by using the Dunn's multiple comparisons. A p-value of <0.05 was considered to be significant.

Results

Dogs with TZL do not express different cytokines in their serum than control dogs or dogs with TZCUS

Serum cytokine measurement was performed on 35 dogs with TZL, 45 dogs with TZCUS, and 32 control dogs with no evidence of TZ cells in their peripheral blood. For a number of cytokines, less than 50% of the dogs tested had detectable serum cytokine levels. This included GM-CSF, IFN-γ, IP10, IL-2, IL-6, IL-7, IL-10, and TNF-α. For IL-15, IL-18, KC and MCP-1, the cases had detectable levels of cytokines in over 50% of dogs within this group, while in all three groups more than 90% of dogs had detectable levels of IL-8. IL-8 is a specific neutrophil chemoattractant cytokine[36] that has been shown to be expressed and produced by neutrophils.[37] Since the sample was from peripheral blood, the neutrophils, rather than T cells, are likely responsible for production of this cytokine. The median and range (min and max) of cytokines for all three groups is summarized in Supplemental Table 3.1. There was no significant difference between cases, TZCUS dogs or controls for any of the measured cytokines/chemokines. In this system there was no evidence of increased IL-10 production in TZL, which if increased would be a possible mechanism of immunosuppression.

Inflammatory cytokines can be induced in TZL cell culture

In cell culture supernatants, detectable production of all cytokines was found in over 50% of samples in at least one of the conditions (no stim vs. stim). Interestingly, TNF-α was detectable in 100% of the cases that were stimulated and 92% of cases with no stimulation. In

examining culture supernatants from dogs with TZL we were unable to detect IL-2, IL-6, and IL-8 above the limits of detection for this assay, and production of IL-7, IP10 and IL-15 was just above the limits of detection with any of the conditions. The other cytokines that T cell can produce, and that we were able to detect, included INF-γ, IL-10 and TNF-α. Cytokine concentrations for these three cytokines in normal dogs and dogs with TZL are summarized in Supplemental Table 3.2**.** The fold change (cytokine concentration in the stimulated sample over cytokine concentration in the non-stimulated sample) for each condition is shown in Fig 3.1. There were no significant differences in IL-10 production in TZL cases in any of the conditions. The median concentration of TNF-α of TZL cases with no stimulation was significantly lower than in TZL cases stimulated with PMA ($p < 0.0001$) (Supplemental Table 3.2). Similarly, there was a significant difference in the median concentration of IFN-γ in TZL cases with no stimulation, or stimulation with beads, compared to TZL cases stimulated with PMA (no stim vs. PMA: $p = 0.0005$ and beads vs. PMA: $p = 0.03$) (Supplemental Table 3.2). There was no difference in PMA stimulated TZL cells with any of the cytokines compared to control cells stimulated with PMA. In the control dogs there was a significant difference in TNF-α production with PMA stimulation versus no stimulation ($p = 0.0013$) but no other significant differences. There was high variability in dogs both in the TZL cases and control cases, and there were fewer samples stimulated with PHA or PMA compared to no stimulation and stimulation with beads. A wide range of cytokine production has been previously detected in the serum of normal dogs, as well as many disease states including lymphoma.[38,39] There was no evidence for immunosuppression in TZL cases through the production of IL-10 *in vivo* or *in vitro*. IFN-γ and TNF-α production could be induced in TZL dogs with PMA. These are considered inflammatory

cytokines, and these cytokines have been shown to be induced with PMA/ionomycin in other species.[40] TNF-α production was also induced in control dogs with PMA/ionomycin. It is uncertain if the production of these cytokines in TZL cases is specific to TZL cells. Since the samples were not sorted originally, we are unable to determine if the production of these cytokines is from neoplastic TZL cells or normal CD45+ T cells in the sample.

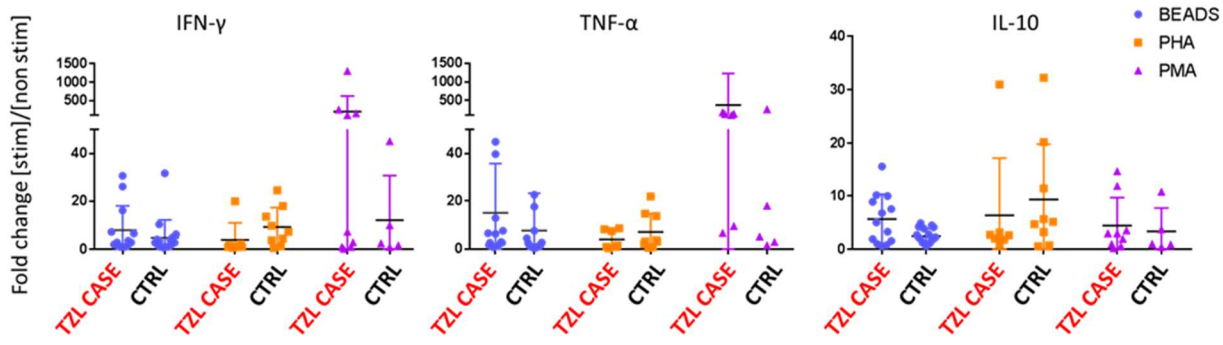

Figure 3.1. The production of IFN-γ and TNF-α in TZL cases is induced by stimulation with PMA. The fold change (concentration of stim/concentration of no stim) for TZL cases (red) and control (CTRL, black) peripheral blood cell culture. The stimulatory methods included anti-CD3/anti-CD28 beads (Beads, blue), PHA (PHA, orange), and PMA/ionomycin (PMA, purple). The supernatants were collected and assayed at 24 hours of culture.

With the canine multiplex the median IL-2 production was 12pg/mL, which is at the lower limits of detection for that assay. We attempted to get a better indication of IL-2 production with the use of an IL-2 specific ELISA. The lower limits of detection for the IL-2 VetSet, ELISA Development Kit (Kingfisher Biotech, Inc.) was only 60pg/mL. We assayed 2 samples of cultured, fresh, normal, control lymph node (LN), 2 samples of control peripheral blood cultures harvested at days 1 and day 3, and 3 TZL peripheral blood cultured samples harvested at days 1 and day 3. These samples included no stimulation, stimulation with PHA (LN only) and stimulation with anti-CD3/anti-CD28 beads. All samples were below the limits of detection. The concentration in LN control samples ranged from 19 – 40pg/mL; the

concentration in control peripheral blood samples ranged from 12 – 20pg/mL; and the concentration in TZL peripheral blood samples ranged from 12.5 – 48.4pg/mL. Since our samples were not within the lower limits of detection of these assays we did not continue to attempt to measure these cytokines with this ELISA

IL-4 was not an available cytokine in the canine multiplex; we attempted to measure this cytokine by ELISA. IL-4 is a Th2 cytokine and has been shown been shown to inhibit IFN-γ gene expression.[41,42] The canine IL-4 ELISA we pursued was not sensitive enough for our culture conditions. The assay has a reported sensitivity of about 100pg/mL. We made several attempts at a standard curve with a sensitivity of less than 100pg/mL, but were unsuccessful at getting a sensitivity reading below 300pg/mL. Even with these efforts, the OD readings for our samples were within the range of background OD readings. The samples we tested included cultured canine peripheral blood and lymph node in control dogs, in addition to dogs with TZL in both stimulated and non-stimulated conditions (Table 3.3). Only 5 samples had an OD in which a concentration could be calculated and these OD readings were at the limits of detection of this assay. Further, these samples were for control lymph nodes only; none of the cultured TZL cases had OD readings within the limits of detection (300pg/ml).

Table 3.3. IL-4 is below the limits of detection in TZL cases and controls. Samples of controls and TZL cases in conditions of non-stimulation and stimulation at 24 and 72 hours, the optical density (OD) at 450 nm, and concentration of IL-4 in pg/mL.*The concentration was calculated based on the standard curve obtained from the date the samples were run. Samples with a concentration below zero were not determined (ND).

CI number	sample type	condition	time of harvest (hr)	OD 450 nm	Concentration* pg/mL
66813	CTRL LN	no stim	24	0.06	308.01
66813	CTRL LN	PHA	24	0.04	189.53

66813	CTRL LN	Beads	24	0.05	269.24
66813	CTRL LN	no stim	72	0.02	124.92
66813	CTRL LN	PHA	72	0.01	70.36
66813	CTRL LN	Beads	72	-0.01	ND
55354	CD4-CD8-TZL	no stim	72	-0.01	ND
55354	CD4-CD8-TZL	Beads	72	0.05	236.04
62932	CTRL BLD	no stim	24	0.00	ND
62932	CTRL BLD	Beads	24	0.00	ND
62933	CTRL BLD	no stim	24	-0.02	ND
62933	CTRL BLD	Beads	24	-0.03	ND
62932	CTRL BLD	no stim	72	-0.05	ND
62932	CTRL BLD	Beads	72	-0.04	ND
62933	CTRL BLD	no stim	72	-0.04	ND
62933	CTRL BLD	Beads	72	-0.02	ND
55395	CD4-CD8-TZL	no stim	24	-0.04	ND
55395	CD4-CD8-TZL	Beads	24	0.02	114.20
55395	CD4-CD8-TZL	no stim	72	-0.05	ND
55395	CD4-CD8-TZL	Beads	72	-0.06	ND

TGF-β production is not induced in TZL

TGF-β gene expression was found to be significantly increased in TZL cases and discussed in Chapter 1. We wanted to measure TGF-β expression in TZL culture supernatants to determine if production of this immunosuppressive molecule could be a mechanism for immunosuppression in this disease. There were no significant differences in TGF-β production in TZL cases with multiple stimulatory methods including PHA, PMA/ionomycin and IL-2 (Fig 3.2), nor were there significant differences in TGF-β production in TZL cases versus the controls. Moreover, there was no correlation between TGF-β concentration and the total lymphocyte count, the white blood cell count, or the TZL cell count. Although we detected increased gene

expression of TGF-β in TZL cases we were unable to confirm induced production of this cytokine *in vitro*.

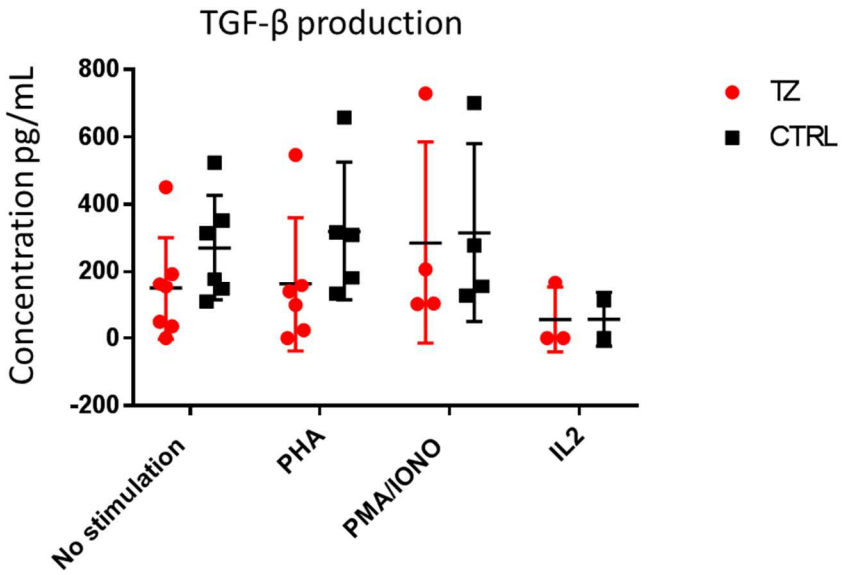

Figure 3.2. TGF-β production is not induced *in vitro* in TZL. The concentration measured by ELISA in pg/mL in cell culture supernatants of TZL cases (red) and control cases (black). These concentrations were adjusted for background TGF-β from culture media containing %10 FBS.

IFN-γ expression by normal T cells is not suppressed by TZL

IFN-γ is a pro-inflammatory cytokine that is produced by Th1 cells and is involved in Th1 differentiation, which can be repressed by GATA3.[43,44] First, we wanted to determine if the induced IFN-γ production identified by the cytokine multiplex was produced by the normal T cells, or the TZL cells. In order to answer this question, we used intracellular flow cytometry to identify which population of cells produced IFN-γ in the presence of PMA/ionomycin. Second, we wanted to determine if TZL cells were able to suppress IFN-γ expression in normal T cells, which would support our hypothesis of an immunosuppressive environment for this disease. We found that stimulation for three days with PHA, followed by 4 hours of stimulation with PMA/ionomycin, produced appreciable levels of IFN-γ in lymph node samples from control

dogs. The expression of IFN-γ was much higher after three days of stimulation compared with 1 or 2 days in two different control canine lymph nodes (Fig 3.3).

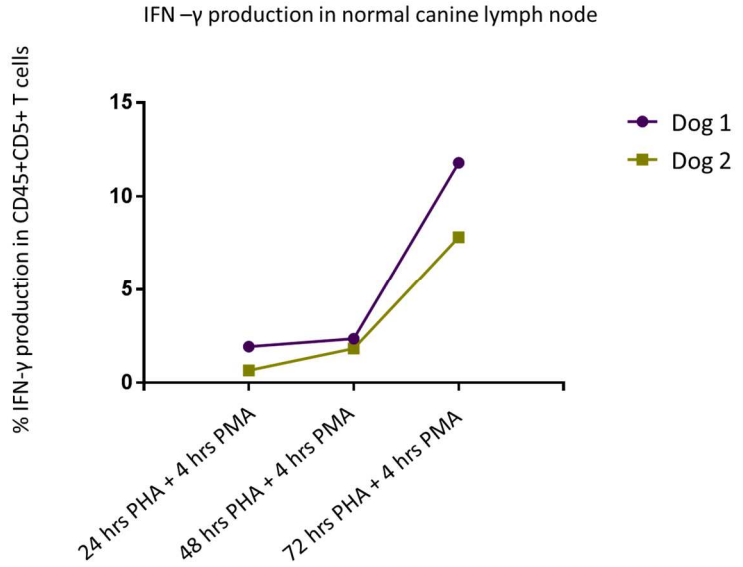

Figure 3.3. IFN-γ production is induced with PMA/ionomycin after 3 days of stimulation in normal canine lymph node tissue culture. Two normal canine lymph nodes that were stimulated for 24, 48, and 72 hours with PHA. At each of these time points the samples were stimulated with PMA/ionomycin for 4 additional hours prior to processing in the presence of Brefeldin A. The y-axis is the %IFN-γ expressing CD5+CD45+ T cells as determined by flow cytometry.

In TZL cases we found very little production of IFN-γ in CD45+ T cells and TZL cells with no stimulation, or with stimulation by PHA alone after three days. There was production of IFN-γ in the CD45+ T cells in all TZL cases when stimulated with PHA followed by PMA/ionomycin (Fig 3.4). There was a statistically significant difference in the CD45+ T cells that received additional stimulation with PMA/ionomycin, compared with those that received no stimulation (Fig 3.4; $p = 0.0009$). In three of the TZL cases there was also production of IFN-γ with the addition of PMA/ionomycin within the TZL cell population but this was not statistically significant.

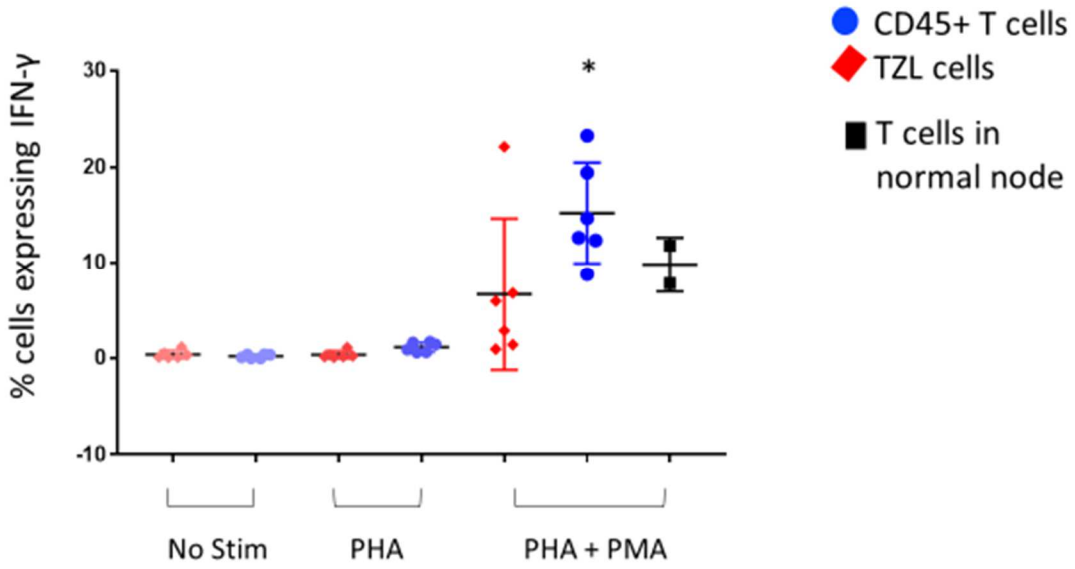

Figure 3.4. IFN-γ production is not inhibited by TZL cells. The percentage of CD45+ T cells (CD45+CD5+, blue) and TZL cells (CD5+CD45-, red) that express IFN-γ (γ-axis) after 3 days in culture with no stimulation or stimulation with PHA. After 72 hours the wells were spiked with Brefeldin A (no stim and PHA) for 4 hours, or Brefeldin A and PMA/ionomycin (PHA+PMA) for 4 hours, and then harvested and processed for intracellular IFN-γ by flow cytometry. The % of T cells expressing IFN-γ in two normal lymph nodes (black) with PHA and PMA/ionomycin is shown for reference of the potential IFN-γ production in normal dogs without TZL. The γ-axis is the %IFN-γ expressing cells as determined by flow cytometry.

There is an example IFN-γ production after three days of stimulation with PHA, followed by stimulation with PMA/ionomycin for 4 hours prior to harvest in a TZL case in Fig 3.5. The percentage of CD45+ T cells producing IFN-γ in TZL cases was higher than in the normal lymph nodes; for CD45+ T cells expressing IFN-γ: mean = 14.9% in TZL lymph nodes versus mean = 9.8% in normal lymph nodes (Fig 3.4). These findings suggest that TZL cells do not suppress the production of IFN-γ in CD45+ T cells *in vitro*.

TZL cells do not inhibit cell proliferation of CD45+ T cells

TZL cells were found to express high levels of TGF-β which has been found to inhibit IL-2 induced T cell proliferation.[45] We have previously shown in Chapter 2 that normal CD45+ T cells are still able to proliferate within culture with TZL cells (CD45- T cells). In proliferation

experiments described in Chapter 2, we noted that the proportion of normal CD45+ T cells within culture conditions increased with stimulatory conditions. Fig 3.6 is an example of one of the stimulatory experiments performed in Chapter 2. The normal T cells were also able to proliferate in all stimulatory conditions, unlike TZL cells which only proliferated in conditions independent of the TCR (Chapter 2, Fig 2.2).

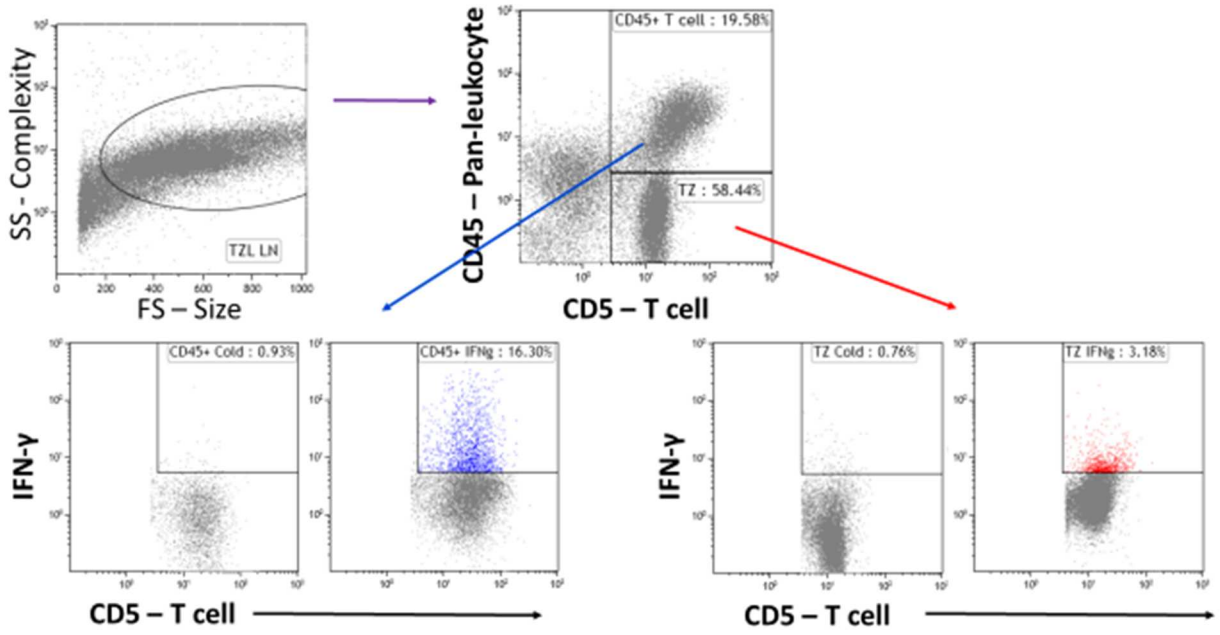

Figure 3.5. Gating strategies for identification of IFN-γ production in normal T cells and TZL cells *in vivo*. An example of IFN-γ production in a TZL case after 3 days of culture with PHA followed by 4 hours of stimulation with PMA/ionomycin. The CD45+ T cells and TZL cells are isolated based on expression of CD45. The CD45+ cold and TZ cold controls are shown and set to less than 1%. These gates are linked to the gates with colored events (blue and red). The % of CD45+ T cells (blue) and TZ cells (red) that are expressing IFN-γ are determined by the percent of cells within the gates set to the cold controls (Normal T cells IFN-γ = 16.30%; TZ cells IFN-γ = 3.18%).

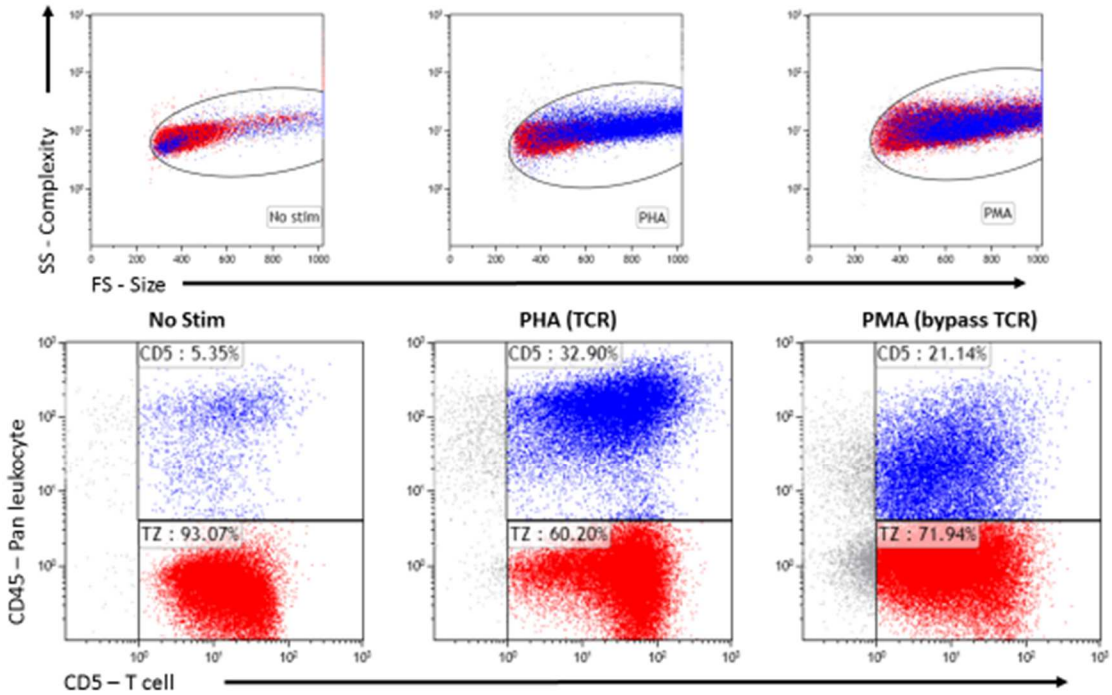

Figure 3.6. Normal T cell proliferation does not appear to be inhibited by TZL cells. Normal T cells (CD5+CD45+) are identified in blue and TZL cells (CD5+CD45-) are identified in red. The proportion of normal T cells increases from 5 to 32% with stimulation by PHA, and to 21% with stimulation by PMA and ionomycin. The top panel shows the complexity of cells on the y-axis and the size on the x-axis. The normal T cells enlarge with stimulation by both PHA and PMA/ionomycin whereas TZL cells only enlarge with PMA/ionomycin.

These findings suggest that TZL cells do not inhibit normal T cells from proliferating in culture. We wanted to investigate this assumption by determining the total cell count of normal T cells in culture. The total cell count of normal T cells increased in 4 of 5 cases stimulated with PHA, and in 8 of 12 cases stimulated with IL-2 (Fig 3.7). In normal peripheral blood, normal T cells increased in total cell count in 2 of 4 cases when stimulated with PHA and 2 of 6 cases stimulated with IL-2. Based on this information we do not have evidence that TZL cells suppress cell proliferation of normal T cells within the same environment *in vitro*.

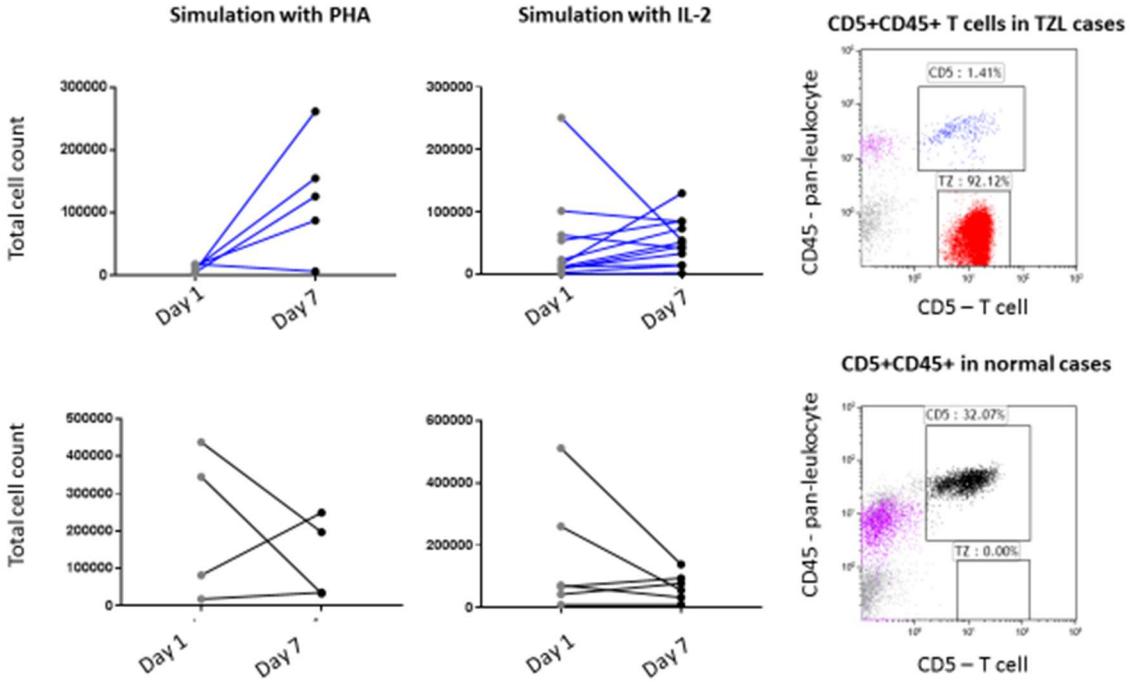

Figure 3.7. TZL cells do not show evidence for suppression of proliferation in normal T cells *in vitro*. The total cell count for normal CD45+ T cells in culture with TZL cells (top) and normal T cells in normal peripheral blood (bottom). The total cell count was determined at 24 hours (day 1) and 7 days after stimulating with either PHA (left) or hrIL-2 (right). The dot plots in the right-most panel are showing the identification of normal T cells that are CD5+CD45+ in TZL cases (top, blue) and CD5+CD45+ in normal blood (bottom, black) with no TZL cells.

Discussion

We did not find differences in serum cytokines in dogs with TZL versus TZCUS dogs or control dogs without TZL (Supplemental Table 3.1). Detection of serum cytokines can be difficult as sample handling can also affect detection. Serum samples are best kept on ice and spun down as soon as possible because many cytokines will begin degrading as soon as the sample is drawn.[48] Time of day, cortisol levels, and nutritional status can all affect serum cytokine concentration.[48] The accuracy of cytokine quantification in serum ELISA and multiplex assays was found to be poor, depending on the cytokine.[49] In serum samples, the detection of IL-2 was low at several tested concentrations;[49] one explanation for this is the presence of soluble IL-2 receptors in circulation.[50] Some of these factors could have affected detection of

cytokines in our samples. In a study of atopic dermatitis, IL-2 was detected in plasma in 9 out of 20 dogs with disease, but the values were below the limits of detection in 6 of those dogs.[51] The T cell growth factor cytokines, IL-2, IL-7, and IL-15 were also difficult to detect in the serum of dogs that received a single IV dose of *Escherichia coli* lipopolysaccharide.[52] We had similar difficulty in detecting T cell growth factor cytokines in TZL cases, TZCUS and control serum samples.

In vitro we did identify that IFN-γ and TNF-α could be induced with stimulation by PMA and ionomycin (Fig 3.1, Supplemental Table 3.2). These are considered inflammatory cytokines and in other mammals, PMA/ionomycin has been shown to induce Th1, inflammatory cytokines more efficiently.[40,53,54] We also attempted to measure IL-2 and IL-4 production in TZL but we did not have a sensitive enough method. IL-4 was detected in the serum in a study of dogs with atopy with no differences between disease and control dogs, and concentrations were in the 100 -200pg/mL range,[51] which is below the limits of detection of the assay we attempted to use. We were unable to optimize our assay to limits of detection below 300pg/mL which was not sensitive enough for expected production in cell culture. It would be interesting to determine if we could detect intracellular IL-4 in TZL cells; however, intracellular IL-4 has been measured in PBMCs of dogs with Leishmania infection with very low production (< 5%).[55] In normal dogs, the maximal expression of IL-4 of PBMCs stimulated with PMA/ionomycin was a mean of < 2% of CD4+ and CD8+ T cells.[56] IL-2 activity in dogs has also been measured using bioassays with canine IL-2 –dependent T cell blasts or murine interleukin-2 dependent T cell lines.[57,58] IL-4 and IL-2 were also detected in dogs with semi-quantitative RT-PCR with mitogen

stimulation, which is a sensitive method to detect cytokine levels.[58,59] These methods may be more successful at detecting IL-2 or IL-4 in our system.

The stimulatory methods can also have an effect on the cytokines produced. For instance, PMA/ionomycin has been shown to increase production of certain cytokines including IFN-γ, TNF-α, IL-2 and IL-6; however, IL-4 and IL-10 production were unaltered and produced at low concentrations.[53] Other studies have had similar findings with respect to stimulation with PMA/ionomycin and production of IFN-γ,[54] TNF-α and IL-10.[60] We were unable to confirm evidence of production of Th2 cytokines or immunosuppressive cytokines with ELISA and canine multiplex methods *in vivo* or *in vitro*.

Our original hypothesis was that TZL may induce immunosuppression in a subset of cases, based on the observation that demodicosis is present in at least 10% of TZL cases.[30,31] TGF-β expression has been found to be higher in the serum of dogs with demodicosis,[61] and we found high gene expression of TGF-β in TZL cases in Chapter 1. However, we were unable to induce TGF-β in our TZL cell culture system, and we did not find a difference in production of this cytokine in TZL cases versus control dogs (Fig 3.2). TGF-β expression in TZL may be affected by post-transcriptional regulation. TGF-β1 has been reported to have evidence for post-transcriptional and/or post-translational regulation as the message levels are not correlated with protein secretion.[62] Increased TGF-β1 expression is quickly induced in activated B and T cells yet secretion increases over several days.[63,64] We measured TGF-β production at 24 hours and we may find more significant differences if measured at a later time point. Alternatively, the *in vitro* culture conditions may not have accurately mimicked the tumor microenvironment

to induce cytokine production. Using serum free media might also aid in detection of small differences in TGF-β production in this disease.

Some of the most effective contributors of Th1 and Th2 differentiation are cytokines. Common examples are IFN-γ potentiating Th1 cells[65] and IL-4 leading to differentiation of Th2 cells.[66-68] Th1 and Th2 subsets potentiate their own differentiation with mutual inhibition of the other subset. For example, IFN-γ suppresses Th2 cell function while IL-4 and IL-10 inhibit development of Th1 cells.[41,69-71] TGF-β has also been shown to inhibit IFN-γ production.[72,73] Furthermore, GATA3 can inhibit IFN-γ production in Th1 cells.[74] Finally, exogenous galectin-1 and galectin-1 condition media has been shown to reduce production of IFN-γ.[19,23] We had previously identified that TZL cell express GATA3, TGF-β, and galectin-1 in Chapter 1. This gene expression profile led us to believe that TZL cells would inhibit the production of IFN-γ in cell culture and this could be a possible mechanism of immunosuppression in this disease. Contrary to what we expected, CD45+ T cells were able to produce IFN-γ in TZL culture, and we didn't find any difference in production of IFN-γ in CD45+ T cells in normal lymph nodes versus TZL lymph nodes (Fig 3.5). In some cases, TZL cells did express IFN-γ but this was not significantly different from cases with no stimulation. Additionally, the percentage of TZL cells expressing IFN-γ was lower than CD45+ T cells. In cutaneous T cell lymphoma in humans, the neoplastic T cells express increased Th2 cytokines and low levels of IFN-γ**.**[20] In this disease benign T cells, when removed from malignant T cells, show enhanced Th1 responses and decreased Th2 response. Similarly, T cells from healthy individuals that were co-cultured with neoplastic CTCL cells exhibited markedly decreased IFN-γ production**.**[20] Although the TZL cells did not suppress the phenotypically normal T cells in culture, it is possible that TZL cells would suppress T cells

from normal dogs and this could be a path to explore in the future. The 'normal' T cells in TZL may not be functionally normal and so we may be missing the possible immunosuppressive effects of TZL cells. However, the neoplastic CTCLs have been shown to produce Th2 cytokines,[20,75,76] which we have not been able to confirm in TZL cells.

As mentioned above TZL cells were found to express high levels of galectins in Chapter 1. Galectin-1 can cause death in activated T cells, whereas galectin-3 can also act intracellularly to prevent apoptosis in T cells.[27,28] Galectin-1 treatment has been shown to shift the immune response toward a Th2 phenotype with inhibition of T cell proliferation.[77-79] These features of galectins suggested that TZL might be able to suppress CD45+ T cells by decreasing proliferation and increasing galectin mediated cell death of these cells. To the contrary, CD45+ T cells were able to proliferate in the presence of TZL cells (Fig 3.6 and Fig 3.7). In this *in vitro* system we found no significant evidence for suppression of CD45+ T cells by TZL cells. Differential sialylation of N-and O-glycans on cell surface is related to susceptibility to galectin induced cell death.[21] Glycotransferases, such as a-2,6 sialyltransferase (ST6Gal1) may modify N-acetyl-lactoasmine sequences and prevent galectin-1 binding.[80] It is possible that TZL cells produce glycotransferases responsible for these additions, and that by-standard T cells are also glycosylated and protected from galectin-1 induced apoptosis. We did measured expression of ST6Gal1 in Chapter 1 and it was increased in TZL cases compared to controls, but not significantly.

We were unable to confirm immunosuppression of normal T cells in TZL using an *in vitro* model, despite clinical evidence and gene expression suggestive of immunosuppression in this disease. It is possible that rather than TZL inducing immunosuppression, chronic inflammation

and an immunosuppressive environment are involved in TZL development. Associations between inflammation and cancer development have been established in mammals, including vaccine-induced soft tissue sarcomas in cats[83] and implant-associated osteosarcoma in dogs.[84] In humans, there is also evidence of unresolved chronic inflammation being involved in promoting tumors though initiation and metastasis in several types of cancer.[11] Non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to reduce the risk of colon cancer development in patients with a genetic predisposition,[85] as well as the incidence of lung cancer in patients that were smokers.[86] Interestingly, a risk factor study of TZL found that dogs that were administration of omega-3 fatty acids were 3 times less likely to develop TZL (Labadie et al., manuscript under review). Since omega-3 supplements decrease inflammation, the protective association of this in TZL might support a role for inflammation in the development of TZL.

In conclusion, we did not find a difference in serum cytokine production in dogs with TZL. *In vitro* we were able to induce production of IFN-γ and TNF-α when stimulated with PMA/ionomycin in TZL cases. Based on lymph node cell culture in TZL the primary source of IFN-γ appears to be from the normal T cells in culture; however we were able to induce lower levels of IFN-γ production in TZL cells. Most strikingly, we did not see inhibition of IFN-γ production in the normal T cells in culture with TZL cells. Additionally, in TZL we did not find evidence for suppression of proliferation in normal T cells. TZL showed increased expression of immunosuppressive molecules, TGF-β, galectin-1 and -3, that are reported to be involved in suppression of proliferation, IFN-γ production, and causing apoptosis of Th1 and Th17 cells; despite the reported consequences of these molecules, we were unable to confirm any of these

features of immunosuppression in our cell culture system. We may need to enhance our *in vitro* system to more closely mimic the *in vivo* microenvironment or immunosuppression in this disease may need to be investigated more thoroughly *in vivo*.

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CHAPTER 4: INCREASED FREQUENCY OF CD45 NEGATIVE T CELLS (T ZONE CELLS) IN OLDER GOLDEN RETRIEVER DOGS[1]

Summary

T zone lymphoma (TZL) is characterized by the clonal expansion of T cells lacking expression of the pan-leukocyte antigen CD45 (TZ cells). A strong breed predisposition is observed in Golden retrievers. This study aimed to confirm aberrant CD45 mRNA expression and determine if Golden retrievers without clinical lymphoma have an increased frequency of circulating TZ cells. Gene expression analysis on confirmed TZL cases showed a significant decrease in CD45 expression compared to normal dogs. Peripheral blood samples from senior dogs, 242 Golden retrievers and 42 non-Golden retrievers, without evidence of lymphoproliferative disease were assessed for the presence of TZ cells by flow cytometry. Thirty-one percent of Golden retrievers had TZ cells compared to 14% of non-Golden retrievers. Thirty-four percent of Golden Retrievers with TZ cells had a clonal T cell receptor gamma (TRG) gene rearrangement. Interestingly 20% of Golden retrievers without TZ cells also had a clonal TRG rearrangement. Golden retrievers may have an increased risk of TZL due to an increased frequency of TZ cells.

Background

Canine lymphoproliferative disease characterization has improved with the use of ancillary diagnostics including immunocytochemistry, immunohistochemistry, flow cytometry, PCR for antigen receptor rearrangement (PARR) and, more recently, gene expression profiling.

[1]Chapter published as: Hughes KL, Labadie JD, Yoshimoto JA, Dossey JJ, Burnett RC, Avery AC. Increased frequency of CD45 negative T cells (T zone cells) in older Golden retriever dogs. *Vet Comp Oncol.* 2018.16(1):E109-E116

Many of the canine lymphoproliferative diseases mimic human disorders and efforts have been made to utilize the World Health Organization scheme when classifying canine lymphoma.[1,2] As these subtypes become more completely defined, some marked breed-specific predilections for different subtypes have become evident. For example, Boxers[3,4] and Golden retrievers[4] are highly represented amongst the group of dogs that develop an aggressive form of T cell lymphoma, histologically characterized as lymphoblastic lymphoma or peripheral T cell lymphoma not otherwise specified (PTCL-NOS).[3] Another example includes a recent study in which small breed dogs were found to be over-represented in the group of dogs that develops B cell chronic lymphocytic leukemia.[5] Breed-specific tendencies to develop particular disorders indicate a high likelihood for genetic risk factors underlying these diseases, and position the dog as a useful model for studying the role of genes in the development of lymphoproliferative disease.

T zone (or clear cell) lymphoma (TZL) is a variant of PTCL-NOS that is described in both humans and dogs.[6,7] TZL is characterized by neoplastic T cells expanding the paracortex and medullary cords and compressing the fading germinal centers.[1] The cells are small to intermediate in size with very rare mitoses.[1,8]

The prevalence of TZL is estimated as 3-14% of all canine lymphomas in two different large-scale studies[1,2] and there is a striking breed predilection in dogs, with 40% of all TZL cases being diagnosed in American Golden retrievers.[9] European Golden retrievers, which represent a discreet genetic group[10] do not appear to be over-represented in TZL.[11,12] The median age of dogs diagnosed with TZL is 10 years old in multiple studies.[9,11] Lymphadenopathy and lymphocytosis are both commonly associated with TZL, and the presence of TZL in the blood

does not appear to indicate a more aggressive clinical course.[9,11,13] The overall median survival time is reported to be 760[11] to 1022 days,[13] consistent with an indolent nature. The disease is often detected as an incidental finding of lymphocytosis and/or lymphadenopathy; interestingly 10 – 50% of cases present with or develop demodectic mange,[13,14] suggesting that the TZL may cause immunosuppression in a subset of cases.

TZL exhibits a unique phenotype: the neoplastic T cells do not express the pan-leukocyte antigen CD45, allowing for reliable diagnosis by immunophenotyping.[9] Recently this was corroborated with immunohistochemistry.[15] Additionally, real-time PCR for CD45 transcript and DNA showed almost complete lack of CD45 gene expression but presence of CD45 DNA.[15] CD45 is a transmembrane protein tyrosine phosphatase that has multiple isoforms that are abundantly expressed on the cell surface of all leukocytes.[16-18] Two major isoforms of CD45 have been recognized in canine T cells, with expression varying based on phenotype and activation status.[19] The T cell receptor (TCR) is closely associated with CD45, and CD45 expression is necessary for T cell response to antigen.[16]

TZ cells express increased levels of the complement receptor CD21,[9,20] which is commonly used to identify B cells in dogs. In previous studies, CD21 expression has been identified by flow cytometry[9,20] and with microarray in TZL,[21] however, expression was not compared to normal T cells in these studies. The neoplastic cells can express CD4+, CD8+, neither subset antigen,[9] or, rarely, both.[11]

Our laboratory is conducting a study of environmental and genetic risk factors for TZL in Golden retrievers. While screening control Golden retrievers (dogs $\geq$ 9 years old with no clinical evidence of lymphoma of any type) we discovered that many dogs had small but detectable

numbers of CD5+CD45- cells (TZ cells) in their peripheral blood. This observation caused us to ask if TZ cells are equally frequent in all dogs over the age of 9, or if this is a breed-specific phenomenon.

The objectives of this study are two-fold. First, we wanted to further validate the phenotype of TZ cells by examining mRNA levels for the proteins that characterize these cells: CD45 and CD21. Then, we wanted to determine the frequency of TZ cells in non-lymphoma bearing older Golden retrievers and other purebred dogs. We hypothesized that TZ cells would be found more frequently in Golden retrievers than other breeds reflecting the breed predilection observed for this form of lymphoma. Validation of this hypothesis would suggest that an early event in the development of TZL is loss of CD45 expression, and would pave the way for mechanistic studies of oncogenesis.

Methods

Selection of cases and controls for gene expression analysis

To investigate the aberrant antigen expression seen in TZL, we measured the levels of CD45 and CD21 mRNA from case and control samples. Case samples included blood or lymph node aspirates from 34 dogs diagnosed with TZL by flow cytometry through the Colorado State University Clinical Immunology (CSU-CI) laboratory between December 2013 and March 2015. These cases were not breed restricted. A diagnosis of TZL included greater than 60% of the lymphocytes characterized as TZ cells (CD5+CD45-cells) in lymph node samples or the presence of ≥5,000 TZ cells/µL in the peripheral blood of dogs with lymphadenopathy or lymphocytosis respectively. These criteria were based on previous publications in which the minimum criteria for the diagnosis of lymphoid malignancy was 60% or greater aberrant cells in a lymph

node,[22,23]or 5000 cells/µL or greater phenotypically homogeneous expansion of lymphocytes in peripheral blood.[5] The control group consisted of T cells purified from lymph node and thymic tissue collected from 8 young, healthy, hound mix dogs that were being utilized for a surgical continuing education course. All of these procedures were approved by the IACUC committee at Colorado State University.

Cell sorting for gene expression studies

TZL cases: TZ cells were purified from blood or lymph node of TZL cases by negatively selecting CD45-expressing cells. Briefly, based on the Miltenyi Biotec protocol, the nucleated cells were re-suspended in MACS buffer (PBS-0.5% BSA-2mM EDTA) resulting in a total volume of 100 µL after addition of the primary antibodies. All antibodies were purchase through Abd Serotec/Bio-Rad. Anti-CD45 PE (Pan-leukocyte, clone YKIX716.13) at a concentration of 0.01 µg/µL was added and the samples were incubated for 10 minutes in the dark at 4^{o}C, and then washed with MACS Buffer. The supernatant was removed and samples were resuspended in 80-90 µL of MACS Buffer and 10µL of anti-PE beads (Miltenyi Biotec, San Diego, CA). The samples were incubated for 10 minutes in the dark at 4^{o}C and washed with MACS Buffer. The supernatant was removed and the cells were resuspended in 500 µL of MACS buffer. For magnetic depletion of CD45+ cells, the 500µL cell suspension was loaded onto an LD column that had been placed into a MidiMACS separator. CD45- cells that were not bound by beads were collected and tested for purity by flow cytometry. Fig 4.1 shows an example of sorted CD5+CD45- (TZ) cells purified by this method. The median purity of the sorted TZ cells was 98%, and only one sample had lower than 90% purity (82%).

Controls: CD4+ and CD8+ T cells were purified from the lymph nodes and thymus of 8 healthy control dogs to be used as normal controls in a series of studies of gene expression profiling in T cell lymphoma. Cells were stained with anti-CD18-PE (Human CD18, clone YFC118.3), anti-CD5-FITC (T cells, clone YKIX322.3) and either anti-CD4-PB (CD4 T cells, clone YKIZ302.9) or anti-CD8-FITC (CD8 T cells, clone YCATE 55.9) and sorted by fluorescence activated cell sorting on a MoFlo cell sorter (Beckman Coulter, Fort Collins, CO). All samples were analyzed for purity, and only those samples with >90% of the desired cells were used for RNA purification.

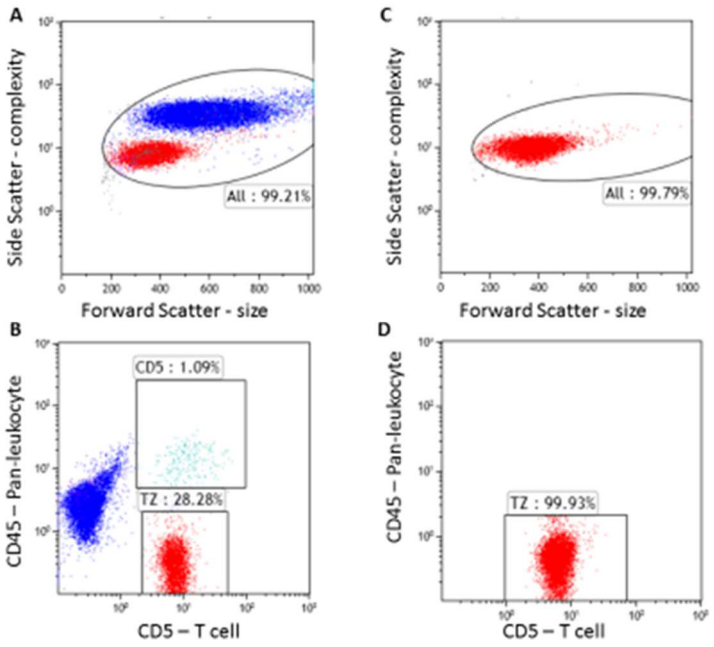

Figure 4.1. Example of TZ cells purified from one dog for gene expression. A. B., Plots from the peripheral blood in a TZL case before sorting for TZ cells, and C.D., are plots from the same sample of peripheral blood after purification of TZ cells. The TZ cells are in the TZ gate, normal T cells are in the CD5 gate. A. C., depict light scatter properties. B. D., depict expression of CD5 and CD45. After the sort the sample is composed of 99.93% TZ cells.

Gene expression using NanoString technology

Expression of CD45 and CD21 was measured using NanoString technology. NanoString technology was employed because it does not require high quantity or quality of RNA, such as RNA derived from convenience clinical samples that have been shipped and then further manipulated once arriving in the laboratory. Only 100ng or less of total RNA is needed, the detection method has a 0.1-0.5 fM detection limit and the assay is reproducible (R^2 = 0.999 average for replicates).[24] Gene expression is measured by counting the number of transcripts for each gene of interest, rather than using relative expression as is done with microarrays, and there is no PCR amplification step.

After sorting, the cell fractions were suspended in RNA lysis buffer prepared according to the Purelink RNA mini kit (Life Technologies, Carlsbad, CA) and stored at -80^{o} C until analysis. RNA samples were analyzed for degradation and concentration using Agilent RNA ScreenTape assay (Agilent Technologies, Santa Clara, CA). Samples had concentrations ranging from 1250 – 129000pg/µL and RNA integrity numbers (RIN) ranging from 2.6 to 7.6. Approximately 100ng of purified RNA per sample was hybridized overnight to the mRNA custom built probes and hybridized reactions were loaded into the nCounter Digital Analyzer (NanoString Technologies, Seattle, WA) through the University of Arizona Genetic Core.

Probes were designed based on the predicted sequence of Canis lupus familiaris CD45. While multiple CD45 isoforms have been annotated in the dog, only two isoforms have been shown to be expressed in the dog. The difference in these two isoforms is the presence or absence of exon 4, which is included in the larger molecular weight form of CD45.[19] Exon 4 is expressed by naïve T cells, but not expressed in the majority of activated and memory T cells in

humans.[16,25] Therefore, two probes were designed to detect CD45 expression (Fig 4.1, Table 4.1). The probe designed for PTPCRb was a pan-CD45 probe predicted to bind to both demonstrated canine isoforms[19] as well as all predicted possible canine isoforms by analogy with mouse and human studies.[18] The second probe, PTPRCa, was predicted only to bind to the higher molecular weight isoform of CD45 (Fig 4.2).

The CD21 probe was designed to detect all three predicted canine isoforms of this protein (Table 4.1). Gene expression was normalized using 6 housekeeping genes (Table 1) which were chosen from a group of 15 housekeeping genes because they exhibited low variability between dogs.

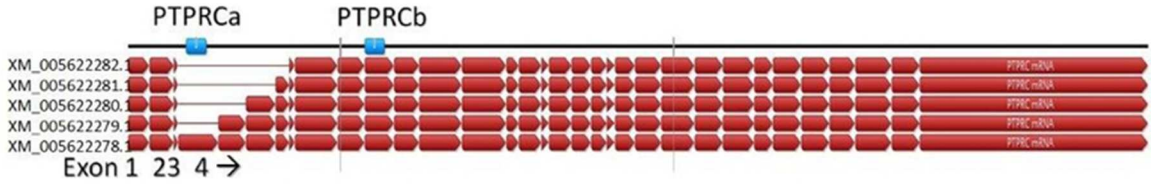

Figure 4.2. CD45 splice variants. Graphic depiction of CD45 mRNA, predicted splice variants and mapping of designed probes for PTPRCa and PTPRCb. Each red arrow represents an exon, and the first 4 exons are enumerated.

Table 4.1. Probe design for CD45, CD21, and housekeeping genes.		
Gene Symbol	**NCBI reference sequence**	**Target region of sequence**
PTPRC (CD45b)	XM_005622278.1	1134-1233
PTPRC (CD45a)	XM_005622278.1	279-378
CR2 (CD21)	XM_005622319.1	2436-2535
Housekeeping genes		
GUSB	NM_001003191.1	1363-1462
SDHA	XM_535807.4	1641-1740
HPRT1	NM_001003357.1	177-276
EEF1G	XM_848484.3	1066-1165
TBP	XM_005627736.1	666-765

POLR2A	XM_852751.1	671-770

Selection of dogs for quantifying CD45-negative T cells in peripheral blood

The frequency of TZ cells in the peripheral blood was assessed in two populations of dogs: Golden retrievers (GRs) and non-Golden retrievers (non-GRs). GR samples were obtained from the control group of a case-control study of risk factors for TZL being conducted by the CSU-CI laboratory. Samples from dogs recruited June 2013 to April 2015 were utilized**.** Criteria for controls for this study included Golden retrievers, 9 years of age or older, with no suspicion or history of lymphoproliferative disease and no lymphocytosis. Other medical conditions, including cancers other than lymphoma/leukemia, did not exclude dogs from the study. Clinical signs were reported by veterinarians based on physical exam findings and history obtained during sample submission. Non-GR dogs were selected from dogs presenting to the Colorado State University Veterinary Teaching Hospital (CSU-VTH) between April 2014 and January 2015. Shih tzus, which were reported to be the second most frequent breed to develop TZL,[9] were excluded from this study and will be part of a separate investigation. Medical records were reviewed to identify purebred dogs, 9 years and older. Dogs with prior diagnosis or evidence of lymphoma/leukemia (lymphocytosis, etc.) were excluded from the study. Age for all dogs was calculated from the date of birth provided to the date of sample received and were rounded to the nearest year. Since we were detecting small numbers of TZ cells, we wanted T cell numbers to be adequate. In order to avoid small numbers of T cells leading to false negatives, absolute lymphopenia identified on the complete blood cell count (CBC) resulted in exclusion from the study.

Immunophenotyping

Routine CBCs were performed by the Clinical Pathology Laboratory at the CSU-VTH and included both automated cell counts (Advia 120 Hematology Analyzer, Siemans, Tarrytown, NY) and blood smear evaluation for assessment of cell morphology and a manual white blood cell differential count.

Immunophenotyping by flow cytometry was performed on peripheral blood collected in EDTA tubes with a panel of antibodies used for cellular labeling, as previously described[9] and listed in Table 2. Antibodies included CD45 (pan-leukocyte), CD21 (B cells) and CD5 (pan-T cell). CD45 expression was assessed in the same staining reaction as CD5 and CD21. All data analysis for flow cytometry was performed with Kaluza software (Beckman Coulter, Brea, CA). Samples were considered positive for TZ cells if the percentage of TZ cells was greater than 1% of the total population of all lymphocytes (T and B cells). If 1% or fewer of the lymphocytes were TZ cells the dog was considered to be negative for TZ cells and was categorized as such in both the GR and non-GR populations.

Table 4.2. Antibody panel for flow cytometry analysis. Unless otherwise noted, all antibodies were purchased from AbD Serotec. Clones are as follows: CD45 = YKIX716.13, CD18 = YFC118.3 (human CD18), CD4 = YKIX302.9, CD8 = YCATE 55.9, CD5 = YKIX322.3, CD21 = CA2.1D6, CD22 = RFB4 (human CD22, purchased from AbCam), CD3 = CA17.2A12, CD14 = TUK4 (human), class II MHC = YKIX334.2, CD34 = 1H6, CD25 = P2A10 (purchased from eBiosciences).

Tube	Antibody Specificity and Fluorochrome
1	M IgG1-FITC/M IgG1-PE/M IgG1-Alexa 647/M IgG1-Alexa 700/M IgG1-PE-750/M IgG1-Pacific Blue
2	CD3-FITC/CD25-PE/CD5-APC/CD8-Alexa 700/CD4-Pacific Blue
3	Class II MHC-FITC/CD22-PE/CD21-Alexa 647
4	Class II MHC-FITC/CD34-PE/CD5-APC—CD14-PE-Alexa 750
5	Class II MHC-FITC/CD18-PE/CD5-APC/CD14 PE-Alexa 750/CD4-Pacific Blue
6	CD5-FITC/CD45-PE/CD21-Alexa 647

Clonality testing

The presence of a clonally expanded lymphocyte population was detected by PARR for the TRG as previously described[26] with modifications noted in supporting information (Table 4.3 and Fig 4.3). The presence of a clonally expanded lymphocyte population was detected by PARR (PCR for clonal antigen receptor rearrangements) as originally described in Burnett et al,[9] but with substantial changes that were established in 2012. These changes reflect increased knowledge of the structure of the T cell receptor gene and gene usage. [27-29]

Genomic DNA was extracted from clinical samples with the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. A "touchdown PCR" protocol was utilized. Initial denaturation and polymerase activation were at 95° for 15:00. Cycling conditions were: 10 cycles of 94° for 0:30, 64-59° for 0:30 (annealing temperature decreased by 0.5° every cycle) and 72° for 1:30, and 30 cycles of 94° for 0:30, 59° for 0:30 and 72° for 1:30 with a final extension of 72° for 7:00. Genescan analysis was carried out on an ABI 3130xl system calibrated for use with Applied Biosystem's five-dye chemistry, the DS-33 Dye Set (G5 filter set)(Life Technologies/Applied Biosystems, Carlsbad, CA). One µL of a ten-fold diluted PCR product was mixed with 14.5µL HiDi formamide and 1µL of GS600LIZ standard for each reaction to be analyzed. Data were analyzed with GeneMarker software (Softgenetics, State College, PA).

The PARR assay was carried out in two separate reactions. The primers for each reaction are shown in Table 4.3. The amplification reaction containing the immunoglobulin gene primers also includes amplification of the rhodopsin gene in order to verify the presence of amplifiable DNA.

The amplification reaction containing the TCR gene primers was carried out in a second reaction. This reaction results in the amplification of 3 V gamma gene families, which are distinguishable by the size of the PCR product and the dye attached to each primer (Table 4.3). A result was considered clonal if amplification of any given family resulted in from 1 – 4 clonal peaks that were 3x the height of the baseline. Examples of results are shown in Fig 4.3. This assay detects clonal T cells in 88% of flow cytometrically[9] confirmed T zone lymphomas (n = 193, unpublished results).

The amount of blood used for each reaction was 200µL and a result was considered clonal if one or two peaks were seen in the electropherogram that were greater than 3x the baseline. Equivocal clonal TRG gene rearrangements were identified when the peak height did not quite reach the 3x threshold, but was nonetheless prominent.

Table 4.3. PCR Primers for the T cell receptor for the PARR assay

Primer name	**Gene targeted**	**Sequence**	**Dye**
TCRG			
JGG1	VG2	CCCTCCTGTTTCCCGGTA	Fam (large product)
DPD	VG7	ARGCCATGTACTACTGTKCCTG	Fam (small product)
JGG12	VG3	CCCCAGGCACTTCAGTCTAC	Ned
JGG7	JG	TAACCCTGAGMAYTGTGCCA	
DPG	JG	TAACCMTGAGCTTTGTGCCA	
DPF	JG	CCTTGTCCAAATATCTTGATCCA	
JGG11	JG	MCTTCTGTAAATWTCTTGATCCA	

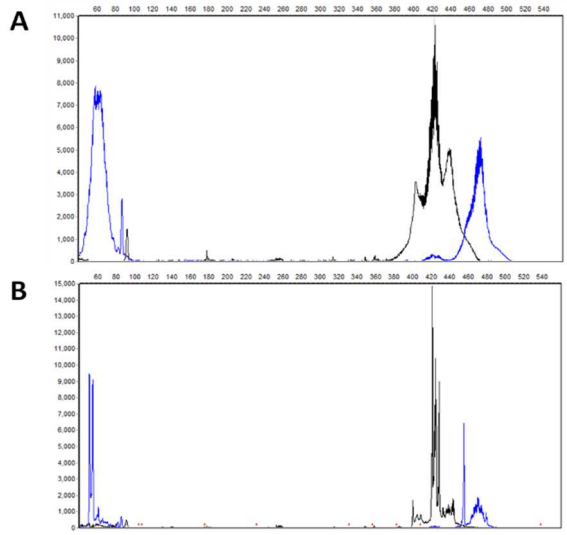

Figure 4.3. Examples of polyclonal (negative) and clonal (positive) PARR results. A. Amplification of a reactive lymph node using the primers described in Table 4.3. The low molecular weight PCR products in the 60 nucleotide range are amplified with V gamma 7 primer and the higher molecular weight PCR products (blue) in the 470 nucleotide range are amplified with V gamma 2 primer. The PCR products in the 420 range (black) are amplified with V gamma 3 primer. B. Blood from a GR with TZ cells in the peripheral blood revealing clonal products detected by all three V gamma primer.

Statistical analysis

nCounter software (NanoString technologies) was used to carry out the normalization of mRNA levels in the gene expression study. To compare gene expression between cases and controls, a Student's *t* test was performed (Prism 6; Graphpad, La Jolla, CA). The gene expression value was reported as the absolute number of transcripts, which was transformed to the signal $\log_2$ ratio (a signal $\log_2$ ratio of 1 is equal to a fold change of 2) to determine the p-value. To compare the prevalence of TZ cells in the peripheral blood of GRs versus non-GRs and the percentage of GRs (with and without TZ cells) with a TRG clonal result, a Pearson's Chi-squared test was performed. The difference in CD21 expression between TZ cells and normal T cells in the same dog was calculated using a paired Wilcoxon test of the log median fluorescence intensity. The scatter size of each population was compared using a paired t-test.

Differences in breed, sex, and hematological characteristics were analyzed using Pearson's Chi-squared tests (Prism 6; Graphpad) Median age between groups was compared using the Mann-Whitney test. Statistical significance was defined as $p< 0.05$.

Results

Gene expression of CD45 and CD21

We first wanted to validate the observation that TZ cells downregulate CD45 and upregulate CD21 by measuring mRNA levels, since expression of these two proteins has been described as unique features of TZL,[9,15,20,21]Forty-eight samples (14 sorted T cell controls and 34 TZL cases) from lymph node, peripheral blood, and thymus (controls only), were analyzed for mRNA counts using NanoString technology.

The results showed that the mean log_2-transformed CD45 mRNA counts in TZ cells was 5.2-8.4 fold (log2 fold change) lower than in controls ($p < .0001$, Table 4.4) depending on the isoform detected. CD21 mRNA counts were more variable among TZL cases, but the median value was 3.3 fold higher than CD21 mRNA counts in controls (Table 4.4) ($p < .0001$). These findings indicate that mRNA levels reflect protein expression, and verify that the antibodies used for these studies are recognizing the appropriate corresponding proteins. Furthermore, the results support previous findings that no variant of CD45 is expressed by the TZ cells.

Table 4.4. Expression of CD45 and CD21 in T zone lymphoma cases and controls.
[a]Normalized mRNA counts. [b]IQR, Interquartile range

	Cases (Median[a])	IQR[b]	Controls (Median)	IQR	log2 fold change	p value case vs ctrl
CD45a	168.8	129.3-491.3	6113.0	4840-7476	-5.2	<.0001
CD45b	124.3	32.75-1165	43190.0	40740-48800	-8.4	<.0001
CD21	814.7	460-1051	83.5	53.7-300.9	3.3	<.0001

Frequency of TZ cells in senior GRs and non-GRs purebred dogs

We enumerated TZ cells in 242 peripheral blood samples from GRs. Seventy-four of these dogs (30.6%) had variable numbers of TZ cells in their peripheral blood. This was significantly higher than the percentage of non-GRs with TZ cells in the peripheral blood ($p = 0.03$). The percentage of TZ cells varied from 2–95% of all T cells, median = 18.1% (Fig 4.4A.,B.,E). The median fluorescence intensity (MFI) of CD21 in TZ cells (MFI = 12) compared to normal T cells in the same dog (MFI = 0.55) was significantly higher (median difference = 7.0, $p < 0.0001$) (Fig 4.5). In GRs, the size of TZ cells (median size = 361) was also significantly larger than normal T cells (median size = 342) with a median difference of 15.21 ($p = 0.0003$, not shown).

Of the 42 non-GRs, there were 6 (14%) dogs with small numbers of TZ cells identified by flow cytometry and 36 dogs without TZ cells. Among the 6 dogs with TZ cells, the proportion of TZ cells ranged from 6-20% of all T cells, median = 17.4% (Fig 4.4C.,D.,E.). In dogs with TZ cells, the MFI of CD21 in TZ cells (MFI = 7.3) compared to normal T cells (MFI = 0.37) was significantly higher (median difference = 11.5, $p < 0.03$) (Fig 4.5). There was no significant difference in the size of TZ cells versus normal T cells in non-GR dogs. There was no significant difference in age, sex, presence of anemia, or presence of thrombocytopenia between the dogs with TZ cells and the dogs without TZ cells in either the GR group or the non-GR group.

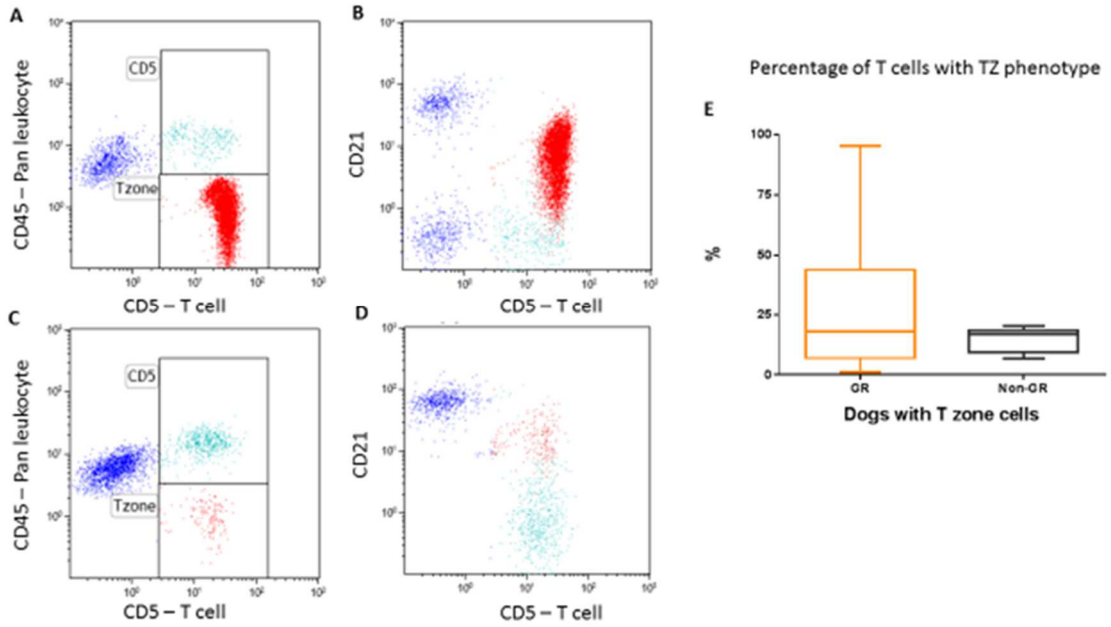

Figure 4.4. Older dogs have variable percentages of TZ cells in their peripheral blood. A.,B. TZ cells from a healthy GR. In this sample the number of TZ cells represented 95% of the total T cell population, the highest percentage of TZ cells detected in non-lymphoma bearing GRs. C.,D. TZ cells in a non-GR, the highest percentage TZ cells detected in the blood of non-GRs. In this sample the number of TZ cells represented 20% of the total T cell population. E. The range of TZ cells in GRs (n = 74, median = 18.1%) versus non-GRs (n = 6, median = 17.4%). The whiskers are set at the minimum and maximum value.

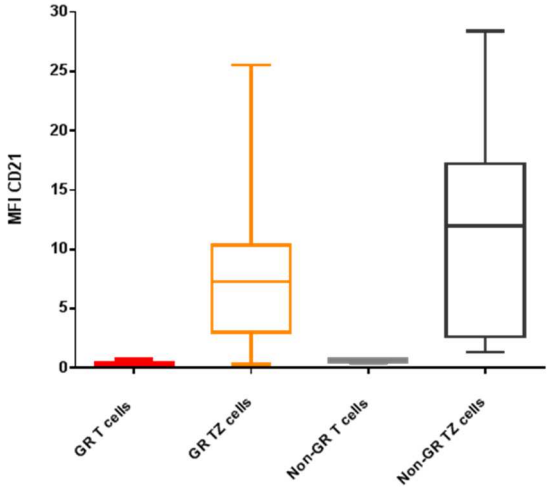

Figure 4.5. TZ cells express higher levels of CD21 than normal T cells. The median fluoresce intensity (MFI) of CD21 in CD45+ T cells (normal T cells) versus CD45- T zone

T cells (TZ cells) in Golden retrievers (GRs) and non-Golden retrievers (non-GRs). In both the GR and non-GR samples, TZ cells express higher levels of CD21 than the normal T cells. The whiskers are set at the minimum and maximum value.

Clonality assessment

PARR was performed in 67 of the 74 cases of GRs with TZ cells (Table 4.5). Twenty-three (34.3%) of the cases had clonal TRG gene rearrangements, one dog (1.5%) had an equivocal result, and 43 (64.2%) were polyclonal. Additionally, 32 (20%) of 157 GRs with no evidence of TZ cells in peripheral blood had clonal TRG gene rearrangement. The percentage of GRs with TZ cells with a clonal TRG gene rearrangement was significantly higher than the percentage of GRs without TZ cells with a clonal TRG result ($p = 0.026$).

All 6 non-GRs with TZ cells had PARR performed, and all had polyclonal TRG gene rearrangements, demonstrating no evidence for clonality (Table 4.5). The PARR assay was performed on 34 of the non-GRs without TZ cells and 2 had a clonal TRG gene rearrangement. The clonal TRG gene rearrangements in these dogs may have represented an emerging T cell lymphoma/leukemia although this cannot be confirmed because both dogs were euthanized shortly after the sample was obtained due to progressive transitional cell tumor of the bladder in one dog and progressive immune mediated hemolytic anemia in the other.

Table 4.5. PARR results for GR and non-GR with and without TZ cells in the blood. n indicates the number of samples available for the PARR assay. The percentage is the number of samples with that result out of the total samples with a PARR result.

	GR with TZ cells		**GR without TZ cells**		**Non-GR with TZ cells**		**Non-GR without TZ cells**	
PARR results	n = 67	%	n = 157	%	n = 6	%	n = 34	%
Clonal TRG gene rearrangement	23	34.3	32	20.4	0	0.0	2	5.9
Equivocal clonal TRG gene rearrangement	1	1.5	1	0.6	0	0.0	0	0.0

Polyclonal TRG gene rearrangement	43	64.2	124	79.0	6	100.0	32	94.1

Follow up studies of GRs with TZ cells

Sequential peripheral blood samples were available for 50 dogs within the entire population of GRs. Twenty-two of these dogs had TZ cells in the first sample, and all of these dogs also had TZ cells in the follow up sample. Twenty-eight dogs had no TZ cells in the first sample. Of these, 12 had TZ cells in the follow up sample and the remaining 16 did not. At the time of this manuscript, none of these dogs had developed evidence of clinical disease, in particular lymphocytosis or peripheral lymphadenopathy. As of August 2018, after a 4 year period, the highest lymphocyte count in any of the 34 GRs with follow-up was 3700 cells/µL. Two of the 35 dogs (5.7%) progressed to clinical disease with peripheral lymphadenopathy. In these two dogs the lymphocyte count has remained within the reference range (dog 1 = 21% of lymph node consisting of TZL cells, highest lymphocyte count 2900 lymphocytes/µL; dog 2 = 43% of the lymph node consisting of TZL cells, highest lymphocyte count 1600 lymphocytes/µL). In dog 1 the time from the initial identification of TZ cells to development of peripheral lymphadenopathy was 3 years and 6 months. This dog had a polyclonal PARR result for the first sample but later developed an identical clonal TRG gene rearrangement in the blood and lymph node at the time of clinical disease. In dog 2 the first sample with TZ cells was 2014 and lymphadenopathy was identified 3 years and 3 months later. This dog also developed a clonal TRG rearrangement with the same clone identified in the blood and lymph node at the time of clinical disease.

In the remaining dogs, PARR was originally performed on 43 of the 50 dogs and the results did not change for 40 of the dogs. In 3 dogs, however, the PARR assay changed from a polyclonal to clonal result in the follow up sample. These 3 dogs had TZ cells in their blood on both occasions.

Discussion

In this study, we have found that the loss of CD45 antigen expression and the increased expression of CD21 by TZ cells is reflected in the expression of the relevant genes (Table 4.4). These findings indicate that the abnormal antigen expression in TZL is not a function of conformational changes in the antigen, or in the case of CD45, differentially spliced protein that is not detected by the antibody used in the analysis and support recent findings from another group.[15] The expression of CD21 in TZL in our study (Fig 4.5) is in concordance with previously identified CD21 expression by flow cytometry,[9,20] and increased CD21 (CR2) gene expression identified in dogs with TZL using the Affymetrix Canine_2.0 gene chip.[21] In our study, the isolation methods for TZL cases differed from the isolation of normal T cells because of the limitations imposed by the use of samples submitted for diagnostic purposes. However, the purity and quality of RNA was similar for both cell types and our results are supportive of other investigations into phenotypic abnormalities of TZL[9,15,20,21] These findings further characterize these aberrancies as occurring at both the protein and mRNA level and support the characterization of this particular subtype of lymphoma in dogs by immunophenotyping. We have demonstrated that 30% of GRs with no evidence of lymphoma/leukemia have circulating TZ cells. These cells can be identified in 14% of older dogs of other breeds. These proportions reflect the distribution of clinical TZL, where GRs represent almost 40% of all cases.[9] A minority

of the TZ cell positive GRs (34%) had clonally rearranged TRG genes (Table 4.5). Additionally, 20% of GRs with no evidence of TZ cells in the peripheral blood had a clonal rearrangement of TRG genes. PARR may be a more sensitive test than flow cytometry when there is only a minor clonal population within a sample. The clonal T cells in this study are suspected to be the TZ cells but since they were not sorted we cannot rule-out a separate emerging T cell lymphoma/leukemia or a reactive clonal population. We favor the hypothesis that the clonal T cells in these dogs may represent a pre-neoplastic state, similar to pre-neoplastic B and T cell disorders in people: monoclonal gammopathy of undetermined significance (MGUS), monoclonal B lymphocytosis (MBL), and T cell clonopathy of unknown significance (TCUS).

MGUS is an asymptomatic plasma cell dyscrasia with low but measurable risk of progressing to multiple myeloma (MM).[30-32] MBL is a monoclonal gammopathy with no evidence of lymphoproliferative disease, but the cells have a chronic lymphocytic leukemia (CLL) immunophenotype with rare progression to CLL (1-2% per year).[33] Genetic risk factors have been identified in both of these disorders and they are thought to represent a pre-neoplastic state.[30,33,34] TCUS may represent a similar process for T cells, but it is difficult to equate the process in people with what we have described here in dogs, because T cell clonality in people is not defined by DNA based clonality assessment but by expansion of a family of T cells that use the same Vb gene, but that do not have identical T cell receptors.[35]

In GRs, genetic risk factors may also contribute to increased frequency of TZ cells as well as progression to overt lymphoma similar to these human syndromes. If the above hypothesis is correct, we might expect to see progression to TZL in a subset of the dogs that carry these cells.

Although we only have follow up on a small portion of these GRs, we did not see significant expansion of TZ cells in any dog. When multiple samples of peripheral blood were available, TZ cells were generally mildly increased and none of these dogs showed progression of disease, such as lymphocytosis or lymphadenopathy. In MGUS, patients are monitored throughout their life as the risk of transformation is considered to be life-long. The risk for development of MM in MGUS is only 1% per year.[30] MBL with a low-count (< 500 clonal B cells/µL)[36] can be identified in about 5% of adults over 40 years old with rare progression to CLL,[33] and in one study, no individuals with a low-count MBL showed progression to lymphoid neoplasia with a median follow-up of 34 months.[37] If there was a similar risk of development of TZL in dogs with TZ cells, a much larger cohort needs to be identified and followed for a longer period of time in order to determine the risk. When we revisited follow-up samples after about 4 years we did find progression to clinical disease (lymphadenopathy) in 2/34 dogs (5.7%). These two dogs are full siblings. We also received samples from 4 other siblings and they all had small numbers of TZ cells. In 2 of the other siblings we have had multiple samples with no evidence of progression and in the other 2 siblings we only had one sample. In MGUS in humans, there is an increased prevalence of MGUS in families with at least one person with a lymphoproliferative or plasma cell disorder.[38] Furthermore, the prevalence of MBL in relatives of familial CLL is 2 – 3 fold higher in individuals over 40 years than the overall population.[39-41] These studies are supportive of genetic factors and/or shared environmental risk factors in this syndrome which is also suggested by our findings. Familial clustering of lymphoma has previously been reported in dogs.[42] It is also possible that there is an environmental factor that contributes to disease

development that has yet to be identified. Recently, it was reported that diagnosis of TZL in GRs showed differential geographic distribution suggesting a possible environmental risk factor.[43]

The ability to recognize potential pre-neoplastic states provides a system in which stepwise progression to neoplastic transformation can be investigated. Determining the significance of TZ cells in GRs and possible environmental factors or disease states which lead to persistence of T cell infiltration and eventual neoplastic development is ongoing. Additionally, identifying diagnostics to recognize pre-neoplastic states in dogs may aid in early diagnosis of more aggressive subtypes of lymphoma. There has been limited research looking at early disease detection markers in dogs with only one study applying clinical screening studies in neoplasia.[44] In this study, blood ferritin levels showed promise as a biomarker for early evidence of disseminated histiocytic sarcoma in Bernese Mountain dogs.[44] Further development and identification of novel pre-neoplastic markers would be extremely valuable since many purebred dogs may have genetic components contributing to higher incidence of certain types of cancer.

The presence of clonal T cells in the blood of older GRs, and a smaller number of non-GRs should be considered when interpreting the results of clonality assays. First, a positive T cell clonality test without supporting clinical data should not be taken to indicate neoplasia, and the assay should not be used as a screening test. Second, the presence of a clonal TRG gene rearrangement in B cell neoplasms reported by some laboratories[45-47] should not necessarily be interpreted as aberrant rearrangements; they may in fact reflect the presence of TZ cells, which would not be identified without flow cytometry.

In conclusion, TZ cells were identified in greater than 30% of GRs, the breed with the highest number of dogs to develop TZL. These cells are readily identified by flow cytometry and aberrant antigen expression is confirmed at the gene expression level. We have found that a higher percentage of GRs without lymphoma have TZ cells in their blood when compared to other purebreds, and this reflects the frequency with which GRs develop TZL. Additionally, the lack of clinical signs and rare progression to disease in these dogs suggests that this is still a pre-neoplastic state despite evidence of clonal TRG gene rearrangement. Given these findings, clonal TRG gene rearrangements should be interpreted with caution in non-clinical older dogs, particularly GRs. Continued prospective analysis of GRs to determine the biological significance of TZ cells and possible identification of other genetic and environmental factors involved in the development of TZL is ongoing.

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CONCLUSION AND FUTURE DIRECTIONS

Determining the cell-of-origin leading to lymphoproliferative diseases can enhance our understanding of pathogenesis, along with identifying potential therapeutic targets. We were specifically interested in characterizing TZL, a subtype of PTCL, by determining the cell-of-origin, its proliferative capacity, immunosuppressive activity, and implications for earlier detection of disease. There are still gaps following our investigation as to the ultimate cell-of-origin in this disease and future endeavors aimed at complete identification of the molecules involved in proliferation or immunosuppression would be fruitful. CD45, absent from TZL cells, is necessary for the TCR response including cytokine production, effector functions, and proliferation.[1-5] While our work did not include determining the mechanism by which this normally abundantly expressed pan-leukocyte protein is lost, certainly future inquiries directed toward this question would be informative. Elucidating the mechanism by which this vital protein is lost could apply to other lymphoproliferative diseases, including cutaneous T cell lymphoma and anaplastic large T cell lymphoma in humans, both of which can lose expression of CD7.[6,7]

Cellular Origin of TZL

Based on our gene expression profiling, TZL is most likely derived from Th2-like cells with immunomodulatory functions or an induced Treg cell that does not express FOXP3. Mouse IL-10 producing Treg cells have been identified which do not express Foxp3 despite having the ability to inhibit proliferation of T cells similar to traditional CD4+ CD25+ Treg cells.[9] However, we did not detect increased expression of Th2 type cytokines, nor were we able to confirm production of the immunosuppressive cytokines, TGF-β or IL-10 *in vitro*.

Future directions

The gene codeset from our investigation was only the beginning; with the inherent plasticity of T cells, a more comprehensive understanding of expression in this disease will come from exploring numerous other genes of interest. For example, GATA3 and TGF-β have been associated with multiple T cell subsets including: Th2, Treg, and Th9 cells.[10-15] Our team has submitted TZL samples for RNA sequencing with pending results. This information will provide a more global view of gene expression in this disease and may further support a Th2 or Treg cellular origin. Still, this data may only complicate the cell-of-origin question while suggesting future pathways for investigation.

There are several pathways which our codeset did not address. For example, we did not determine the expression of STAT5[16] or other transcription factors which have been implicated in Th2 differentiation including Dec2 and Gfi-1.[17] Furthermore, It could be useful to determine the phosphorylation status of transcriptional regulators; exploring their associated pathways in TZL. Gene expression could be similar between TZL cases and controls, however phosphorylation status of STAT, cJun, AKT, MYC, ERK, and p38 proteins may differ. The BD Phosflow™ T cell activation kit is available for flow cytometric assessment of phosphorylation status of p38, ERK1/2, Stat1, Stat3, Stat5, Stat6 in humans and antibodies may certainly cross-react with canine samples. Alternatively, western blot could be used to investigate phosphorylation status.

Functional Pathways in TZL

We attempted to identify proliferative and functional mechanisms of TZL cells. Gene expression analysis revealed increased expression of the immune modulatory molecules TGF-β,

galectin-1, and galectin-3, which are involved in suppression of proliferation,[18] IFN-γ production,[18,19] and cause apoptosis of Th1 and Th17 cells.[20] However, we were unable to confirm any of these features of immunosuppression in TZL cells *in vitro*. In addition to the challenge of identifying effector functions and cytokine production in TZL, we also had difficulty inducing proliferation in these cells. We were eventually able to induce proliferation of TZL cells through by-passing the TCR. CD25 (IL-2R) expression was variable in our gene expression data, but has previously been identified to be increased in TZL[21] with IL-2 playing a role in priming Th2 cells.[22] IL-2 was found to induce proliferation in a subset of cases, suggesting IL-2 may contribute to proliferative mechanisms in TZL.

Future Directions

Although IL-2 was identified as a potential growth factor for TZL cells, other mechanisms may be acting in vivo. Our investigation did not include B cell signaling or inflammatory cell signaling in TZL cell cultures. Future studies looking at TZL cell proliferation mechanisms in the lymph node may provide some insight. Lymph node aspirates would include the microenvironmental B cells and dendritic cells which may play a role in TZL cell activation. From gene expression data, we know TZL cells express CD40L (TNFSF5) and Notch at significantly higher levels than controls and both of these molecules can be involved in T cell activation.[23,24] Direct interaction between CD40 on APCs and CD40L on TZL cells, or between jagged and delta-like ligands on APCs and Notch on TZL cells, could be involved in signaling pathways for this disease. These interactions could be studied using cell cultures from lymph node samples. Adding artificial antigen presenting cells to culture, such as K562 cells which have been used to expand T cells in dogs,[25] could be another way to enhance proliferation in TZL. Alternatively,

culturing TZL cells with additional growth factors such as IL-15 and/or IL-7 may elicit a greater response.

Since CD45 is necessary for T cell proliferation and cytokine production in mice and human cell lines,[1,5,26] investigating the mechanism by which CD45 is lost in TZL, may provide insight into alternative signaling pathways in this disease. Interestingly, CD45 DNA is present while the mRNA is absent in TZL,[27] which suggests transcriptional regulation may be involved in the development of this disease. Sequencing the CD45 gene could be useful to suggest possible mechanisms for decreased transcription, such as a stop codon or splice variant leading to termination of transcription. Furthermore, epigenetic changes in transcription could contribute to decreased expression in TZL. Bisulfite sequencing to determine the methylation pattern for CD45 could elucidate a mechanism for decreased transcription.

As a result of this work, we identified several molecules which may be involved in the pathogenesis of TZL including GATA3, TGF-β, and galectins. IL-2 signaling may be an important aspect of cell signaling in this disease. The IL-2 signaling pathway or other signaling pathways could be a potential therapeutic target since conventional multidrug chemotherapy may not improve survival in this disease. Our studies and future investigations including RNA sequencing, protein phosphorylation and epigenetic patterns of CD45 would be consistent with prior attempts to determine cellular origin and consider functional implications in human B cell tumors, which are well characterized, but remain elusive with T cell lymphoproliferative diseases.

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