Characterizing and Targeting the Interferon-gamma Signaling Pathway in T cell Large Granular Lymphocyte Leukemia

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A Dissertation presented to the Graduate Faculty of the
University of Virginia in Candidacy
for the Degree of Doctor of Philosophy

Department of Pathology

University of Virginia
May 2018

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Dissertation Abstract

T cell large granular lymphocyte leukemia (T-LGLL) is a rare, chronic hematological malignancy characterized by clonal proliferation of cytotoxic CD8+ T cells due to defective apoptosis. Currently there is no cure and the main treatment options are broad immunosuppressant therapies for management of symptoms. The vast majority of T-LGLL patients require treatment at some point and thus there are efforts to identify and evaluate novel and specific therapeutics for T-LGLL. One such area of investigation is the regulation of interferon-gamma (IFN-γ) signaling. IFN-γ is an inflammatory cytokine that is associated with worse disease progression and symptomology in multiple cancers and autoimmune diseases. Excess IFN-γ, in the absence of infection, inhibits proliferation and induces apoptosis of healthy peripheral blood mononuclear cells (PBMCs), contributing to disease state. As a result of this, it is crucial to reduce IFN-γ production in cancers, including T-LGLL, where patients have significantly elevated circulating levels of this pro-inflammatory cytokine compared to healthy donors.

Calcitriol, the active form of vitamin D, has shown promise as an inhibitor of IFN-γ production. Thus, we first turned our attention to evaluating calcitriol for use as an IFN-γ inhibitor in T-LGLL. We initially assessed the effects of 24 h calcitriol treatment on TL-1 cells, the patient-derived cell line model of T-LGLL (**Chapter 3**). We found that calcitriol significantly decreased IFN-γ secretion and activation of signal transducer and activator of transcription 1 (STAT1), a transcription factor that becomes activated via phosphorylation of tyrosine residue 701 (p-STAT1) in response to IFN-γ. STAT1 also typically promotes transcription of IFN-γ. As a result of calcitriol treatment, p-STAT1 and IFN-γ inhibition occurred while the vitamin D receptor (VDR), a nuclear receptor and transcription factor, increased on the protein level in TL-1 cells. We next sought to

elucidate the mechanism behind calcitriol-mediated reduction in IFN-γ production and whether VDR upregulation was required for this effect (**Chapter 4**). We found that calcitriol reduced IFN-γ intracellular protein and mRNA transcript levels and p-STAT1 protein levels within 4 h. Moreover, calcitriol-mediated IFN-γ reduction was independent of p-STAT1 levels but required VDR upregulation. Our results suggested that p-STAT1 and IFN-γ levels were regulated independently of each other, indicating a dysregulation of the canonical IFN-γ signaling pathway. Thus, in an effort to better understand the regulation of IFN-γ, we sought to characterize the IFN-γ-mediated signaling pathway from IFN-γ signaling to transcription of IFN-γ (**Chapter 5**).

Previous studies demonstrated that T-LGLL cells have a deficiency in suppressor of cytokine signaling 1 (SOCS1), a negative regulator of IFN-γ-mediated signaling. SOCS1 is typically induced in response to IFN-γ, allowing for a tightly controlled signaling process. However, despite high IFN-γ output, T-LGLL cells exhibit significantly lower SOCS1 levels compared to normal donor cells. Therefore, T-LGLL cells are likely to be unresponsive to IFN-γ production, allowing an unchecked production of the inflammatory cytokine as seen in other cancers. We found that TL-1 cells have a significantly lower surface protein and mRNA transcript level of the IFN-γ receptors (IFNGR) compared to Jurkat T cells, our positive IFN-γ responsive cell line. IFN-γ did not induce Janus kinase 2 (JAK2) or STAT1 phosphorylation or established IFN-γ-mediated gene targets, including IRF-1 and SOCS1, in TL-1 cells. This further demonstrated a lack of responsiveness to IFN-γ. We found that STAT5b, but not STAT1 or STAT3, played a role in regulating IFN-γ transcript levels.

Taken together, the decrease in IFNGR levels is a plausible explanation for the excessive IFN-γ production and lack of negative regulation observed in T-LGLL. This pathway can be targeted effectively using calcitriol to significantly reduce IFN-γ production. As calcitriol inhibits IFN-γ independently of STAT1, calcitriol is able to reduce IFN-γ regardless of its interactions with STAT1, providing a potent therapeutic to reduce inflammation. This thesis is the first study to evaluate IFN-γ signaling and a treatment aimed at specifically targeting IFN-γ in T-LGLL. Future studies are needed to assess the efficacy of calcitriol in T-LGLL patients in the clinic.

In loving memory of Mrs. Concettina (Connie) Runzo (AKA Nanny) (1928-2014) and Dr. Susan Nyland (1957-2017).

Nanny was a second mother to me growing up and loved and supported me more than words could ever describe. Her battle with cancer inspired me to pursue biomedical sciences. Nanny was more than a grandmother to me, but also a true source of strength, friendship, and compassion. From mailing me packages of food to make sure I would eat in undergraduate and graduate school to sending me sweet cards to brighten my day, I was beyond fortunate to have had such a close relationship with Nanny.

Susan took a leap of faith with me after only knowing me for a few days by encouraging me to join the Loughran lab. She checked in on me every single day, danced around the lab with me on weekends, brought her dogs to visit me when I needed puppy snuggles, and made me laugh even when experiments failed (usually by singing or demonstrating her Zumba moves). She was a bright, optimistic mentor who never doubted my capabilities as a scientist.

These two incredible women served as my mentors, my support system, and inspiration. Both phenomenal women passed away during my PhD, but their contributions to my career and personal life will never be forgotten and I will be eternally grateful for their support and love.

Dedication Page

I would like to dedicate my work to my mom (Michele Kulling), my dad (Mark Kulling), my fiancé (Ryan Larkin), my late grandfather (Thomas Runzo AKA Pappy), and my spunky Yorkshire terrier (Gracie Mae Daisy) for their never-ending love, sacrifices, and support throughout my life and educational endeavors.

Acknowledgments

I would first like to thank my advisor, Dr. Thomas Loughran, who provided me with support, guidance, and resources to grow as a scientist and succeed. No matter how busy you are, you always made time to meet and give advice. Whenever I asked you about a publication or grant application, you always told me to "go for it." Through your mentoring, you made me a better scientist and writer. Thank you for always believing in me and encouraging me even when the odds were not in my favor.

Additionally, I would like to thank my mentors Dr. David Feith and Dr. Kristine Olson. Dave, thank you for continually pushing me to be the best I can be, providing me with constructive critiques so that I can improve, making me laugh with your love of pink and sparkles, and always being there to join in for brainstorming experimental ideas. You've given so much time and energy, including responding to my questions or ideas on weekends even, and I truly thank you for that. Kristine, thank you for allowing me to join the exciting new vitamin D team during my first year and really taking me under your wing. You taught me everything I know about cell culture and western blots and always included me in opportunities. I was lucky to have you as a scientist role model and also a really good friend that I could talk to about anything. I enjoyed spending time with you in and out of lab. You have always been there for me and made me want to come to lab every day even when experiments failed. To the rest of the Vitamin D Dream Team (Thomas Olson, Cait Hamele, Rebecca Rainbow, Katy Carter, Mariella Toro, Su-Fern Tan, Katie Moosic) and the rest of Loughran Lab (Wendy Dunton, Matt Schmachtenberg, Bryna Shemo, Andrea Hines, Alex Wendling, Shubha Dighe, Jennifer Pearson, Tiffany Wang, Jun Yang, Jeff Xing, Holly Davis) thank you for your support, suggestions, and listening to all of my presentations a million times! I hope that you will continue to clap for everyone at the end of each lab meeting presentation. Also, thank you, Mindy Borszich, for your kindness, cheerfulness, and support throughout the years. Your e-mails and kind words always made me smile.

I would also like to thank my committee (Dr. James Mandell, Dr. Adam Goldfarb, Dr. Janet Cross, Dr. Ulrike Lorenz, and Dr. David Kashatus) for their guidance and support throughout the years, with experiments and with my career. With the mix of clinical and basic science expertise from my committee, I was able complete an exciting project that I hope will translate into the clinic someday. I appreciated how invested you all were when applying for clinical fellowships and how excited you were when I was accepted into a program. Your support means so much to me. Additionally, thank you, Dr. Cross, for supporting clinical rotations through the Pathology graduate program. Being able to rotate in clinical microbiology solidified my career choice. And to Dr. Poulter, thank you for allowing me to continue to rotate with you, volunteering to be my mentor in clinical microbiology, long after the pathology rotation ended. Your faith and time invested in me means the world to me and I am incredibly thankful for all of the hours I spent in clinical microbiology with you and for the ability to pursue projects. I will have quite the shoes to fill as a Clinical Microbiology Director someday.

Of course, I need to extend a sincere thank you to my undergraduate research lab (the Hairston Lab) at Cornell University who believed in the freshman biology major with zero research experience or bench work practice. Thank you, Dr. Nelson Hairston and Dr. Brooks Miner, for being incredible mentors and really supporting my desire to pursue an independent research project. I will never forget the impact you both had on my scientific career.

To all of my friends (especially Lauren, Ruth-Anne, Amanda, Katie, Sarb, Breanna) who supported me throughout graduate school, from Charlottesville and afar, thank you! In particular, I would like to thank my fiancé, Ryan Larkin. Ryan has been a huge positive part of my life and support system for the past 7 years. He supported me when I first started doing research as an undergraduate and even when chasing my dreams put us at long distance for over 4 years. I will be forever grateful for the number of red eye flights taken for less than 48 hours together and his positive attitude every time he stepped off the plane or even when I had to go into lab during his short visits here. Thank you for taking me on fun adventures, staying on Facetime with me when I had to go into lab at 4am so I wouldn't be alone, making me smile and laugh during tough graduate school times, encouraging me, and always being there for me.

Lastly, I would like to acknowledge and thank my parents, my biggest fans. My parents made sure I knew I could be anything I wanted to be and, although neither particularly enjoys reading science or understands what I study, they were always so supportive and eager to hear about my work. They sacrificed a lot to allow me to receive the best education that I could, even if that meant living across the country from each other so I wouldn't have to change schools during critical times. Through all of my overnight time courses and nonstop experiment days, my mom has been there for me and been my biggest champion and best friend, visiting me when I couldn't go home for holidays or when I really just needed a visit (and home cooked meals). She embraced me, pink sparkles and all, for who I am and taught me that the sky really isn't the limit. Thank you, Mom.

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Chapter 1

Large Granular Lymphocyte Leukemia & Vitamin D

Partially adapted from: **Kulling PM**, Olson KC, Olson TL, Feith DJ, and Loughran TP Jr. Vitamin D in Hematological Malignancies and Disorders. Eur J Haematol. 2016;98(3):187-97.

1.1. Funding and Abbreviations

Funding: PMK is supported by the Immunology Training Grant T32AI007496-21 and the UVA Wagner Fellowship. Bess Family Charitable Funds and LGL Leukemia Foundation provided support for the preliminary vitamin D studies in TPL laboratory. Research reported in this section was supported by the National Cancer Institute of the National Institutes of Health under award number R01CA098472 and R01CA178393 (to TPL). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Abbreviations: large granular lymphocyte (LGL), large granular lymphocyte leukemia (LGLL), natural killer cell (NK), Janus kinase (JAK), signal transducer and activator of transcription (STAT), peripheral blood mononuclear cell (PBMC), soluble Fas (sFas), interleukin (IL), Fas ligand (FasL), interferon-gamma (IFN-γ), interferon-gamma receptor (IFNGR), T cell receptor (TCR), human T cell leukemia/lymphoma virus (HTLV), human immunodeficiency virus (HIV), suppressor of cytokine signaling (SOCS), chronic lymphocytic leukemia (CLL), systemic lupus erythematosus (SLE), myelodysplastic syndrome (MDS), rheumatoid arthritis (RA), methotrexate (MTX), cyclosporine A (CsA), human leukocyte antigen (HLA), Killer cell immunoglobulin-like receptors (KIR), Src homology 2 (SH2), nuclear factor-κB (NF-κB), IL-15 receptor α (IL-15Rα) and soluble IL-2R, vitamin D response elements (VDRE), vitamin D receptor (VDR), ultraviolet radiation (UVR), retinoid X receptor (RXR), overall survival (OS), relapse free survival (RFS), chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL), non-Hodgkin's lymphoma (NHL), diffuse large B cell lymphoma (DLBCL), Cutaneous T cell leukemia (CTCL), Sézary syndrome (SS), mycosis fungoides (MF), immune thrombocytopenic purpura (ITP), autoimmune hemolytic anemia (AIHA), B cell Hodgkin's lymphoma (B-

HL), B cell non-Hodgkin's lymphoma (B-NHL), diffuse large B cell lymphomas (DLBCL), Burkitt's lymphoma (BL), aplastic anemia (AA), Single nuclear polymorphisms (SNPs), tumor-associated macrophages (TAMs), acute lymphoblastic leukemia (ALL), extracellular signal-regulated kinases (ERK), mitogen-activated protein (MAP), glycogen synthase kinase 3 (GSK3), T cell acute lymphoblastic leukemia (T-ALL), experimental allergic encephalomyelitis (EAE)

1.2. Large Granular Lymphocyte Leukemia

1.2.1. Normal & Malignant Large Granular Lymphocyte Biology

Large granular lymphocytes (LGL), ranging from 15-18 μm in size, characteristically exhibit a large cytoplasm with azurophilic granules and a round nucleus containing mature chromatin (1, 2). In healthy individuals, LGL cells comprise 10-15% of normal peripheral blood mononuclear cells (PBMCs) (3). Following an encounter with an antigen, such as a virus, LGL cells rapidly clonally expand, increasing the circulating population by over 50,000-fold (4-7). During this process, cytotoxic functions, including release of granzyme B, perforin, and interferon-gamma (IFN-γ) occur (5, 8). This kills off infected cells, thereby preventing the spread of virus (5, 8). Once the antigen is cleared, the LGL cell population contracts through activation induced cell death (AICD) mediated by the Fas/Fas Ligand (FasL) pathway (3, 5). However, defects in this process can occur, leading to a permanently expanded clonal LGL population. In the case of large granular lymphocyte leukemia (LGLL), the expanded clonal population is resistant to apoptosis, despite high levels of FasL and no known mutations in the Fas receptor gene (3). As patients have elevated soluble Fas (sFas), it is hypothesized that sFas competes with Fas receptor by acting as a soluble decoy receptor, leading to apoptosis resistance (4). This is further supported by the finding that serum, which contains high levels of multiple sFas variants, blocks Fas dependent apoptosis of leukemic cells (9). The elevated levels of c-FLIP further contribute to decreased apoptosis in T-LGLL cells through inhibition of Fas signaling (10). As a result of these defects in apoptosis, LGLL cells continue to perform cytotoxic functions in the absence of infection (3, 4), contributing to a damaging inflammatory environment (11-13).

LGLL is a rare lymphoproliferative malignancy of the cytotoxic T cells (T-LGLL) or

natural killer (NK) cells (NK-LGLL) (1, 3). Together, these diseases make up 2-5% of chronic lymphoproliferative disorders in western countries and 5-6% in Asia (2). LGL leukemia is estimated to occur at a rate of 0.2-0.72 per 1,000,000 individuals (14, 15). The median age at diagnosis for T-LGLL is over 60 years (1, 14), with most studies demonstrating equal occurrence in males and females (1, 15) and races (15). However, LGLL occurred more frequently in males over the age of 65 in the Netherlands (14) and females were likely to be diagnosed on average three years earlier than men (15).

The indolent form of LGLL is most common, comprising 85-90% of cases (1, 3, 12). Indolent LGLL is a chronic cancer with an overall survival of greater than 10 years (1). One study found that the 5-year and 10-year survival rates were 74% and 68%, respectively, with no significant differences in survival rates between chronic T-LGLL and NK-LGLL (14). Poorer survival was observed in patients over the age of 60 (15) and death often occurs from infection and sepsis rather than the cancer itself (12, 16). As indolent T-LGLL is the most common type of LGLL, I will focus solely on this form of LGLL.

1.2.2. T-LGLL diagnosis and symptomology

T-LGLL is often diagnosed during a routine complete blood cell count. The first criteria for T-LGLL diagnosis is an elevated number of persisting circulating LGL cells (17). This elevated count is typically defined as greater than 2×10^9 per liter (1), but can be as low as 0.5×10^9 per liter or lower if additional criteria including presence of a clonal T cell receptor gene rearrangement (2). Next, flow cytometry is used to determine the immunophenotyping of the elevated LGL cells. T-LGLL cells are most commonly CD3+, TCR$\alpha\beta$+, CD5^{dim}, CD8+, CD16+, CD27-, CD28-, CD45RO-, CD45RA+, and CD57+,

although markers vary between patients (1) and LGL clones within the same patient (18). Thus, deviation from these makers does not exclude T-LGLL (1). For example, there are rare cases of TCR$\gamma\delta$ (19, 20) and CD4+ T-LGLL (21). However, overall, T-LGLL cells have a mature post-thymic, terminal-effector memory, and activated T cell phenotype (2, 3, 17). In addition to confirming characteristic markers of T-LGLL, TCR clonality must be assessed, typically by TCR γ-polymerase chain reaction analysis (2). This allows for differentiation between a reactive LGL population and T-LGLL as T-LGLL cells exhibit TCR clonality.

Symptomology is not used to confirm or exclude T-LGLL diagnosis as 1/3 of T-LGLL patients are asymptomatic (12). Common symptomatic manifestations of T-LGLL include anemia (50%) (13), thrombocytopenia (20%) (16), neutropenia, and other cytopenias, which can lead to bacterial infections and fatigue (12). T-LGLL may also occur with or precede other malignancies and disorders such as myelodysplastic syndrome (MDS), monoclonal gammopathy of unknown significance, pure red cell aplasias (PRCA), B cell neoplasms, additional hematological and solid tumors (12), and polyclonal hypergammaglobulinemia (16). Co-occurring autoimmune diseases are found in roughly 40% of T-LGLL patients (3). These autoimmune diseases include, but are not limited to, systemic lupus erythematosus (SLE) (12, 16), Sjogren's (12, 16), Hashimoto's Thyroiditis (3, 12), autoimmune hemolytic anemia (16), polymositis, and most commonly, rheumatoid arthritis (3, 12).

Rheumatoid arthritis (RA) occurs in 36% of T-LGLL patients compared to less than 1% of the general population (3). There are many shared clinical features of RA and T-LGLL. Both diseases exhibit inhibitory and activating NK receptors on LGL cells,

pro-inflammatory cytokine production, similar seroreactivity to viral peptides, defective apoptosis processes, overexpression of cytotoxic effector molecules, and clonally expanded population of T cells with the effector or memory phenotype (22). There is also a subset of T-LGLL patients with RA that have Felty's syndrome, defined by the co-occurrence of RA, neutropenia, and splenomegaly (3). Even in the absence of T-LGLL, Felty's syndrome patients had an increased population of LGL cells with the CD8+ phenotype (22). Taken together, T-LGLL is hypothesized to share a common pathogenesis with RA and Felty's syndrome.

1.2.3. Potential Drivers of T-LGLL

The cause of T-LGLL is unknown, but familial cases suggest a shared environmental component (23). Furthermore, there is support for a retroviral agent in the pathogenesis of T-LGLL. Human T cell leukemia virus I (HTLV-I), a well-established leukemia virus, is known to induce monoclonal proliferation of T cells, which increases the risk of adult T cell leukemia (24, 25). Despite the rarity of HTLV infection in T-LGLL (26, 27), the vast majority of T-LGLL patients exhibit seroreactivity to peptides derived from HTLV (28-30). On average, elevated HTLV-specific IgG was detected in 35-51% of T-LGLL patient serum (28-30) compared to less than 1% of normal healthy donors (28). In particular, T-LGLL patient serum demonstrated seroreactivity to the HTLV-I transmembrane envelope protein, p21e, and more specifically the BA21 region (30). The BA21 region is a 34 amino acid sequence to which roughly 60% of HTLV infected patient serum have specific IgG (30). This reactivity is not unique to T-LGLL as diseases that co-occur with T-LGLL, including RA and aplastic anemia, exhibit this HTLV reactivity pattern, supporting a common viral component. Both RA and T-LGLL patient serum contain high levels of IgG specific for the HTLV-I envelope region, but are not infected

with the virus (31). Furthermore, 35% of patients with aplastic anemia, myelodysplastic syndrome, and paroxysmal nocturnal hemoglobinuria also demonstrate reactivity to BA21 (28). Importantly, these diseases are associated with bone marrow failure and increased T cell activation resembling T-LGLL (28). BA21 reactivity did not correlate with transfusions status or treatment with intravenous immunoglobulin, Epoetin Alfa, cyclosporine, prednisone, anti-thymoglobulin, or methotrexate (MTX) (28). Based on pooled risk determinations, bone marrow failure patients with increased BA21 reactivity were 128 times more likely to have LGLL, demonstrating a correlation between BA21 reactivity and LGLL development (28). This reactivity observed for T-LGLL appears to be unique to HTLV, as bovine leukemia virus and primate T cell lymphoma/leukemia virus, two oncogenic retroviruses, were not associated with T-LGLL (32). The viral antigen theory is further supported by the CD8+ T cell terminal memory differentiation phenotype of T-LGLL cells. The observed CD45RA+ expression and absence of CD62L expression indicates repeated antigen stimulation (10).

There is also support for a genetic component for T-LGLL development. The vast majority of T-LGLL patients exhibit a normal karyotype (1). However, a few documented cases of chromosomal abnormalities, trisomy 8 and trisomy 14, have been reported (33). In regards to human leukocyte antigen (HLA) types, there is no association between HLA type and T-LGLL. In patients with both T-LGLL and RA, HLA-DR4 is present in the majority of these patients, but this allele occurs in the minority of T-LGLL patients without RA (16). This suggests that HLA-DR4 does not play a role in the pathogenesis of T-LGLL alone.

However, mismatch between activating or inhibitory receptors and their

respective ligands is well documented in T-LGLL. Killer cell immunoglobulin-like receptors (KIR) are important immunological mediators found on NK cells and some T cells, particularly CD8+ memory T cells (34). One KIR, KIR3DL2, suppresses cytotoxic function, including the production of IFN-γ, when bound to its ligand (34, 35). This acts as a regulatory mechanism for controlling inflammatory output (35). When the ligand is mismatched, self-tolerance is reduced and cytotoxic T cell response is increased. This mismatch has been found at a significantly higher frequency in T-LGLL patients due to the absence of the A3 and A11 alleles encoding the KIR3DL2 ligand (35). In contrast to KIR3DL2 and its ligand, HLA-C2 when interacting with its ligand 2DS1, promotes a cytotoxic response (35). This activating function is often controlled in healthy individuals through mismatch of 2DS1 (35). However, T-LGLL patients have a lower frequency of mismatch of the HLA-C2 ligand, 2DS1, compared to normal healthy donors (35), leading to an increase in cytotoxic activity and lack of inhibition of this activating pathway (35). Taken together with the lack of suppression by the mismatched KIR3DL2 ligand, T-LGLL cells are skewed to be constitutively cytotoxic with increased IFN-γ production, further contributing to the inflammation.

Somatic mutations are rather common in T-LGLL, with mutations in signal transducer and activator of transcription 3 (STAT3) occurring in 30-50% of T-LGLL patients (3, 36-38). STAT3 mutations occur on exons 20 and 21, which encode a Src homology 2 (SH2) domain (36, 39). The mutations promote dimerization and activation of STAT3 (36). The increase in hydrophobic protein surfaces, as a result of mutations, promotes localization of STAT3 and subsequent transcriptional activity (36, 40). The most common STAT3 mutations are Y640F (17%), D661V (9%), D661Y (9%), and N647I (4%) (36). These STAT3 mutations were also found to occur in 43% of Felty's

syndrome patients, further supporting a common driver or shared disease state between the two diseases (41). STAT3 mutations have also been found in diffuse large B cell lymphomas, ALK-negative anaplastic large cell lymphomas, and peripheral T cell lymphomas (39).

In T-LGLL, patients with STAT3 mutations experienced a higher frequency of neutropenia (38) and pure red cell aplasia (42). However, Y640F STAT3 mutations conferred a favorable response to MTX compared to patients without the mutation, with 100% of T-LGLL patients with Y640F STAT3 mutations experiencing a therapeutic response after completing a full course of immunosuppressant therapy (40). Moreover, T-LGLL patients with STAT3 mutations had a better overall survival (43). Multiple STAT3 mutations occur in roughly 17% of T-LGLL patients, with the co-occurrence of RA being more common in this patient population (44). Although more infrequent, STAT5b mutations occur in 2% in the T-LGLL population, with N642H and Y665F being the most common mutations (3, 39). Patients with STAT5b mutations tend to have a more aggressive disease progression (12) and these mutations occur at a higher rate in CD4+ T-LGLL (45).

1.2.4. T-LGLL Treatment Options

At some point, 42-45% of T-LGLL patients require systemic treatment (14, 15). Current treatment options for T-LGLL are immunosuppressant therapies since there is a wide variation in disease symptoms and lack of approved drugs for specifically targeting T-LGLL cells. Course of treatment is based on presence of symptoms, including neutropenia or anemia, or co-existing autoimmune diseases (8). If patients are asymptomatic, a “watch and wait” approach is adopted. If treatment is initiated, the most

common options are the immunosuppressant therapies MTX (10 mg/m^2 per week), cyclosporine A (CsA, 3 mg/kg per day), or cyclophosphamide (100 mg per day); MTX is normally the first line of treatment (2) with cyclophosphamide administered next if a response is not observed (40). Response is assessed no sooner than 4 months following treatment initiation (2) and there is currently no evidence for cross-resistance between the three lines of treatment (12).

The overall response rate for these immunosuppressant therapies is estimated to be 50% with complete response observed in 21% of patients on MTX, 33% of patients on cyclophosphamide, and less than 5% of patients on CsA, which is typically given as a final line of therapy (2). MTX is associated with toxicities including infection, pneumonitis/pulmonary infiltrates, fatigue, hypoxia, and dyspnea while gastrointestinal bleeding and increased serum glutamic oxaloacetic transaminase have been noted with cyclophosphamide use (40). Bone marrow toxicity and development of additional hematological malignancies are observed with long term use of cyclophosphamide; therefore, the treatment length is typically no more than 12 months (3). If symptoms are not controlled by any of these three treatments, purine analogs, including 2'deoxycoformycin and fludarabine, may be prescribed (12). Given the high proportion of T-LGLL patients that require treatment at some point, the discovery of novel targeted therapies is warranted.

1.2.5. Dysregulated Pathways in T-LGLL: The IFN-γ-STAT Signaling Pathway as a Potential Drug Target

Ultimately, T-LGLL therapeutics should be developed to specifically target dysregulated pathways in T-LGLL with the goal of eradicating the clonal T-LGLL

population while avoiding damage to healthy PBMCs. There are several aberrantly activated pathways in T-LGLL leukemia including the Ras-Raf-1-MEK1-ERK, MAP kinase, phosphatidylinositol 3-kinase-Akt, sphingolipid, and nuclear factor-κB (NF-κB) pathways (4). However, the most commonly studied dysregulated pathway is the Janus kinase (JAK)-STAT pathway.

The JAK-STAT pathway, summarized in **Figure 1.1**, is activated by cytokines, which bind to their respective receptors. This causes activation of the JAK proteins, JAK1, JAK2, JAK3 or TYK2, depending on the cytokine (46). These tyrosine kinases then phosphorylate the receptors and recruited STATs (46). Once the STATs are activated, they form homo- or heterodimers and translocate into the nucleus, where they promote transcription of their gene target based on the cytokine stimulus (46). There are seven STAT proteins in total: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, STAT6 (46). STAT1 (47-49) and STAT3 activation (50-52), in particular, are considered to be pro-tumorigenic and thus are often desirable targets for cancer therapies (53-55).

Although STAT3 mutations are common in T-LGLL, patients lacking the mutations still experience constitutive STAT3 activation (p-STAT3) (56) and increased STAT3 transcriptional activity (39). One explanation for the hyperactivation of STAT3 is the high production of IL-6 by T-LGLL patient PBMCs (57). When T-LGLL cells were treated with an IL-6 receptor blocking antibody or IL-6 was neutralized, p-STAT3 levels were dramatically reduced, implicating IL-6-mediated signaling in continual promotion of p-STAT3 (57). Furthermore, suppressors of cytokine signaling 3 (SOCS3), a negative regulator of STAT3, was found to be significantly reduced in T-LGLL patient cells, preventing inhibition of the IL-6-STAT3 signaling pathway (57). This suppression of

SOCS3 and increase in IL-6 production is found in other cancers (58-60).

IL-2 and IL-15, which share a common β and γ receptor chain, are also implicated in the pathogenesis of T-LGLL and are well documented to activate STAT1, STAT3, STAT4, and STAT5 (46). IL-15 receptor α (IL-15Rα) (61) and soluble IL-2R (62) are both elevated in T-LGLL, explaining T-LGLL cell responsiveness. Routine supplementation with IL-2 or IL-15 is necessary for survival and maintenance of the TL-1 cells, a patient-derived cell line model of T-LGLL (63). IL-15 (61, 64) and IL-2-mediated (64, 65) signaling also promote proliferation of T-LGLL cells. The most compelling evidence for the role of IL-15-signaling in T-LGLL pathogenesis stems from mice overexpressing IL-15. IL-15 transgenic mice developed a fatal lymphocytic leukemia, with expansion of both NK and CD8+ T cells (66). Moreover, transfer of IL-15-treated cells into mice induced development of LGLL with an increase in centrosome aberrancies, aneuploidy, aurora kinases, and DNA hypermethylation (67). IL-15 is also associated with rheumatoid arthritis (68, 69) and celiac disease (70), diseases that can be associated with T-LGLL. Despite sharing receptor chains, overexpression of IL-2 does not induce LGLL in mice (71). Given the importance of IL-15-mediated signaling in T-LGLL and associated disease states, IL-15 receptor blocking antibodies have been developed and evaluated in preclinical and Phase I clinical trials (72, 73). Although no toxicity was observed, the antibodies were not effective and thus additional efforts are dedicated to creating novel ways to target this pathway.

Another upregulated component of the JAK-STAT pathway in T-LGLL is STAT1. T-LGLL patient cells have constitutively activated STAT1 (p-STAT1) (56) despite no known STAT1 mutations in the SH2 domain (74). This is particularly problematic as

STAT1 is known to promote transcription of and become activated by IFN-γ (46), a pro-inflammatory type II interferon cytokine (75). Activated T cells are the main sources of IFN-γ (76) and production of this inflammatory cytokine is tightly regulated. However, when IFN-γ production is not controlled, excess IFN-γ is associated with worse disease outcome, development of certain autoimmune diseases and cancers, and damage to healthy PBMCs (77-86). As T-LGLL is a disease of chronically activated T cells, it is not surprising that there is a significantly elevated IFN-γ level in T-LGLL patient serum (40). The excess circulating IFN-γ may explain the occurrence of neutropenia in T-LGLL patients as IFN-γ is known to upregulate FasL and promote Fas-mediated apoptosis of immature myeloid cells, leading to neutropenia (13). Elevated levels of IFN-γ are also a driver of aplastic anemia (79), a disease that often co-occurs with T-LGLL (3).

The excessive production of IFN-γ and hyperactivation of STAT1 are thought to result from a dysfunctional STAT1-IFN-γ pathway, with its hypothesized effects on T-LGLL summarized in **Figure 1.2**. The normal IFN-γ signaling pathway is shown in **Figure 1.3**. Response to IFN-γ is mediated through IFN-γ receptor (IFNGR) surface protein levels, which is found on most cell types including T cells (75). There are two IFNGRs, IFNGR1 and IFNGR2, that are unique for IFN-γ signaling (75). IFNGR1, a high affinity receptor, directly interacts with IFN-γ while IFNGR2, a low affinity receptor, determines downstream signal transduction (76). Both receptors are necessary for IFN-γ-mediated signaling (76). After IFN-γ, a noncovalent homodimer, binds to IFNGR1, activation via autophosphorylation of JAK1 and JAK2 occurs (75, 76, 87, 88). The JAKs then phosphorylate STAT1, inducing STAT1 homodimer formation and translocation into the nucleus (76). STAT1 promotes transcription of its gene targets, which includes SOCS1,

a negative regulator of IFN-γ signaling. SOCS1 then inhibits signaling at the IFNGR, preventing further IFN-γ-mediated gene induction (89). This negative regulation is particularly important to prevent further transcription of IFN-γ and apoptosis of IFN-γ responsive cells. T-LGLL cells have significantly reduced SOCS1 mRNA levels, further suggesting a defect in IFN-γ negative regulation (40).

In addition to SOCS1 regulation, IFNGR can be modulated in response to TCR activation (90), infection (91, 92), stimulation with other cytokines (93) or activating agents (94), or receptor internalization through endocytosis (75, 93). These processes reduce cellular sensitivity to IFN-γ, diminishing IFN-γ-mediated effects including proliferation, apoptosis, or inhibition of differentiation (95). This allows some tumor cells to avoid surveillance by the immune system as seen in basal cell carcinoma (96). Downregulation of the IFNGR is also implicated in the promotion of disease progression (91). As IFN-γ is damaging to healthy PBMCs and promotes an inflammatory environment, it is critical to limit IFN-γ production in T-LGLL.

The JAK-STAT pathway can be targeted in several diseases using FDA-approved IL-6, IL-6R, or JAK inhibitors (97). Small-molecule inhibitors, including ruxolitinib and tofacitinib, have been successful in specifically targeting JAKs in the clinic for arthritis and myelofibrosis, respectively (97). Ruxolitinib inhibits JAK1 and JAK2 while tofacitinib inhibits JAK1 and JAK3 (97) with an observed reduction in p-STAT1 and p-STAT3 (98). Siltuximab and tocilizumab target IL-6 and IL-6R, respectively (97). Use of JAK inhibitors is not always desirable as JAKs function upstream of many critical pathways, causing off target effects. Thus, it would be beneficial to target specific transcription factors and cytokines that promote disease progression, such as STAT1,

STAT3, and IFN-γ, in the case of T-LGLL.

Despite considerable research efforts, specific STAT inhibitors or targeting of IFN-γ is not yet available for clinical use. STAT1 and STAT3 are difficult to target therapeutically as these proteins are intracellular transcription factors without enzymatic activity (97), unlike the JAKs, which are kinases. STAT3 inhibitors are being developed and evaluated in clinical trials currently, with these drugs often disrupting STAT3 dimerization and DNA-binding activity (97). There are fewer studies regarding specific targeting of STAT1 or IFN-γ, which is crucial given that STAT1 and IFN-γ typically function in a canonical signaling pathway and are both considered pro-tumorigenic. To compensate for the lack of pharmaceutical approaches for specifically inhibiting STAT1 and IFN-γ, we began to evaluate calcitriol, the active form of vitamin D, as this compound has been shown to decrease p-STAT1, p-STAT3, and IFN-γ in multiple disease models. Unlike current immunosuppressant therapies and pharmacological inhibitors, calcitriol is a natural compound that has minimal toxicity and high efficacy in clinical trials.

1.3. Vitamin D as an Anti-Cancer Therapeutic

1.3.1. Vitamin D Background

Vitamin D is a steroid that has long been implicated in calcium homeostasis and bone mineralization (99, 100). Vitamin D can be obtained through supplements, dietary intake, and ultraviolet radiation (UVR) exposure (101). UVR catalyzes the conversion of 7-dehydrocholesterol to pre-vitamin D_3 in the skin, which further undergoes modification to vitamin D_3 (102). This form of vitamin D can also be obtained in certain foods in the western diet but to a lesser extent (102). Vitamin D_3 is transported through the

bloodstream to the liver through interaction with the vitamin D binding protein (102). $25(OH)D_3$, the circulating form of vitamin D, is synthesized in the liver through hydroxylation by CYP2R1 (102). From there, $25(OH)D_3$ travels to the kidney where CYP27B1 converts it to $1,25(OH)_2D_3$, the active form of vitamin D, referred to as calcitriol (102). Certain immune cells also possess active CYP27B1, allowing them to convert the circulating $25(OH)D_3$ to active calcitriol (101, 103). Once in its active form, calcitriol can exert its effects on various tissues and biological processes. Calcitriol will bind the vitamin D receptor (VDR), to induce a conformational change in the protein, which permits binding to the retinoid X receptor (RXR) (104). The VDR-RXR heterodimer then acts as a transcription factor by binding vitamin D response elements (VDRE) in DNA, causing transcription or repression of target genes (104). The metabolism of vitamin D is shown in **Figure 1.4**.

Aside from its classical roles in healthy individuals, vitamin D has been shown to exhibit numerous anti-cancer and immune-modulating properties (99, 102, 104). In multiple cancers and autoimmune diseases, calcitriol inhibits proliferation, induces apoptosis, decreases angiogenesis, and sensitizes cells to chemotherapy (104). Thus, considerable efforts have been dedicated to understanding the mechanism of vitamin D action and its full range of effects on tumor cells. Given that chemotherapy and other current anti-cancer agents have serious undesirable side effects, there is a push to investigate vitamin D use in the clinic since its main toxicity is hypercalcemia, which can be circumvented by using vitamin D analogs (104). Calcitriol and its analogs have been explored in Phase I and Phase II clinical trials for multiple cancers including prostate cancer, pancreatic cancer, and hepatocellular carcinoma (104).

Vitamin D actions on solid tumor cancers have been extensively characterized and reviewed (99, 102, 104). However, recently there has been a surge in vitamin D related studies on hematological disorders, both autoimmune and cancers, as there have been numerous *in vitro* and clinical studies with vitamin D. Clinical studies demonstrated that vitamin D analogs are well tolerated in patients with MDS (105, 106), supporting their use in the clinic. Moreover, 11/19 MDS patients experienced a clinical response after treatment with vitamin D analogs or calcitriol (106).

1.3.2. Vitamin D Serum Levels

25(OH)D_3 levels in hematological disorders and malignancies

Serum 25(OH)D_3 levels are used as an indirect measure of vitamin D levels in patients (107). Often serum levels are categorized as sufficient, insufficient, and deficient, with the exact levels required for each determined by the diagnostic laboratory (107). Thus, there is some uncertainty about what levels of vitamin D are needed to maintain good health, although the Endocrine Society defines vitamin D deficiency as 25(OH)D_3 serum levels below 20 ng/mL and insufficiency as 25(OH)D_3 levels of 21-29 ng/mL (108). Vitamin D deficiency is relatively common in the healthy population with deficiency occurring at a higher rate in Native Americans, African Americans, and women with a body mass index greater than 30 according to one study in the United States (107). One study found that 72.8% and 19.6% of African Americans and Native Americans, respectively, had insufficient levels of 25(OH)D_3, while only 11.3% of healthy European-Americans were 25(OH)D_3 deficient (107).

Even with vitamin D deficiency occurring in the general healthy population, lower 25(OH)D_3 levels are associated with development of certain cancers and severity of

cancer prognosis. Overall in hematological cancers, lower serum 25(OH)D_3 is associated with worse disease, higher malignant cell burden, and poor response to therapy based on a cohort of 105 patients with various leukemias, of which 72% were vitamin D deficient or insufficient (109). A meta-analysis of seven published studies encompassing 2,643 patients with hematological cancers showed that low serum 25(OH)D_3 levels were significantly correlated with shortened overall survival (OS) and relapse free survival (RFS) (110). Thus, the relationship between circulating 25(OH)D_3 levels and disease state in several hematological cancers and disorders is being investigated and will be discussed below.

The Philadelphia chromosome, which results from a reciprocal translocation of chromosomes 9 and 22, is characteristic of chronic myeloid leukemia (CML), and is also seen in some patients with acute lymphoblastic leukemia (ALL) (111). Sufficient 25(OH)D_3 levels were positively correlated with molecular response to treatment in Philadelphia chromosome positive leukemia patients (109). There was an observed protective effect of higher 25(OH)D_3 serum levels against development of chronic lymphocytic leukemia (CLL), although no correlation was detected between 25(OH)D_3 status and overall risk of lymphoid cancer development in this study (112). The prevalence of serum 25(OH)D_3 deficiency in 97 newly diagnosed intensively treated AML patients was recently determined (113). This study revealed that 65% of these patients did not have adequate circulating vitamin D levels, with 35% and 30% categorized as insufficient and deficient, respectively (113). Furthermore, patients that had serum levels below the accepted normal range had an increased association with worse RFS (113). This finding is supplemented by a different study that demonstrated a significant decrease in circulating 25(OH)D_3 levels in acute leukemia patients experiencing relapse

compared to those in complete remission (109). Moreover, low vitamin D levels were associated with worse OS after azacitidine treatment in AML and more episodes of febrile neutropenia and hospitalization (114). Similarly, 30.5% of newly diagnosed CLL individuals were found to be 25(OH)D_3 insufficient, and there was a significant association between 25(OH)D_3 insufficiency and shortened time-to-treatment and overall survival (115).

A study of 983 newly diagnosed non-Hodgkin's lymphoma (NHL) patients, comprised of NHL subtypes of diffuse large B cell lymphoma (DLBCL) and T cell lymphoma, found that 44% of these patients had insufficient circulating 25(OH)D_3 levels (116). These low 25(OH)D_3 serum levels were associated with shorter event-free survival (EFS) and OS in DLBCL and T cell lymphoma, while there was no correlation with 25(OH)D_3 insufficiency and EFS in the other NHL subtypes (116). Vitamin D deficiency is also associated with shorter survival in follicular lymphoma, another type of NHL (117). When DLBCL patients were treated with rituximab, patients with vitamin D deficiency had shorter EFS (118). Rituximab-mediated cellular cytotoxicity was improved in vitamin D deficient patients supplemented with vitamin D (118). This suggests that vitamin D supplementation may improve patient outcomes in the clinic and should be further investigated.

Cutaneous T cell leukemia (CTCL) patients with either Sézary syndrome (SS) or mycosis fungoides (MF) also have low 25(OH)D_3 circulating levels, defined as less than sufficient levels, comparable to the decreased levels in patients with other cancers (119, 120). Even when these patients were supplemented with vitamin D_2, there was no observed difference in response to treatment (119).

Serum 25(OH)D_3 levels have been determined in non-cancerous hematological disorders. In immune thrombocytopenic purpura (ITP), lower serum levels were negatively correlated with platelet count (121). In the case of primary autoimmune hemolytic anemia (AIHA), patients that underwent multiple treatments had lower serum 25(OH)D_3 levels compared to patients that only underwent one line of therapy (121). Across several hematological disorders, including AIHA, ITP, Evan's syndrome, and chronic idiopathic neutropenia, serum 25(OH)D_3 levels were decreased compared to healthy donors (121).

Since the main source of vitamin D is UV-B exposure, several studies have been conducted to determine early life sun exposure and development of certain cancers, including NHL, which has limited known risk factors. One study investigated whether early sun exposure could play a role in developing NHL and found that there was a significantly decreased risk of developing NHL in individuals with increased sun exposure between 13 and 21 years of age (122).

Although there are ample studies regarding vitamin D status in hematological malignancies and disorders, the majority of these studies are retrospective. Overall, higher 25(OH)D_3 levels were positively correlated with response to treatment in hematological malignancies (109). Deficiency in 25(OH)D_3 was associated with more aggressive disease state (109, 113, 116-118) and was more common in individuals with hematological diseases (115, 119-121). These studies provide valuable correlative information regarding current vitamin D status and disease state, but do not allow for assessment of causality. Thus, carefully designed prospective studies are necessary to

more accurately define the role of vitamin D status in hematological disease pathogenesis.

1.3.3. VDR

Expression

VDR is necessary for anti-cancer effects of vitamin D (102), and therefore, its levels may predict response to vitamin D treatment. This prompted measurement of VDR levels for various cancers. VDR was found to be highly expressed in 80% of B cell Hodgkin's lymphoma (B-HL), but only 17.4% of B cell non-Hodgkin's lymphoma (B-NHL) (123). Of the subtypes of HL and B-NHL examined, nodular lymphocyte predominant Hodgkin's lymphoma and diffuse large B cell lymphomas (DLBCL) had the highest proportion of samples expressing VDR, respectively (123). B-CLL and CTCL with SS were found to highly express VDR (120, 124). On the contrary, VDR and CYP27B1 mRNA expression was decreased in tumor associated macrophages in Burkitt's lymphoma (BL) (125). In a small sampling of only four ALL patients, none had detectable levels of VDR (126). Thus, VDR protein levels vary across cancer types, suggesting a potential role of VDR in disease.

Recent studies explored the role of vitamin D in the pathogenesis of acquired aplastic anemia (AA), an autoimmune cytopenia that can be associated with lymphoproliferative neoplasms, including LGLL (127). Surprisingly, untreated AA patients did not have altered circulating $25(OH)D_3$ serum levels compared to healthy donors, but had significantly decreased VDR mRNA expression levels (128). Thus, VDR downregulation is hypothesized to contribute to the hyperimmune status of AA (128). On the contrary, in other autoimmune cytopenias and hematological disorders, specifically

AIHA, ITP, Evan's and CIN, VDR expression levels were higher in the disease state compared to normal donors (121).

Overall, the majority of cells involved in hematological diseases highly express VDR. This suggests that these cells may be responsive to vitamin D treatment, which may lead to anti-tumor effects. For those diseases that exhibit decreased VDR levels, the ability to restore VDR levels should be investigated in an effort to increase sensitivity of these cells to vitamin D treatment.

SNPs

Single nuclear polymorphisms (SNPs) that have been found in VDR are often associated with different disease prognosis. A summary of the known SNPs is illustrated in **Figure 1.5**, created using the UCSC Genome Browser (129). One VDR SNP, rs10783219, was associated with a significantly lower complete remission rate, shorter RFS, and decreased OS in AML (113). For NHL, SNPs with significant disease association have been discovered in VDR including rs3819545, rs2239186, and rs886441 (122). There was also a CYP24A1 SNP, rs2762939, associated with NHL (122). Furthermore, these SNPs were associated with development of specific subtypes of NHL. The VDR SNPs rs3819545 and rs2239186 correlated with the indolent types of NHL: follicular lymphoma (FL), chronic lymphocytic leukemia (CLL), and small lymphocytic lymphoma (122). Interestingly, there were no reported polymorphisms associated with RXR or CYP27B1 for NHL (122). Although increased sun exposure was shown to decrease the risk of developing NHL, this trend was only true for those NHL individuals that were homozygous for the wild-type VDR rs4516035, a VDR germline variation associated with development of DLBCL (122). One case-control study found

that VDR SNP rs1544410 is at a higher frequency in AA patients compared to healthy controls (130). Furthermore, VDR SNPs were associated with different severities of AA, with rs1544410 and rs7975232 correlating with nonsevere AA and rs2228570 coinciding with severe AA (130). Given the multitude of studies, VDR SNPs may serve as predictive indicator of disease prognosis and risk of hematological disease development. Additional studies are required to better understand the biological consequences of these SNPs and correlate the various VDR SNPs with more hematological diseases.

1.3.4. Effects on Healthy Immune Cells

Since many hematological disorders affect PBMCs, which include T cells, B cells, natural killer (NK) cells, monocytes, and dendritic cells, many studies have investigated the effects of vitamin D on these healthy cells as well as those involved in cancers and autoimmune diseases. For example, vitamin D and its analogs inhibited proliferation and TNF-α production in PMBCs from healthy donors and Crohn's patients (131, 132) as well as blocked NF-κB activation in PBMCs from Crohn's patients (132). Vitamin D exerts a variety effects on specific PBMC subtypes as outlined below.

<u>T cells</u>

T cells are direct targets of vitamin D in addition to being indirectly modulated by vitamin D through dendritic cells and B cells as reviewed in later sections (133). Activated T cells have high levels of VDR while naïve T cells have very low or undetectable levels of VDR (133-135). T cell activation increases levels of VDR and CYP27B1, peaking at 48 hours post-activation, contrary to CYP24A1 which is not detectable at the time of isolation or post-activation (133). VDR levels further increase with calcitriol (133) or 25(OH)D_3 treatment (135). Interestingly, when treated with

calcitriol repeatedly for a longer time course, T cells experience a modest increase in CYP24A1 (133). As CYP24A1 is responsible for the catabolism of calcitriol to $1,24,25(OH)_3D_3$, which has a significantly lower binding affinity for VDR (104), T cells are able to convert $25(OH)D_3$ to its active form, calcitriol (135-137), and are also able to then break down calcitriol into a less active form (133).

Various subsets of T cells respond differently to calcitriol treatment. One study examined the response of CD45RO+ and CD45RA+ T cells to calcitriol. Proliferation of CD45RO+ T cells was reduced by calcitriol treatment, while CD45RA+ T cell proliferation was unchanged (138). In both subsets of T cells, IFN-γ and IL-2 production was decreased (138), matching several accounts of cytokine reduction in the literature. Additional studies found that IFN-γ and IL-10 production in T cells was significantly inhibited when treated with calcitriol at the time of activation or when VDR levels were maximal two days after activation (133). However, in long term culture, calcitriol was required every two days to substantially maintain reduced IFN-γ and IL-10 production (133). Calcitriol inhibited IL-2-induced IFN-γ production in human T cells (139) and IL-2, IFN-γ and IL-10 production in mice (140). VDR itself has been implicated in controlling proliferation and IFN-γ and IL-17 production in CD8+ T cells (141). When VDR is knocked out, CD8+ T cells exhibit increased proliferation and production of IFN-γ and IL-17 compared to wild-type CD8+ T cells (141).

The direct and indirect effects of calcitriol on CD4+ T cells have been extensively studied. Activated, but not naïve, CD4+ T cells are able to convert $25(OH)D_3$ to calcitriol (135). Furthermore, calcitriol was found to stabilize VDR, prevent its degradation and extend its half-life in CD4+ T cell (135). When CD4+ CD25- T cells were stimulated and

treated with calcitriol, IFN-γ, IL-17, and IL-21 pro-inflammatory cytokine production decreased while expression of CTLA-4 and FoxP3 and production of IL-10 increased (142). Proliferation of CD4+ T cells was not significantly altered by calcitriol treatment (142). However, when these CD4+ T cells were treated with both calcitriol and IL-2, the cells mimicked adaptive regulatory T cells in their ability to suppress the proliferation of resting CD4+ T cells (142).

B cells

VDR is found in activated B cells but not detectable in naïve B cells (134), which appears to be a common theme among multiple cell types. Calcitriol treatment inhibits proliferation (126, 143) and promotes apoptosis of activated B cells (126). For example, calcitriol and vitamin D analogs inhibited the proliferation of IL-2/*Staphylococcus aureus* stimulated B cells (144). Furthermore, calcitriol inhibits the production of plasma cells and post-switch memory B cells (143) as well as the production of immunoglobulin, potentially by reducing IL-2 receptor levels (145). Although VDR and CYP24A1 are at low levels and undetectable, respectively, in freshly isolated B cells, calcitriol increases both VDR and CY24A1 expression in B cells regardless of their activation state (143). Defined intracellular changes have been documented when B cells are treated with calcitriol, which include a decrease in NF-κB activation in naïve B cells through impaired NF-κB p65 binding to the p105 promoter (146). While calcitriol can act on individual immune cells, it is evident that this can also modify how cells interact with each other. For example, calcitriol-treated B cells exhibited reduced co-stimulatory molecule expression, leading to a decrease in ability to activate T cells as evidenced by a decrease in T cell cytokine production and proliferation (147).

Dendritic cells

Murine and human dendritic cells (DCs) have measurable levels of CYP27A1, CYP2R1, and CYP27B1, indicating that these cells can metabolize vitamin D (103). In addition, transcriptionally active VDR has been detected in these cells (103). Previous work has shown that calcitriol treatment of human and mouse DCs decreases T cell proliferation, possibly by decreasing IFN-γ output by DCs (103). Calcitriol was also found to decrease maturation, activation, differentiation, and survival of DCs, leading to a suppression of T cell activation and proliferation (148). Taken together, calcitriol dampens the immune response through inhibition of the ability of DC to activate T cells, preventing autoimmunity and hyper-responsive T cells.

NK cells

The effects of calcitriol treatment on NK cells, a type of cytotoxic lymphocyte, are currently unclear as contradictory findings have been reported. One study found that calcitriol acts on early NK progenitors to decrease NK cell development, proliferation, differentiation, and IFN-γ production of stem cell-derived NK cells, which created a smaller NK cell population that was largely immature with reduced function (149). Interestingly, mature NK cells were unaffected by these parameters (149). Cytotoxic capabilities were inhibited at the level of NK cell activation, while these cytotoxic functions were unaffected in NK cells that were in the effector phase, suggesting that calcitriol inhibits cytotoxic functions of NK cells during the activation stage only (150). IL-2 and IFN-γ production were decreased upon calcitriol treatment, while supplementation of exogenous IL-2 reversed this inhibition (150). On the other hand, another study found that calcitriol increases the cytotoxicity of NK cells through IFN-γ production, which enhanced killing capabilities of primary NK cells against leukemia and solid tumor cell

lines (151). It was determined that the mechanism for susceptibility to NK cell-mediated killing was due to downregulation of the microRNAs miR-302c and miR-520c within tumor cells, which increased the transcription of the NKG2D ligands, MICA, MICB, and ULBP2 (151). Upregulation of these NKG2D ligands serves as a danger signal to the immune system, targeting these tumor cells for destruction (151).

Macrophages

The primary role of macrophages in cancer progression is less clear as macrophages have the ability to be pro-tumorigenic and anti-tumorigenic, depending on their exerted functions (125). Despite their ability to promote metastasis, macrophages are able to exhibit antibody-dependent cellular cytotoxicity and participate in phagocytosis (125). Some tumor-associated macrophages (TAMs) are unable to execute these cytotoxic functions, leading to escape of cancerous cells. This process may be a result of vitamin D deficiency, which has been demonstrated in Burkitt's lymphoma (BL). Inflammatory M1 macrophages release cathelicidin, an antimicrobial peptide, which kills the B cell lymphoma cells through targeting the BL cell mitochondria (125). However, this process is dependent on vitamin D and, interestingly, BL patients have fewer M1 and more M2 macrophages (125). These M2 macrophages were anti-inflammatory, exhibited reduced production of cathelicidin, and could not kill BL cells (125). When these M2 macrophages were treated with calcitriol, cathelicidin production was induced and rituximab-mediated cytotoxicity was restored (125). This held true for treating M2 macrophages from patients *in vitro* and for treating $25(OH)D_3$ deficient individuals with vitamin D (125).

1.3.5. Vitamin D effects on malignant hematological cells

Vitamin D exerts numerous effects on malignant hematological cells (**Figure 1.6**). It has been well established that vitamin D induces differentiation in AML cells as well as apoptosis in other leukemia and lymphoma cell lines. However, the supplementation dose required to achieve this unfortunately often induces hypercalcemia *in vivo* (152). Thus, significant efforts have been made to develop vitamin D analogs with increased specificity and potency; investigate combinatorial treatments that utilize vitamin D to enhance sensitivity to chemotherapy treatments; and utilize alternative vitamin D delivery methods that would circumvent the negative side effect yet inhibit proliferation and promote differentiation or apoptosis of cancerous cells.

Calcitriol & Analogs

Calcitriol and its noncalcemic analogue MC903 induce differentiation and inhibit proliferation in SU-DHL4 and SU-BUL5 B-cell lines representative of follicular NHL possessing the common 14;18 translocation (153). The concentrations required to demonstrate these effects *in vitro* were higher than the physiological range (153). Interestingly, there was still an observed clinical response to calcitriol in patients with follicular NHL, even at lower calcitriol doses than required for effects in the cell lines (153). This suggests that calcitriol acts on the CD4+ T cells that are implicated in the induction of lymphoma cell proliferation rather than the tumor cells themselves (153). In another type of NHL, DLBCL, treatment of the cell line models with calcitriol and vitamin D analogs induced necrosis (144). Calcitriol also inhibits growth of malignant B cell progenitors from acute lymphoblastic leukemia (ALL), while leaving viability and differentiation state unchanged (126).

EB1089, a calcitriol analog, has been well studied in solid tumor models and has

been shown to induce apoptosis in a cell cycle independent mechanism in B cell chronic lymphocytic leukemia (B-CLL) patient cells (124). Bcl-2 and Mcl-1 protein levels as well as extracellular signal-regulated kinases (ERK) activity were decreased in B-CLL apoptotic cells while p38 mitogen-activated protein (MAP) kinase and caspase-3 were activated in these apoptotic cells (124). B-CLL patient cells exhibited similar responses to EB1089 regardless of their current treatment regime (124). This apoptosis was unique to the malignant cells, as they were significantly more sensitive to EB1089 treatment compared to B cells from healthy donors (124). This finding demonstrates that the calcitriol-induced apoptosis is selective for the malignant cells, leaving the normal cells unharmed.

EB1089 negatively affects NCI-H929 myeloma cell growth and viability (154). Growth arrest was achieved through induction of G_1 phase cell cycle arrest, while apoptosis involved increased activity of caspase 3 protease and p38 MAP kinase (154). PARP protein degradation, p44 ERK activity inhibition, and decreased Bcl-2 levels were observed during apoptosis (154).

Calcitriol analogs with side chain modifications, PRI-1906 and PRI-1907, increased differentiation of blast cells from patients with AML compared to calcitriol (155). However, CD14 protein levels were increased upon either analog or calcitriol treatment (155). Interestingly, these analogs had increased effectiveness in blasts from patients with mutated NPM1 or a normal karyotype, compared to patients with mutated FLT3 receptor, whose cells exhibited the least blast differentiation (155).

Vitamin D_2

Although many studies utilize calcitriol and its analogs, vitamin D_2, also known as ergocalciferol, is being studied in animal and cell models since ergocalciferol has less toxicity compared to vitamin D_3. Ergocalciferol was investigated in the HL-60 AML cell line (156). This vitamin D compound induced apoptosis through a reactive oxygen species mechanism leading to a loss of mitochondrial membrane potential, production of reactive oxygen species compounds, depletion of glutathione (GSH), release of cytochrome c, increase in pro-apoptotic proteins, decrease in anti-apoptotic proteins, and Fas receptor induction (156).

Synergism with anti-cancer therapies

Synergism of drug combinations is a principle that could be exploited in regards to vitamin D combinatorial treatment. Many studies have shown that combining a vitamin D analog with a cancer drug produces a powerful effect *in vitro*. The combination of calcitriol with azacitidine was tested in the AML cell lines HL-60 and MOLM13 (114). The combination significantly inhibited proliferation more than either drug alone (114). Another study combined glycogen synthase kinase 3 (GSK3) inhibitors with a low concentration of calcitriol and demonstrated that multiple AML cell lines, including HL-60, OCI-AML3 and Mono-mac3, differentiated in response to treatment (157). This promotion of differentiation was greater than calcitriol alone or GSK3 inhibitors combined with an additional FDA-approved GSK3 inhibitor in the HL-60 cell line (157). When treated with the GSK3 inhibitor, the majority of primary AML patient cells tested were sensitized to calcitriol-induced differentiation (157). In the AML cell lines, an increase in irreversible growth arrest in G_0-G_1 phase of the cell cycle and decreased expression of cyclin A were observed with the GSK3 inhibitor and calcitriol treatment (157). The role of GSK3 in this sensitization to calcitriol-induced differentiation was confirmed by utilizing

GSK3B knockdown cells, which exhibited increased differentiation and VDR transcriptional activity compared to GSK3B wild-type cells (157). GSK inhibition in combination with calcitriol led to an induction of VDR serine-208 phosphorylation, a site that affects VDR transcriptional activity, and increased JNK activation, potentially elucidating the mechanism of action of these combined treatments (157). Furthermore, treatment of a mouse model of human AML with the combination of the GSK3 inhibitor and calcitriol prolonged animal survival (157). In addition to the GSK3 inhibitor and calcitriol synergistic effects, the effects of vitamin D_2 analogs in combination with plant polyphenol carnosic acid have been investigated. This combination inhibited G_1-S cell cycle transition and induced cell differentiation in multiple AML cell lines (158).

Combinational treatments have been explored for CTCL. Treatment of the MyLa cell line, a model of CTCL, with 25(OH)D_3 or calcitriol alone did not affect proliferation. However, a combination treatment of 25(OH)D_3 or calcitriol and bexarotene, an RXR agonist, inhibited proliferation (120). The role of vitamin D sufficient and insufficient levels was demonstrated in this experiment, as 33 nM of 25(OH)D_3, representative of insufficient vitamin D, did not significantly increase apoptosis of the MyLa cell line and CTCL patient samples (120). A dose of 100 nM of 25(OH)D_3 was necessary to see effects, with greater apoptosis observed when bexarotene was added (120). These results suggest further investigation into the use of combinatorial treatments with vitamin D to achieve better therapeutic effect.

Alternative vitamin D delivery methods

An additional method to circumvent hypercalcemia and create a more specific cancer target is to alter the calcitriol delivery method. Calcitriol was packaged into a

liposome that would deliver it to the HL-60 AML cell line or primary peripheral blood cancer cells from AML patients (159). Proliferation was inhibited by 65% when treated with 48 nM of calcitriol using liposomal delivery (159). Liposomal delivery of the vitamin D stereoisomer analog, MC1288, achieved this inhibition at a log lower dosage of 4.8 nM (159). Additionally, the effects were specific to AML cells as proliferation was not significantly altered in normal T cell lines (159). Differentiation of HL-60 cells was induced as evidenced by increased CD14 expression and decreased CD33 expression (159).

Resistance to calcitriol treatment

Although calcitriol treatment shows promise in many leukemia and lymphoma cell lines, mouse models, and primary patient cells, there are cell lines that are resistant to the effects of calcitriol. For these malignancies, the mechanism behind this resistance has been studied in an effort to perturb this pathway and restore calcitriol sensitivity. KG1, an AML cell line, is resistant to the differentiating effects of calcitriol contrary to numerous observations of calcitriol sensitivity in other AML cell lines (160, 161). This cell line exhibited very low VDR gene expression due to degradation of VDR mRNA (160). Additionally, KG1 had constitutively active STAT1 and increased levels of interferon stimulated genes (ISGs) resulting from a constitutively active fusion protein FOP2-FGR1 (160). KG1 exhibited restored sensitivity to calcitriol and VDR expression was increased when FOP2-FGR1 was disrupted (160).

Calcitriol and the JAK-STAT pathway

Dysregulation of the Janus kinase (JAK)-signal transducers and activators of transcription (STAT) pathway is common in leukemias and lymphomas including LGLL

(56, 162), T cell lymphomas (163), AML (51, 160), B-CLL (164), and T cell acute lymphoblastic leukemia (T-ALL) (165). This aberrant activation can be due to an increase in JAK-STAT promoting cytokines, repression of endogenous JAK-STAT regulators, activating mutations in the JAKs or STATs themselves (166), or fusion proteins as observed in KG1 (160).

Inhibition of the JAK-STAT signaling pathway leads to apoptosis of several types of cancer cells, including LGLL (56). Thus, investigations into potential JAK-STAT pharmacological inhibitors are underway. Unfortunately, limited potency of STAT inhibitors and negative side effects currently prevent the clinical use of STAT inhibitors (53). Vitamin D may serve as a novel JAK-STAT inhibitor as vitamin D has been found to reduce STAT3 activation in the mouse model of experimental diabetes (167) and experimental allergic encephalomyelitis (EAE) (168) as well as human esophageal squamous cell carcinoma cell lines (169). Furthermore, vitamin D decreases STAT1 activation and pro-inflammatory cytokine output in the mouse model of EAE, correlating with a lesser disease state and improved symptomology (168). In hepatocellular carcinoma pro-inflammatory cytokines are also reduced, likely through a p27(kip1) gene dependent mechanism (170).

Similarly, calcitriol alters pro-inflammatory cytokine production in PBMCs from AA patients. One study found that IFN-γ, TNF-α, IL-10, IL-17A, and TGF-β1 levels were elevated in AA PBMCs, but calcitriol treatment significantly decreased the production of IFN-γ, TNF-α, and IL-17A while increasing TGF-β1 (128). Calcitriol also inhibited the differentiation of Th17 and Th1 cells while increasing Th2 cells (128). Calcitriol decreased T-bet mRNA levels in AA patient cells (128), supporting a shift from Th1

phenotype. Several autoimmune and hematological disorders share a similar cytokine profile of higher IFN-γ, IL-10, IL-6, and IL-17, as observed across ITP, AIHA, Evan's syndrome, and CIN (121). Although this study did not measure changes in cytokine profile after vitamin D treatment, anti-erythrocyte autoantibody production was decreased following vitamin D treatment in patients with AIHA (121), suggesting that vitamin D may decrease the autoimmune nature of autoimmune cytopenias.

1.3.6. Vitamin D Summary

With the discovery of vitamin D deficiency in many hematological disorders, understanding the role of vitamin D in the progression and treatment of such disorders is critical. Treatment with vitamin D or its analogs has shown promise in primary patient cells and cell lines in numerous hematological disorders and malignancies. Interestingly, the exact effects of calcitriol vary for different cell types. In general, calcitriol suppresses pro-inflammatory cytokine production, curtails proliferation, and inhibits antibody production in normal lymphocytes. In malignant cells, calcitriol inhibits proliferation, induces apoptosis, promotes differentiation, sensitizes malignant cells to anti-cancer therapies, and enhances cell cycle arrest. The exact mechanism(s) responsible for these anti-cancer effects is not yet known and should be determined in an effort to better understand how calcitriol exerts its effects.

Furthermore, vitamin D and its analogs have been well tolerated in the clinic (105, 106) and show promise in clinical trials for MDS (106). These studies have shown the potential of vitamin D as a combinatorial treatment. However, the adequate serum vitamin D level in humans and which form of vitamin D is the most appropriate to use for everyday supplementation versus cancer treatment needs to be determined.

Regardless, further investigations are warranted to better elucidate the effects of calcitriol on abnormal cells and to determine its potential utility in the clinical management and treatment of hematological diseases, particularly in T-LGLL, which has not been previously explored.

1.4. Project Rationale

Elevated IFN-γ production is a problem in many cancers and autoimmune diseases, including T-LGLL. IFN-γ induces apoptosis of healthy PBMCs while promoting growth and survival of cancer cells. Despite the mounting support for a negative role of IFN-γ in cancer progression and disease state, there are no therapies available for T-LGLL that are designed to specifically target IFN-γ production. Thus, the overall goal of my thesis work is to develop a novel therapy that can reduce IFN-γ production in T-LGLL without serious side effects. The long-term clinical goal of my work is that this therapy would be a standard T-LGLL therapy that improves patient symptomology with minimal off target and side effects. I approached this goal in three ways: 1) Evaluate an existing IFN-γ reducing agent, calcitriol 2) Elucidate the mechanism of action of calcitriol, and 3) Characterize the IFN-γ signaling pathway in T-LGLL to learn where this pathway can be manipulated.

T-LGLL is a pro-inflammatory leukemia with high STAT protein activation; therefore, we first sought to evaluate calcitriol as a treatment for T-LGLL (**Chapter 3**). Using primary T-LGLL patient PBMCs and the T-LGLL patient-derived cell line, TL-1 (63), we assessed changes in viability, cytokine output, and STAT1 and STAT3 phosphorylation (activation), focusing on components related to the IFN-γ signaling pathway. We next elucidated the mechanism behind calcitriol-mediated effects in T-

LGLL (**Chapter 4**), to better understand how calcitriol affects IFN-γ signaling. Finally, we characterized the dysregulated IFN-γ signaling pathway in T-LGLL to explain the increase in IFN-γ production and provide additional potential therapy targets (**Chapter 5**). This thesis work has provided a basis for vitamin D as a potential therapeutic for T-LGLL and contributed to further understanding of the actions of vitamin D in leukemic T cells with dysregulated IFN-γ-signaling pathways.

1.5. Figures

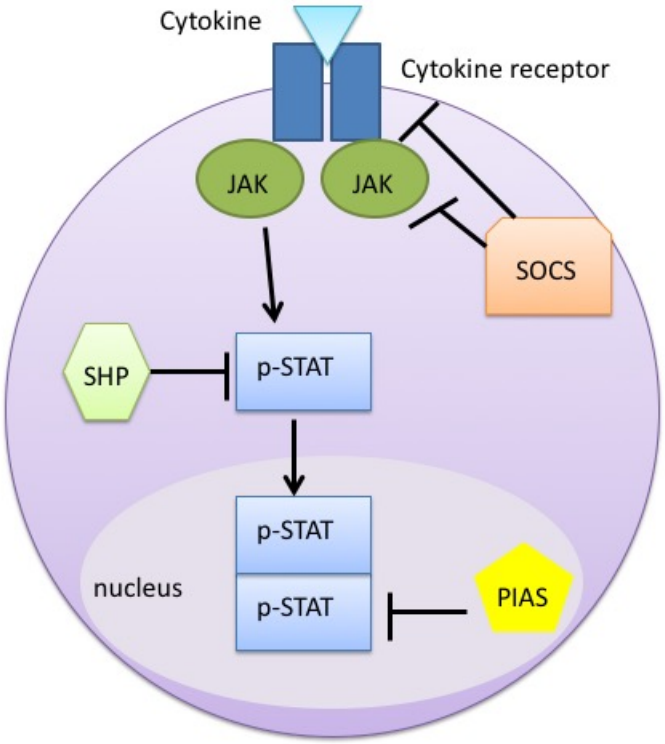

Figure 1.1. Simplified depiction of the positive and negative regulators of the cytokine-induced JAK-STAT signaling pathway. In normal JAK-STAT signaling, a cytokine binds to its respective receptor, inducing activation of the JAKs. This leads to phosphorylation of STATs, which heterodimerize or homodimerize and move into the nucleus to promote transcription of their gene targets. SOCS proteins inhibit activation of the JAKs or cytokine receptors while SHP proteins dephosphorylate STAT proteins in the cytoplasm. PIAS proteins specifically act on phosphorylated STATs in the nucleus. A detailed description of the JAKs and STATs can be found in the text and is reviewed in depth in Abroun S, Saki N, Ahmadvand M, Asghari F, Salari F, Rahim F. STATs: An Old Story, Yet Mesmerizing. Cell J. 2015;17(3):395-411.

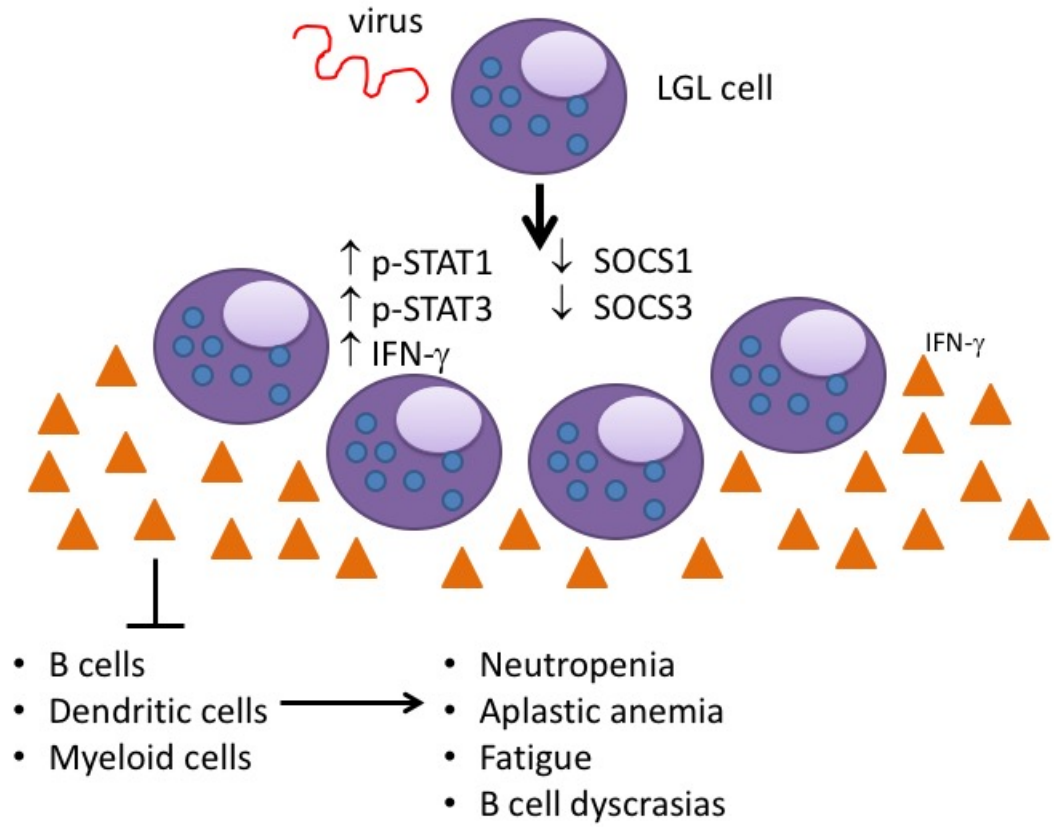

Figure 1.2. Proposed working model for increased IFN-γ production in T-LGLL. Based on our current understanding of T-LGLL, we hypothesize that a virus induces activation of LGL cells, leading to increased proliferation and decreased apoptosis. These changes are accompanied by constitutively activated STAT1 and STAT3 as well as elevated IFN-γ serum levels. Negative regulators of JAK-STAT signaling, SOCS1 and SOCS3, are dramatically reduced. This allows an uncontrolled production of IFN-γ levels which has been well documented to inhibit B cells, dendritic cells, and myeloid cells. As a result of this, neutropenia, aplastic anemia, fatigue, and B cell dyscrasias can occur, as seen in other disease models. See text for relevant citations. Genetic factors, including STAT3 mutations, play a role in T-LGLL develop and symptoms and are reviewed in the text in detail.

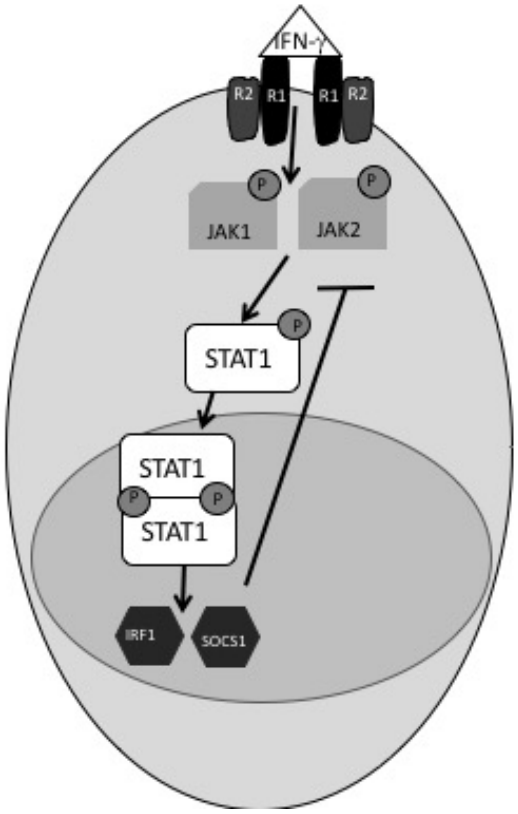

Figure 1.3. Normal IFN-γ signaling. In normal cells, the IFN-γ ligand binds to the IFNGR1. IFNGR2 participates in signal transduction leading to phosphorylation and activation of JAK1 and JAK2 and STAT1. p-STAT1 homodimerizes and moves into the nucleus to promote transcription of its gene targets including IRF-1 and SOCS1. SOCS1 is a negative regulator of IFN-γ and inhibits JAK2 activation, preventing further IFN-γ-mediated signaling. STAT1, as well as STAT3 and STAT5, are known to also support transcription of IFN-γ and thus, controlling activation of these STATs prevents transcription of excess IFN-γ.

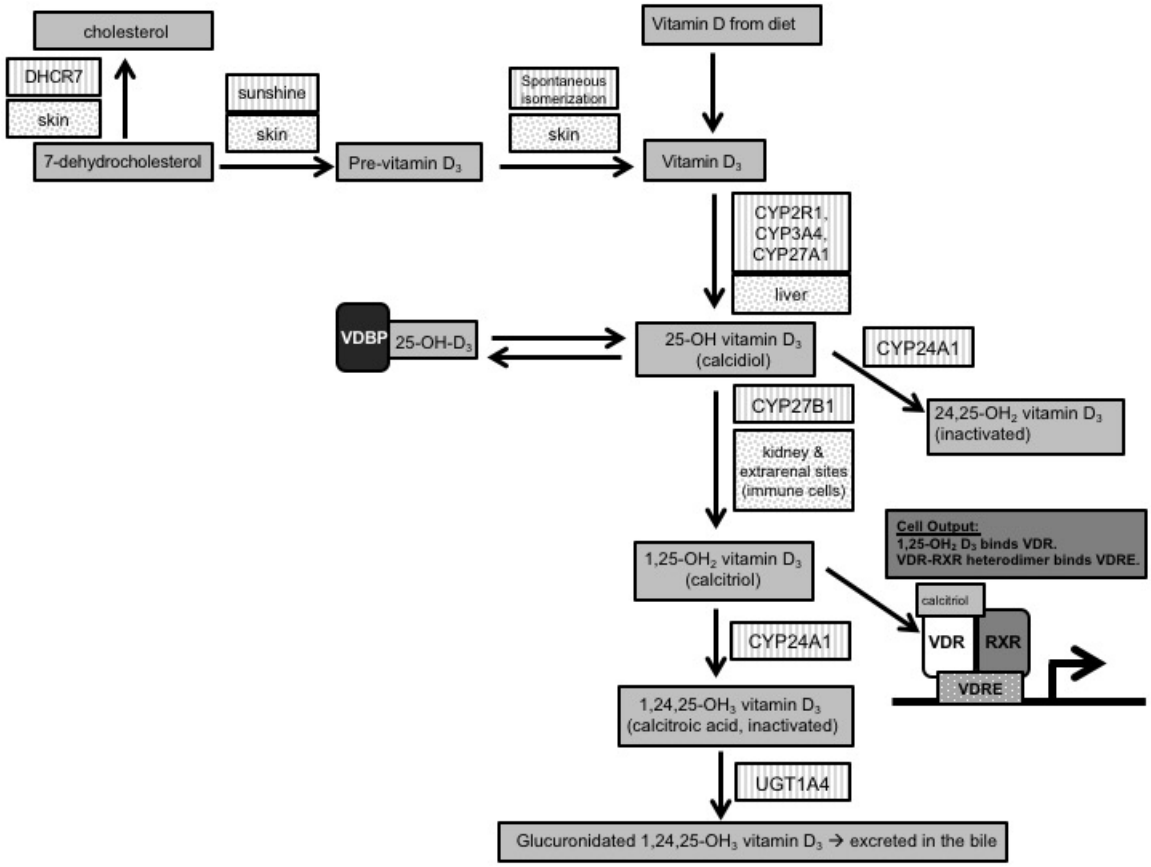

Figure 1.4. Vitamin D metabolism. Light gray boxes show different metabolites in the vitamin D pathway, with the arrows showing the direction(s) of metabolism. Dot-patterned and hatched boxes show the location or enzyme/mechanism for metabolism, respectively. VDBP is vitamin D binding protein, VDR is vitamin D receptor, RXR is retinoid X receptor, and VDRE is vitamin D response element. Figure created by Dr. Kristine Olson based on Jolliffe DA, Walton RT, Griffiths CJ, Martineau AR. Single nucleotide polymorphisms in the vitamin D pathway associating with circulating concentrations of vitamin D metabolites and non-skeletal health outcomes: Review of genetic association studies. J Steroid Biochem Mol Biol. 2016;164:18-29.

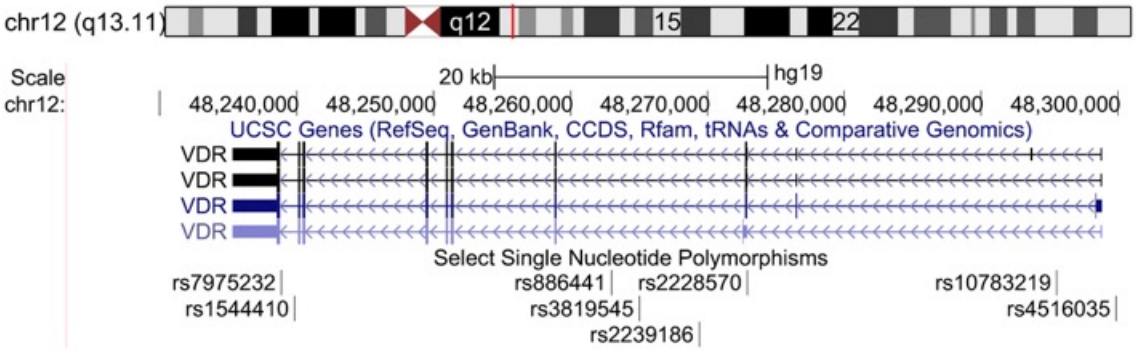

Figure 1.5. Visual representation of polymorphisms in VDR. Figure was captured from UCSC Genome Browser (https://genome.ucsc.edu) utilizing the GRCh37/hg19 human genome assembly. Chromosome cytoband, chromosome coordinates, and relative position of exons (wide bars), introns (line with arrow), and untranslated regions (narrow bars) of four isoforms of VDR are shown. Eight germline single nucleotide polymorphisms are noted. Published in Kulling PM, Olson KC, Olson TL, Feith DJ, and Loughran TP Jr. Vitamin D in Hematological Malignancies and Disorders. Eur J Haematol. 2016;98(3):187-97.

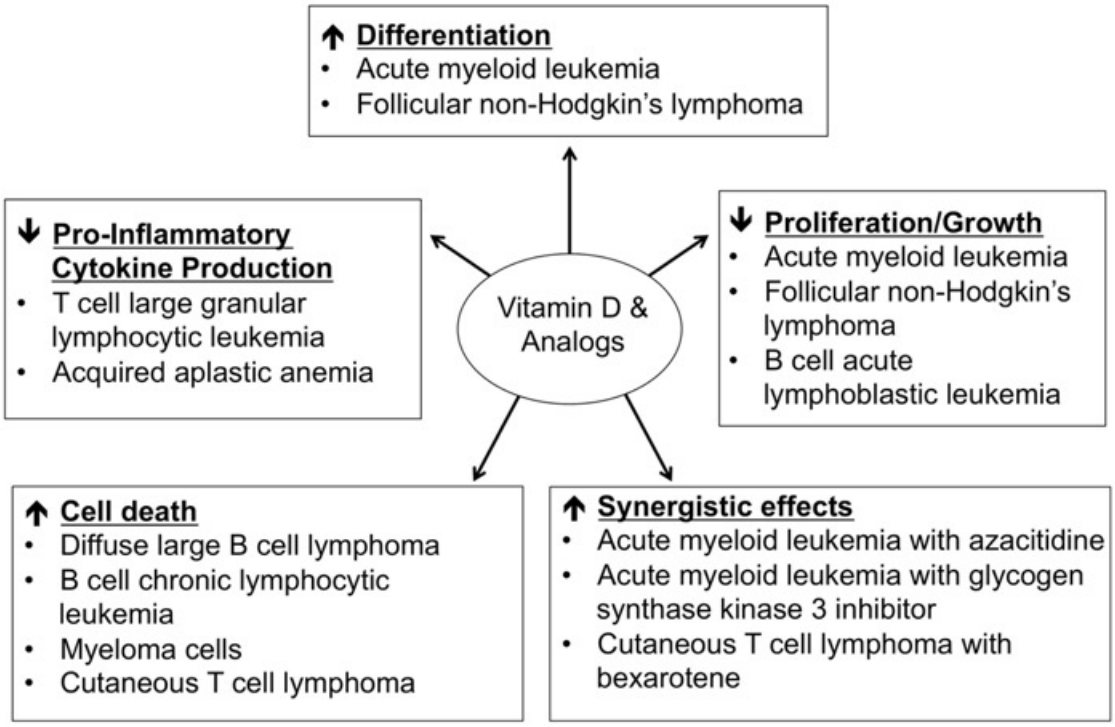

Figure 1.6. Summary of anti-cancer actions of vitamin D in hematological disorders and malignancies. See text for references. Published in Kulling PM, Olson KC, Olson TL, Feith DJ, and Loughran TP Jr. Vitamin D in Hematological Malignancies and Disorders. Eur J Haematol. 2016;98(3):187-97.

Chapter 2

Materials & Methods

Human subjects

Primary patient PBMCs were isolated from confirmed T-LGLL patients. Criteria for inclusion encompassed expanded population of circulating T-LGL cells, immunophenotyping of cell surface markers, and T-LGL clonality (2). Patients were excluded from this study on the basis of current immunosuppressant treatment or previous autoimmune disease diagnosis, unless otherwise indicated. Information regarding gender, age, T-LGLL cell purity at the time of diagnosis, and T cell receptor type for Chapter 3 and Chapter 4 can be found in **Table 3.1** and **Table 4.1**, respectively. All specimens analyzed in this study were obtained from human subjects consented to the LGL Leukemia Registry at the University of Virginia (IRB-HSR#17000 "Large Granular Lymphocyte Leukemia Registry") and studied under the University of Virginia IRB #17070 "Pathogenesis of Large Granular Lymphocyte Leukemia." STAT3 mutational profiling was performed as previously described (74).

Primary cells

PBMCs from either T-LGLL patients or health donors were isolated by Ficoll-hypaque gradient separation as described previously (171). Cell viability was determined by Trypan blue exclusion assay. For normal healthy donor samples, blood was purchased from Virginia Blood Services. Naïve CD8+ T cells were isolated from buffy coats of three normal donors using MagniSort Human CD8 Naïve T cell Enrichment Kit (eBioscience, Cat #8804-6812-74). After obtaining a cell count, each donor's cells were plated at a density of 2 million cells/mL of RPMI 1640 supplemented with10% FBS. One half of the isolated naïve CD8+ T cells were treated with 5 mg/mL of PHA. The other half remained untreated to preserve the CD8+ T cells in their naïve state. As a large number of CD8+ T cells were obtained from one normal donor, a portion of their CD8+ T cells,

both the PHA-treated and non-treated cells, received either media-only, ethanol (vehicle control), or 10 nM calcitriol immediately upon plating with or without PHA. All CD8+ T cells regardless of their treatment condition were maintained at 37°C, 5% CO_2 for 24 h before their protein was harvested using RIPA lysis buffer with protease and phosphatase inhibitor cocktails as described in the western blot section of the methods. The same procedure was used for primary T-LGLL PBMCs used for experiments except these were plated at a density of 1 million cells/mL. For cells treated with calcitriol, cells were maintained in RPMI 1640 supplemented with 10% FBS at 37°C, 5% CO_2 at a density of 1 million cells/mL for 24 h with or without calcitriol. Protein was harvested using RIPA lysis buffer with protease and phosphatase inhibitor cocktails as described in the western blot section.

Reagents

Human IFN-γ1b, premium grade (Cat #130-096-484), human CD119-APC flow cytometry antibody (clone: REA161) (Cat #130-099-920), human IFN-β1a, research grade (Cat #130-107-888), and IL-2 (Cat #130-097-743) were purchased from Miltenyi Biotec. PE anti-human IFN-γ R β chain [2HUB-159] flow cytometry antibody (Cat # 308504) and cell staining buffer (Cat #308504) were purchased from Biolegend. DAPI Solution (Cat #564907) was purchased from BD Pharmigen. OneComp eBeads (Cat #01-1111-42) were purchased from eBiosciences. IL-15 was purchased from Peprotech (Cat #200-15). Radioimmunoprecipitation assay buffer (RIPA) (Cat #R0278), lectin (phytohemaglutinin; PHA, from *Phaseolus vulgaris*, Cat #A6419), and protease and phosphatase inhibitor cocktails (Cat #P8340, Cat #P5726) were purchased from Sigma Aldrich. Calcitriol (1,25-$(OH)_2$ vitamin D_3) was purchased from Cayman Chemical (Cat #71820) and used at 100 nM unless otherwise noted. EB1089 was purchased from R&D

Systems (Cat #3993). 25(OH)D_3 was purchased from Cayman Chemical (Cat #9000683). FBS (Cat #97068-085) was purchased from Seradigm. Clarity enhanced chemiluminescence (ECL) reagent (Cat #170-5061) and PVDF membrane and filter paper (Cat #170-4274), were purchased from BioRad. RPMI 1640 (Cat #10-00-CV), Pierce bicinchoninic acid (BCA) protein assay kit (Cat #PI23225), NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Cat #78833), and SuperSignal West Femto Maximum Sensitivity Substrate (Cat #4096) were purchased from ThermoFisher Scientific. Polyacrylamide gels (4-12%; Cat #NW04125BOX) were purchased from Life Technologies. The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethozyphenyl)-2-(4-sulfonphenyl)-2H-tetrazolium (MTS) Cell Proliferation Colorimetric Assay Kit was purchased from BioVision (Cat #K300-2500). ON-TARGETplus Pooled Human STAT1 siRNA, ON-TARGETplus Pooled Human STAT3 siRNA, ON-TARGETplus Pooled Human STAT5b siRNA, ON-TARGETplus Pooled Human VDR siRNA (Cat #L-003448-00-0005) as well as ON-TARGETplus Control Non-Targeting Pool siRNA (Cat #D-001810-01-05) were purchased from Dharmacon. Ruxolitinib (Cat #S1378) was purchased from Selleckchem.

Cell culture

Media for all experiments was RPMI 1640 supplemented with 10% FBS. The TL-1 cell line, a model of T-LGLL, was previously derived from a T-LGLL patient by our laboratory and immortalized with the retroviral HTLV-2 *tax* gene (63). This cell line was authenticated using short tandem repeat DNA profiling of both cell line and patient DNA (Genetica DNA laboratories). For Chapter 5, the Jurkat T leukemia cell line (172) was also used. TL-1 cell medium was supplemented with IL-2 at 200 U/mL and all cells were maintained at 37°C, 5% CO_2. Jurkat T cells did not receive maintenance cytokine. Cells

were plated at a density of 1 million cells/mL, unless indicated. For all IFN-γ treatment and IFNGR experiments, TL-1 cells were IL-2 deprived for 10 h prior to starting the experiment. Ruxolitinib was dissolved in DMSO. For cytokine signaling experiments, cells were treated with 10 ng/mL of human IFN-γ or 5000 U/mL of human IFN-β dissolved in ultra pure DNAse and RNAase free water. For Chapters 3 and 4, calcitriol, 25(OH)D_3 and EB1089 were dissolved in 100% ethanol with the final percentage of ethanol in the media less than 0.1%. Cells were treated with varying doses of calcitriol, 25(OH)D_3, and EB1089, then harvested, lysed, and protein extracted at the designated time points between 0 to 24 h. For TL-1 cell experiments using IL-15 (Chapter 4), cells were deprived of IL-2 for 10 h before being supplemented with IL-15 at 75 U/mL for 24 h. Calcitriol or ethanol was added at the time of IL-15 stimulation.

siRNA-mediated knockdowns

All siRNA knockdown experiments utilized the Invitrogen Neon Transfection System 100 µL Kit (Cat #MPK10096). For Chapter 4, TL-1 cells were plated at 2.5 million cells/mL and treated with 50 nM, 100 nM, or 200 nM VDR siRNA or 50 nM scramble siRNA for 48 h. For Chapter 5, TL-1 cells were plated at 2.5 million cells/mL and treated with 100 nM STAT1, 50 nM of STAT3, 100 nM of STAT5b, or dose matched scrambled siRNA for 48 h. Jurkat T cells were plated at 0.5 million cells/mL and were treated with 200 nM STAT5b siRNA or 200 nM scrambled siRNA for 48 h. After 48 h, protein was harvested from a subset of TL-1 cells from each condition to assess knockdown status and viability. The MTS Cell Proliferation Colorimetric Assay Kit was used to assess cell viability after 24 h of calcitriol treatment. The reaction was incubated at 37°C, 5% CO_2 for 1 h, and formazan product was detected on a plate reader (Cytation3 Imaging Reader) at 492 nm. Data were normalized to the scrambled

treatment. All conditions were done in quadruplicate. The remaining TL-1 cells from each condition were then counted and re-plated at 1 million cells/mL in fresh media and IL-2, in addition to calcitriol or ethanol vehicle for 24 h. Supernatant was harvested for cytokine analysis by Luminex (Millipore Sigma, Cat #HCYTOMAG-60K-06) and cells were lysed to obtain protein for western blotting.

RNA extraction and quantitative PCR

For IFN-γ and IFN-β-induced signaling experiments, TL-1 and Jurkat T cells were treated with 10 ng/mL of IFN-γ or 5000 U/mL IFN-β or water for 6 h before RNA harvest. For STAT knockdown experiments, TL-1 and Jurkat T cells were treated as described in the siRNA-mediated knockdown section and then RNA was extracted 48 h after siRNA transfection (Chapter 5). Western blot analysis was performed on protein lysates from the same conditions to assess knockdown status. In Chapter 4, TL-1 cells were treated with calcitriol or vehicle control for 24 h following 48 h of STAT siRNA treatment. For all experiments, cells were harvested and lysed with Invitrogen TRIzol Reagent (Cat #15596018) and stored at -80°C. RNA was isolated using Zymo Research Direct-zol MiniPrep kit (Cat #R2050) and quantified using Invitrogen Qubit RNA Broad Range Assay kit (Cat #Q10210) and Invitrogen Qubit 2.0 Fluorometer (Cat # Q32866). Clontech RNA to cDNA EcoDry Premix (Double Primed) (Cat #639548) was used to reverse transcribe the extracted RNA. BioRad iTaq Universal SYBR Green Supermix (Cat #1725121) and PrimePCR SYBR Green Assay primers were used for all qPCR reactions: STAT1 (Cat #10025636, qHsaCED0043612), IFNGR1 (Cat #10025636, qHsaCID0013339), IFNGR2 (Cat #10025636, qHsaCID0008956), IRF-1 (Cat #10025636, qHsaCED0044080), SOCS1 (Cat #10025636, qHsaCED0002534), IFNG (Cat #10025636, qHsaCID0017614), UBC (Cat #10025636, qHsaCED0023867), VDR

(Cat #10025636, qHsaCID0023190), and CYP24A1 (Cat # 10025636, qHsaCID0007605). Each sample was loaded in triplicate for each primer and each condition had three independent biological replicates. Cycle number and annealing temperature were followed according to the Prime PCR protocol. Results were normalized to vehicle or scrambled control.

Cell Fractionation Assay

Cells were fractionated according to the NE-PER™ Nuclear and Cytoplasmic Extraction Reagents protocol. Briefly, 3 million cells were pelleted after treatment. The cells were washed with PBS then cytoplasm was obtained after lysis with CERI (protease and phosphatase inhibitors added) and CERII buffers. NER buffer was used to obtain the nuclear fraction. After quantification of the fractions, 25 µg of protein was loaded onto a gel for western blot analysis.

Western blot

Treated cells were washed with PBS then lysed in RIPA buffer with protease and phosphatase inhibitors. Protein content was quantified using the BCA assay. Proteins were electrophoresed on a 4-12% polyacrylamide gel and then transferred to a PVDF membrane, blocked with non-fat dried milk or BSA, and incubated with primary antibody at overnight at 4°C according to manufacturer recommendations. Cell Signaling Technology primary antibodies used in these studies were: STAT1 (Cat #9175), Phospho-STAT1 (Y701) (Cat #7649), STAT3 (Cat #9139), Phospho-STAT3 (Tyr 705) XP (Cat #9145), STAT5 (Cat #9363), Phospho-STAT5 (Tyr694) (Cat #9351), JAK2 XP (Cat #3230), Phospho-JAK2 (Tyr1007) (Cat #4406), Vitamin D3 Receptor (Cat #12550), IFN-γ XP (Cat #8455), PIAS1 XP (Cat #3550), PIAS3 XP (Cat #9042), SOCS1 (Cat

#3950), SOCS3 (Cat #2932), Histone H3 (Cat #4499), HSP90 (Cat #4877) and β-actin (Cat #3700). After the membrane was washed, it was incubated with secondary antibody (Cell Signaling Technology, anti-rabbit IgG-HRP linked #7074 or anti-mouse IgG-HRP linked #7076) for 1 h then treated with ECL or Femto substrate. Images were captured with a BioRad ChemiDoc MP instrument and analyzed using Image Lab software (BioRad).

Protein half-life determination.

TL-1 cells were pre-treated with calcitriol or ethanol for 1 h prior to creating time 0 lysates or adding cycloheximide (10 μg/mL). Cells were then lysed to obtain protein at 0.25, 0.5, 1, 2, 4, and 6 h following cycloheximide treatment. Viability was assessed using MTS at 6 h after cycloheximide treatment and was unchanged (data not shown).

Cytokine analysis

After 48 h treatment with siRNA (Chapter 4 & 5) or 24 h treatment with calcitriol (Chapter 3 & 4), 25(OH)D_3, or EB1089 (Chapter 4), aliquots of the conditioned media were collected and stored at -80°C prior to analysis by the UVA Flow Cytometry Core using the Luminex MAGPIX bead-based multiplex analyzer. All conditions were done in triplicate.

Flow cytometry

TL-1 and Jurkat cells were treated with Human Fc Receptor Binding Inhibitor Purified (Cat #E05904-1648) from eBiosciences and antibodies according to manufacturer protocols. Cells were analyzed using the BD Biosciences LSRFortessa. Appropriate fluorescence minus one (FMO) and compensation controls were used.

Living cells were gated followed by singlets and green fluorescent protein (GFP) status. TL-1 cells are GFP+ and Jurkat T cells are GFP-, requiring compensation for potential spectral overlap. Median fluorescent intensity of the IFNGR1 and IFNGR2 was determined and compared. Results were analyzed using FCS Express 9 (De Novo Software).

Statistical analysis

Data were analyzed using GraphPad Prism software version 7. An unpaired 2-tailed Student's t-test was utilized and P-values of <0.05 were considered significant. For experiments that normalized the control to 1, a Student's t-test was performed to a hypothetical value of 1.

Chapter 3

Calcitriol inhibits the JAK-STAT signaling pathway in T cell large granular lymphocyte leukemia

Only including experiments performed by Kulling, indicated figures in this section were adapted from: Olson KC, **Kulling PM**, Olson TL, Tan SF, Rainbow RJ, Feith DJ, and Loughran TP, Jr. Vitamin D decreases STAT phosphorylation and inflammatory cytokine output in T-LGL leukemia. Cancer Biol Ther. 2017;18(5):290-303. doi: 10.1080/15384047.2016.1235669. PubMed PMID: 27715403

3.1. Acknowledgements, Funding, and Abbreviations

Acknowledgements: We thank Alexander Wendling and Shubha Dighe for support with blood processing to obtain PBMCs and Holly Davis for LGLL Registry support. We thank Cait Hamele and Lindsay Chatfield for support with research STAT mutation testing and Kabir Ahluwalia for technical assistance. We also thank Mike Solga in the UVA Flow Cytometry Core for performing the Luminex assays. The authors wish to extend a special thank you to the LGLL patient who inspired this project.

Funding: This work was funded by the National Cancer Institute of the National Institutes of Health under award numbers R01CA098472, R01CA178393, and P01CA171983 (to TPL), the Bess Family Charitable Fund (to TPL), the LGL Leukemia Foundation (to TPL), the Immunology Training Grant T32AI007496-21 (to PMK), and the UVA Wagner Fellowship (to PMK).

Abbreviations: acute myeloid leukemia (AML), experimental allergic encephalomyelitis (EAE), interferon gamma (IFN-γ), interleukin (IL), Janus kinase (JAK), large granular lymphocyte (LGL), large granular lymphocyte leukemia (LGLL), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), peripheral blood mononuclear cell (PBMC), phytohemagglutinin (PHA), retinoid X receptor (RXR), signal transducer and activator of transcription (STAT), Src homology 2 (SH2), vitamin D receptor (VDR), vitamin D response element (VDRE)

3.2. Highlights

- T-LGLL patient PBMCs, TL-1 cells, and normal activated CD8+ T cells have high detectable protein levels of VDR.
- Calcitriol does not act on naïve CD8+ T cells as evidenced by undetectable protein levels of VDR in naïve CD8+ T cells.
- Calcitriol significantly reduces p-STAT1 and p-STAT3 in the nuclear and cytoplasmic compartments in TL-1 cell line after 24 h. VDR is increased in the nuclear compartment alone.
- Secretion of IFN-γ and IL-9 was significantly decreased while IL-4 secretion was increased in TL-1 cells following 24 h of calcitriol treatment.
- Calcitriol does not affect TL-1 cell viability.
- Overall, calcitriol promotes a switch from pro-inflammatory cytokine production to anti-inflammatory cytokine production.

3.3. Introduction

T cell large granular lymphocytic leukemia (T-LGLL) is a rare incurable leukemia characterized by clonal expansion of CD8+ cytotoxic T cells resembling CD45RA+ terminal effector memory cells (1-4). T-LGLL often co-occurs with inflammatory autoimmune diseases including rheumatoid arthritis (1). T-LGLL is hypothesized to result from chronic antigen stimulation, leading to a constitutively activated subset of T cells with defective apoptosis (1). Current treatment options utilize single agent immunosuppressants, such as methotrexate, cyclophosphamide, and cyclosporine A for management of symptoms (1). Despite good clinical responses, T-LGLL is not typically cured by such an approach.

Current research efforts aim to develop treatments that target highly activated pathways in T-LGLL. One such pathway is the JAK-STAT pathway (39). In JAK-STAT signaling, a cytokine binds to its respective receptor, activating JAKs which, in turn, activate STAT proteins through phosphorylation. These STAT proteins form heterodimers or homodimers and move into the nucleus to promote transcription of their gene targets, including additional cytokines or STAT proteins (46). In T-LGLL cells, STAT1 and STAT3 are constitutively activated through phosphorylation of their tyrosine residue 701 (p-STAT1) and 705 (p-STAT3), respectively (56). p-STAT1 and p-STAT3 can form heterodimers in T-LGLL cells, with the direct impact on transcription and downstream signaling unknown (56). STAT1 (47-49) and STAT3 (50-52, 173) are considered pro-tumorigenic in multiple autoimmune diseases and cancers, making them desired targets for cancer therapies (53-55). Moreover, there are activating STAT3 mutations in a portion of T-LGLL patients (36, 37, 40, 42), supporting a role of STAT3 in T-LGLL pathogenesis. This notion is further supported by experiments demonstrating

that drug inhibition of STAT activation, specifically STAT1 and STAT3, induces apoptosis of T-LGLL patient cells (56).

The constitutive activation of STAT1 and STAT3 is hypothesized to contribute to the T-LGLL inflammatory environment, particularly through their potential interactions with IFN-γ, a type II interferon. STAT1, and rarely STAT3, become activated in response to IFN-γ and both are known transcription factors of IFN-γ, contributing to higher levels of IFN-γ in many cell types. As expected with chronically activated cytotoxic T cells, T-LGLL patients have significantly elevated IFN-γ serum levels compared to healthy controls (40, 61).

Excess IFN-γ contributes to cancer progression and worse disease symptomology in multiple cancers and autoimmune diseases. IFN-γ promotes tumor growth in ovarian cancer (77) and correlates with worse disease state in melanoma (174), B cell chronic lymphocytic leukemia (175), and Alzheimer's disease (82). Excessive IFN-γ production negatively impacts normal peripheral blood mononuclear cells (PBMCs) by inhibiting proliferation, reducing antigen presentation, and inducing apoptosis (81, 83, 84, 86, 176). Elevated IFN-γ secreted by cytotoxic lymphocytes contributes to the development, progression, and severity of chronic myeloid leukemia (177). In multiple sclerosis, production of IFN-γ by stimulated CD3+ CD8+ T cells correlates with fatigue and depression (178). In aplastic anemia, a disease that may co-occur with T-LGLL (127), IFN-γ is directly involved in the disease development through inhibition of myeloid progenitors and lineage differentiation (79). Thus, it is critical to limit IFN-γ production in diseases, including T-LGLL.

Targeting the key players of JAK-STAT signaling, particularly STAT1, STAT3, and IFN-γ, is a goal of T-LGLL research. Unfortunately, current STAT targeting agents exhibit considerable side effects and limited potency in clinical development for other diseases (53). However, a patient from Dr. Loughran's LGL Leukemia Registry contacted the lab to propose investigation of a natural JAK-STAT pathway inhibitor, vitamin D. This patient was voluntarily supplementing with high doses of vitamin D and documented an improvement in complete blood cell counts and symptomology, including neutropenia. This prompted our investigation into the use of vitamin D as a potential T-LGLL therapy.

Vitamin D is a steroid that is obtained through consumption of fatty foods and ultraviolet radiation exposure (107). Although foods in the western diet are fortified with vitamin D, deficiency is rather common (107) and is linked to the development and severity of certain diseases (113) including several types of leukemia (109, 113, 115). Moreover, vitamin D supplementation often alleviates symptoms or inhibits pro-tumorigenic pathways, including JAK-STAT (167, 169). Calcitriol reduced IFN-γ production and STAT1 and STAT3 activation in the experimental allergic encephalomyelitis (EAE) mouse model of multiple sclerosis (168). This treatment also correlated with improved symptoms and a less severe disease state (168). Calcitriol reduced STAT3-mediated IL-6 signaling in esophageal squamous cell carcinoma (169) and downregulated STAT3 signaling in experimental periodontitis (167). In aplastic anemia, calcitriol treatment of PBMCs decreased IFN-γ production (128), the hypothesized driver of this disease (79). These findings suggest that calcitriol could potentially reduce activated STAT1 and STAT3 and IFN-γ production in T-LGLL as seen

in other diseases. In addition to its effects on the JAK-STAT pathway, calcitriol induces apoptosis, inhibits proliferation, promotes differentiation, and sensitizes cancer cells to chemotherapy, with minimal toxicity (179). Many of these effects require the vitamin D receptor (VDR), a nuclear receptor, which after binding with calcitriol, undergoes a conformational change and acts as a transcription factor (180).

Therefore, we aimed to evaluate the effects of calcitriol on T-LGLL patient cells and the TL-1 patient-derived cell line, TL-1. VDR was detectable at the protein level in the majority of T-LGLL patient PBMCs and TL-1 cell line. We found that calcitriol significantly reduced p-STAT1 and p-STAT3 in the TL-1 cell line after 24 h of treatment, matching our findings in activated CD8+ T cells. This was accompanied by a significant increase in VDR and a decrease in IFN-γ secretion in TL-1 cells. p-STAT1 and p-STAT3 were inhibited in the nucleus and cytoplasm compartments while VDR was increased in the nucleus alone. Calcitriol did not reduce viability in TL-1 cells, suggesting a mechanism for JAK-STAT inhibition independent of cell death. Taken together, calcitriol inhibits the JAK-STAT pathway and upregulates VDR in TL-1 cells. Our results support further investigation into the use of calcitriol as a JAK-STAT pathway inhibitor for T-LGLL.

3.4. Results

T-LGLL patient PBMCs have high levels of VDR protein

As VDR is necessary for many anti-cancer properties of calcitriol (99, 104), we first sought to determine whether T-LGLL patient cells have detectable protein levels of VDR. Primary PBMCs were isolated from T-LGLL patients (**Table 3.1**) and VDR protein levels were assessed using western blot. At the time of collection, the majority selected T-LGLL patients (8/10) were not currently on any immunosuppressant treatments. Although VDR is not present in resting peripheral T and B cells (134), we found that VDR was detectable in 9/10 T-LGLL patients (90%) (**Figure 3.1**). STAT3 Y640F patients, on average, had higher p-STAT3 and total STAT3 levels compared to STAT3 WT patients (**Figure 3.1**). However, VDR levels did not correlate with p-STAT3 or STAT3 levels or STAT3 mutational status (**Figure 3.1**).

Activated, but not naïve, CD8+ T cells respond to calcitriol treatment

To better understand how calcitriol acts on normal T cells compared to T-LGLL cells, we isolated naïve CD8+ T cells from three healthy donors. We proceeded to activate half of the T cells using phytohaemagglutinin (PHA) in order to compare naïve and activated CD8+ T cells. Naïve CD8+ T cells did not have detectable p-STAT1, p-STAT3, or VDR protein levels (**Figure 3.2A**), as supported by the literature (134, 181). On the contrary, activated CD8+ T cells exhibited robust protein levels of p-STAT1, p-STAT3, and VDR (**Figure 3.2A**). Calcitriol reduced the activation of STAT1 and increased VDR levels in the activated CD8+ T cells, while there was no observed effect on levels of p-STAT1 and VDR in naïve T cells after 24 h (**Figure 3.2B**). Our results confirm that VDR is found in activated T cells, but not naïve T cells, explaining the presence of VDR in T-LGLL cells, which are constitutively activated T cells.

Calcitriol reduces p-STAT1 and p-STAT3 while increasing VDR protein levels in TL-1 cells

Based on our findings that nearly all of the primary T-LGLL patient PBMC samples had detectable levels of VDR and that calcitriol reduces p-STAT1 and p-STAT3 in activated T cells, we next sought to determine whether calcitriol altered p-STAT1, p-STAT3, or VDR levels in T-LGLL. We chose to first evaluate the effects of calcitriol in TL-1 cells using a range of commonly published calcitriol dosages (0.1-100 nM) compared to vehicle (ethanol) and no treatment control for 24 h. Calcitriol reduced p-STAT1 at all concentrations, with a significant decrease at 100 nM calcitriol (**Figure 3.3A, Figure 3.3C**). p-STAT3 was also significantly reduced with 10 nM calcitriol treatment (**Figure 3.3B, Figure 3.3D**). The inhibition of STAT1 and STAT3 activation correlated with a significant increase in VDR protein levels (**Figure 3.3A, Figure 3.3E**). Thus, TL-1 cells responded to calcitriol treatment.

The reduction of p-STAT1 and p-STAT3 by calcitriol occurs in the nucleus and cytoplasm in TL-1 cells

We next investigated where the reduction in p-STAT1 and p-STAT3 occurred at the subcellular level. STAT1 and STAT3 become phosphorylated in the cytoplasm following cytokine stimulation (46); in the case of the TL-1 cell line, IL-2 induces activation of these STATs . After dimerizing, the STAT proteins translocate into the nucleus to promote transcription of their gene targets. Therefore, the reduction in p-STAT1 or p-STAT3 could occur in either the cytoplasm or nucleus or both. We treated TL-1 cells with 10 nM calcitriol or ethanol, the vehicle control, or left cells untreated for 24 h. Nuclear and cytoplasmic fractionating was performed to isolate these cellular compartments. Histone

H3 was used as a marker for the nuclear portion and heat shock protein 90 (HSP90) was used for the cytoplasmic compartment. We found that p-STAT1 and p-STAT3 were reduced in both the nucleus and cytoplasm, while VDR was increased in the nucleus alone (**Figure 3.4A, Figure 3.4B**). As p-STAT1 and p-STAT3 are decreased in the nucleus and VDR is increased in the nucleus, transcriptional activity of these proteins is decreased and increased, respectively, suggesting effects of calcitriol on downstream signaling.

Calcitriol significantly reduces IFN-γ production in TL-1 cells

Given the decreased p-STAT1 and p-STAT3 levels in the nucleus, we measured the subsequent effect on cytokine production, as p-STAT1 and p-STAT3 are known to induce transcription of cytokines, including IFN-γ (46, 182, 183). TL-1 cells were treated with 10 or 100 nM of calcitriol or the ethanol vehicle control for 24 h before conditioned media was collected and sent for cytokine analysis using the Luminex platform. Both 10 and 100 nM calcitriol significantly reduced IFN-γ secretion ($p<0.005$) in TL-1 cells within 24 h compared to ethanol, the vehicle control (**Figure 3.5A**). We next wanted to determine whether calcitriol also reduced other inflammatory cytokines, including IL-9 and macrophage inflammatory protein 3 alpha (MIP-3α). IL-9 is associated with inflammatory diseases including ulcerative colitis (184) and psoriasis (185), while MIP-3α is correlated with inflammation and recurrence of hepatocellular carcinoma and poorer response to therapy (186). In TL-1 cells, calcitriol indeed significantly reduced IL-9 ($p<0.05$) (**Figure 3.5B**) and modestly reduced MIP-1α (**Figure 3.5C**) secretion. To examine whether the decrease in inflammatory cytokines was accompanied by an increase in anti-inflammatory cytokines, we also measured levels of IL-4 secretion, in the presence and absence of calcitriol. IL-4 is an anti-inflammatory cytokine that has been

shown to reduce severity of isofamide-induced hemorrhagic cystitis (187) and inhibit tumor necrosis factor alpha (TNF-α) (188) and IL-1β production (189), inflammatory cytokines associated with worst disease state. 100 nM calcitriol significantly increased IL-4 production ($p<0.01$) in TL-1 cells after 24 h (**Figure 3.5D**). Taken together, calcitriol switches the TL-1 cytokine production from pro-inflammatory to anti-inflammatory.

Modulation of STAT activation and cytokine production are not due to changes in TL-1 cell viability

Calcitriol induces apoptosis and decreases cell survival of many malignant cell types (190). To assess whether the changes in cytokine production or STAT activation were due to decreases in cell viability, we treated TL-1 cells with a range of calcitriol dosages for 24 h and measured viability using the MTS assay. TL-1 cell line maintained relatively consistent viability across dosages compared to the ethanol treatment (**Figure 3.6**), ruling out cell death as a cause of cytokine secretion changes.

3.5. Discussion

Our study demonstrated that VDR is highly expressed in primary T-LGLL patient PBMCs (**Figure 3.1**), activated CD8+ T cells (**Figure 3.2**), and the TL-1 cell line (**Figure 3.3**). Although VDR is not necessary for all calcitriol effects, expression of VDR is necessary for any VDR-mediated actions of calcitriol (191). However, malignant cells often downregulate VDR levels (192), avoiding the apoptotic or negative effects of calcitriol on cytokine production. Inactivation or deficiency of the VDR-mediated pathway promotes tumor progression and metastasis in pancreatic cancer (193). In breast cancer, VDR expression was negatively correlated with aggressiveness (194) and with metastasis in clinical specimens as higher VDR expression inhibited the pro-metastatic effects of macrophages (195). In CD8+ T cells in particular, VDR controls CD8+ T cell proliferation and inflammation with specific knockout of VDR in CD8+ T cells inducing colitis (141). Thus, VDR levels are beneficial for controlling cytotoxic effects of CD8+ T cells and responding to calcitriol. It is particularly promising that T-LGLL cells and the TL-1 cell line have robust VDR levels, as this means that the VDR pathway may not be entirely dysregulated.

With VDR highly expressed by 90% of T-LGLL patient PBMCs and TL-1 cell line, it is likely that these cells are capable of responding to calcitriol. Calcitriol reduced p-STAT1 and p-STAT3 in the TL-1 cell line (**Figure 3.3, Figure 3.4**). IFN-γ secreted levels were also significantly decreased (**Figure 3.5**). At the same time, VDR levels were significantly increased with calcitriol treatment (**Figure 3.3, Figure 3.4**). This raises the question of whether VDR engagement is necessary for reduction in p-STAT1, p-STAT3, or IFN-γ in T-LGLL. The necessity of VDR is supported by previous studies that found that VDR knockout in CD8+ T cells increased IFN-γ production (141) and VDR directly

binds to IFN-γ promoter to inhibit IFN-γ production (180). However, there are less data available regarding the role of VDR in regulating STAT activation. VDR has been shown to directly bind to STAT1 in multiple studies (196, 197), releasing STAT1 upon calcitriol and IFN-α treatment in hepatitis C infection (197). Moreover, VDR knockout mice had significantly increased p-STAT3 and significantly decreased p-STAT1, suggesting that VDR does play a role in regulating activation of both STAT proteins (198). We chose to focus on STAT1 and IFN-γ for our additional studies as these two proteins typically function in a canonical pathway. We addressed the relationship between VDR, STAT1, and IFN-γ in **Chapter 4**.

Calcitriol reduced both p-STAT1 and p-STAT3 in TL-1 and T-LGLL cells, matching findings in the EAE mouse model (168). However, this relationship is not universal as STAT1 and STAT3 can also be reduced independently of each other with calcitriol treatment (167), suggesting multiple regulatory mechanisms for reducing STAT1 and STAT3 activation. The cellular location of regulation is not clear as p-STAT1 and p-STAT3 are reduced in both the nucleus and cytoplasm in our studies (**Figure 3.4**). However, observed reduction in the nuclear compartment would directly impact STAT1 and STAT3-mediated transcription of target genes, which is a therapeutic goal. As STAT1 and STAT3 are implicated in the pathogenesis of T-LGLL (56), reduction of STAT1 and STAT3 gene targets is desirable. This inhibition of STAT activation could be potentially explained in several ways. First, calcitriol could increase activity of cytoplasmic phosphatases, including SHP-1 and SHP-2, which act directly on STAT1 and STAT3 to remove their activating tyrosine phosphorylation (46, 199). This would lead to a reduction in p-STAT1 and p-STAT3, but not total protein levels, matching our observations. Second, calcitriol may reduce the ability of the cytoplasmic JAKs to

phosphorylate the STAT proteins, causing a reduction in phosphorylated STATs in the cytoplasm and a subsequent decrease in STATs translocated into the nucleus. This inhibition of JAK activity could be explained by degradation of the JAKs or increased activity of SOCS1 and SOCS3, which directly inhibit JAK-mediated STAT activation (199). For reduction of STATs originating in the nucleus, protein inhibitors of activated STATs (PIAS) could play a role as PIAS1 and PIAS3 inactivate phosphorylated STATs specifically (199). In fact, PIAS3 activity is directly correlated with STAT3 activation and patient survival in malignant mesothelioma (173). PIAS activity would lead to an initial decrease in activated STATs in the nucleus, but not cytoplasm. However, due to the role of STATs in transcribing activating cytokines, reduction of STATs in the nucleus could lead to decreased cytokine-mediated activation of STATs in the cytoplasm. Another possibility is that calcitriol may reduce stability or increase proteasomal degradation of STAT1 or STAT3 in the nucleus or cytoplasm, thereby reducing their activation. This is unlikely, however, as total STAT1 and STAT3 levels remain constant across treatment conditions. We characterize the requirements for calcitriol-mediated reduction of STAT1 in **Chapter 4** and elucidate the relationship between STAT1 and STAT3 in **Chapter 5**.

There are multiple potential mechanisms to explain the decrease in p-STAT1 and IFN-γ. STAT1 and IFN-γ typically function through a canonical signaling pathway, with IFN-γ activating STAT1 (75, 76, 87-89) and STAT1 often transcribing IFN-γ (182, 183). Thus, calcitriol could conceivably act by three mechanisms in TL-1 cells: 1) Calcitriol inhibits STAT1 activation, reducing IFN-γ transcription 2) Calcitriol inhibits IFN-γ production, preventing activation of STAT1 and 3) p-STAT1 and IFN-γ are reduced through independent mechanisms. Cell death is not responsible for the observed changes (**Figure 3.6**), ruling out this particular mechanism. This calcitriol-mediated

mechanism is addressed in **Chapter 4**.

One of the main goals of cancer therapy is to target the cancer cells, while avoiding damage to the healthy PBMCs. Because naïve CD8+ T cells do not have detectable VDR protein (200) (**Figure 3.2**), little or no VDR protein is in the cell. Therefore, naïve T cells do not respond to calcitriol treatment. This allows the naïve T cells to become activated and proliferate upon antigen stimulation (201). Moreover, our results confirm the abundance of VDR in activated CD8+ T cells (**Figure 3.2**). VDR is critical for mounting an effective immune response to pathogens, a process which calcitriol does not inhibit (202, 203). In fact, lower vitamin D serum levels and therefore less VDR signaling is associated with development of infectious diseases (204), implicating vitamin D levels in protecting against the detrimental effects of infection. VDR controls CD8+ T cell proliferation (141), promoting a normal CD8+ T cell expansion and preventing autoimmunity while promoting an effective immune response to pathogens. VDR knockout mice exhibit defects in CD8+ T cell differentiation and survival, leading to a reduction in granzyme B production (205). Moreover, in the absence of VDR, the antigen-specific CD8+ T cell population is markedly reduced and localization to non-lymphoid tissues is inhibited (205). Thus, in normal healthy PBMCs, activated CD8+ T cells have high levels of VDR while naïve CD8+ T cells do not have detectable VDR, matching our findings. Taken together, T-LGLL and TL-1 cells closely resemble normal activated CD8+ T cells with prominent activation of STAT1 and high levels of VDR (**Figure 3.1, Figure 3.3**). Calcitriol reduced p-STAT1 and p-STAT3 while increasing VDR protein levels in activated CD8+ T cells (**Figure 3.2**) and the TL-1 cell line (**Figure 3.3**).

Calcitriol also significantly reduced pro-inflammatory cytokine production (IFN-γ,

IL-9, and MIP-3α) and increased anti-inflammatory cytokine production (IL-4) in the TL-1 cell line (**Figure 3.5**). Calcitriol and other forms of vitamin D have been shown to significantly reduce IFN-γ in multiple disease models and primary patient cells (168, 206-210). Calcitriol also inhibits IL-9 production through preventing the development of T helper type-9 (Th9) cells, producers of IL-9 (211), or through promotion of a regulatory state of CD4+ T cells (212). The involvement of Th9 cells is unlikely responsible for the reduction of IL-9 in TL-1 cells as TL-1 cells are fully differentiated activated CD8+ T cells, but do support our findings that calcitriol can reduce IL-9 production as well as IFN-γ (212). Like TL-1 cells, calcitriol induced IL-4 secretion while reducing IFN-γ in the EAE mouse model (207). This switch from pro-inflammatory to anti-inflammatory cytokine production correlated with a suppression of symptomology and general inflammation (207). Although we cannot assess the impact of the changes in cytokines on overall T-LGLL symptomology *in vitro*, we plan to explore the correlation between cytokine levels and symptomology in T-LGLL as a future direction (described in **Chapter 6**). Our current hypothesis is that calcitriol re-programs TL-1 cells to be less inflammatory through reduction in p-STAT1, p-STAT3, and IFN-γ, which should lead to a reduction in B cell abnormalities and neutropenia, as these conditions are promoted by IFN-γ production.

In conclusion, calcitriol is an effective inhibitor of p-STAT1, p-STAT3, and IFN-γ in T-LGLL cells and the TL-1 cell. The mechanism for STAT deactivation and IFN-γ reduction should be further pursued in order to increase effectiveness of treatment and to consider combinatorial synergistic treatments with existing T-LGLL treatments. With the abundance of VDR protein in the majority of patients sampled, calcitriol shows promise as a potential T-LGLL therapy. Given our findings, calcitriol should be evaluated further in T-LGLL clinical studies.

3.6. Figures & Tables

Patient	other information	age	sex	TCR	LGL phenotype and purity	ANC	STAT3	Treatment
1	transf/dep until CTX	64	M	αβ	CD2+CD3+CD8+ 67%	900	WT	no; prev. on CTX
2		69	F	αβ	CD3+CD8+ 55%, CD3+CD56+ 45%	980	WT	no
3	asymptomatic	61	M	γ	CD3+CD8+ 55%	7000	WT	no
4		59	F	β	CD8+ 43%	1800, 2100 #	WT	no
5		51	F	γ	CD3+CD8+ 96%,CD3+CD57+ 67%	420	WT	CsA
6	asymptomatic	38	F	αβ	20-30%, bone marrow	1200	Y640F	no
7		46	M	αβ	CD3+CD8+ 56%, CD3+CD16+ 34%, CD3+CD57+ 51%	1000	Y640F	no
8	RA	34	F	*	CD3+ 93%, CD8+ 32%	200	Y640F	Plaquenil, pred.
9	stable disease	64	F	no data	CD3+CD8+ 30%	3538	Y640F	no
10		23	F	αβ	CD3+CD8+ 63%, CD3+CD57+ 29%	1100	Y640F	no

Table 3.1. Clinical Data Summary. Relevant information for patient PBMCs used in Figure 3.1. The patient age at the time of sample collection is noted. Clinical flow cytometry data correspond to laboratory diagnostic tests performed on the date of blood collection or closest date for which this information is available. TCR and flow data are from peripheral blood unless noted otherwise. For one patient (Patient #8), TCR positivity is sporadically noted (*). Relevant immunosuppressant LGL therapy use is indicated; other medications may not be listed. Abbreviations: cyclosporine (CsA), prednisone (pred.), rheumatoid arthritis (RA), transfusion dependent (transf/dep).

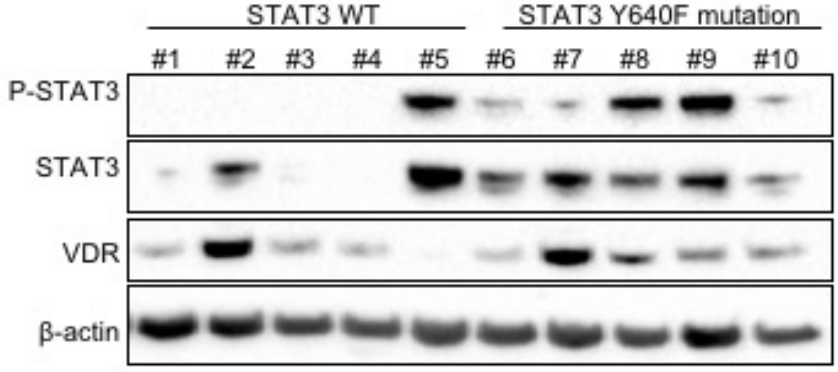

Figure 3.1. VDR and p-STAT1 are detectable in the majority of T-LGLL patients. Protein lysates were prepared from PBMCs from 10 T-LGLL patients, 5 of which had STAT3 Y640F mutational status. Lysates were probed for p-STAT1, STAT1, VDR, and β actin as a loading control.

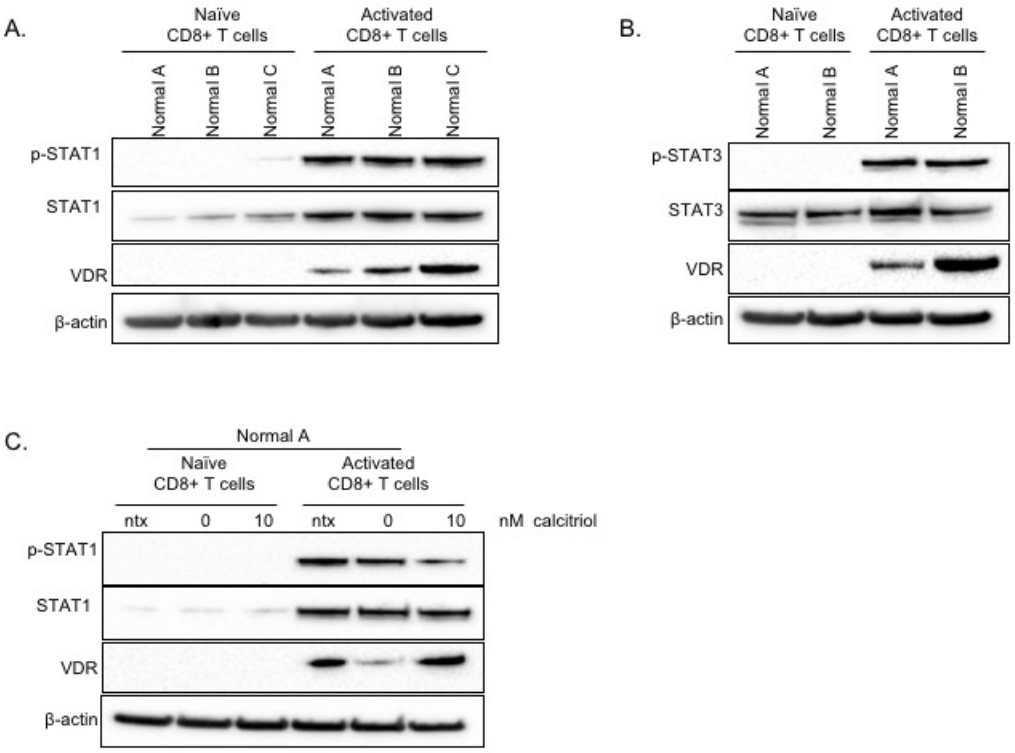

Figure 3.2. Activated CD8+, but not naïve CD8+ T cells, have detectable VDR, p-STAT1, and p-STAT3 and are responsive to calcitriol. Naïve CD8+ T cells were isolated from three healthy donors. Half of these CD8+ T cells were then activated using PHA to create an activated CD8+ T cell group for comparison. Naïve CD8+ T cells were compared to activated CD8+ T cells for levels of (**A**) p-STAT1, STAT1, and VDR as well as (**B**) p-STAT3, STAT3, and VDR. For one patient (**C**), we were able to treat naïve and activated CD8+ T cells with calcitriol, ethanol, or leave untreated for 24 h and measured changes in p-STAT1, STAT1, and VDR. β actin was used as a loading control for all western blot experiments performed. Published in Olson et al. Cancer Biol Ther. (2017)

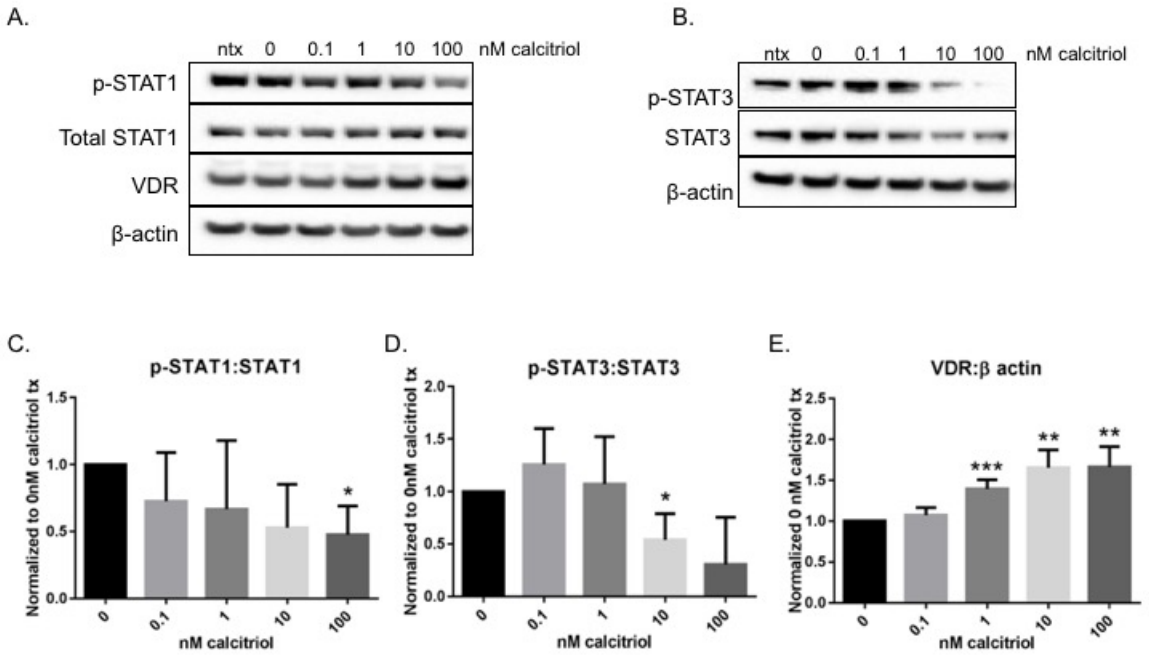

Figure 3.3. Calcitriol reduces IL-2-induced p-STAT1 and p-STAT3 and increases VDR in TL-1 cells. TL-1 cells, supplemented with IL-2, were treated with increasing dosages of calcitriol (0.1 – 100 nM), ethanol (vehicle control), or were untreated for 24 h before protein was harvested. **A, B**. Representative western blots of (**A**) p-STAT1, STAT1, and VDR or (**B**) p-STAT3, STAT3, and VDR (**B**) are shown. β actin was used as a loading control. (**C**) p-STAT1:STAT1, (**D**) p-STAT3:STAT3, and (**E**) VDR:β actin were quantified and normalized to the vehicle control. A student's T test was performed to assess statistical significance. *= $p<0.05$, **= $p<0.01$, ***= $p<0.005$, ****= $p<0.001$. n=3 biological replicates (+/-Stdev) performed by P Kulling, K Olson, and R Rainbow. Published in Olson et al. Cancer Biol Ther. (2017)

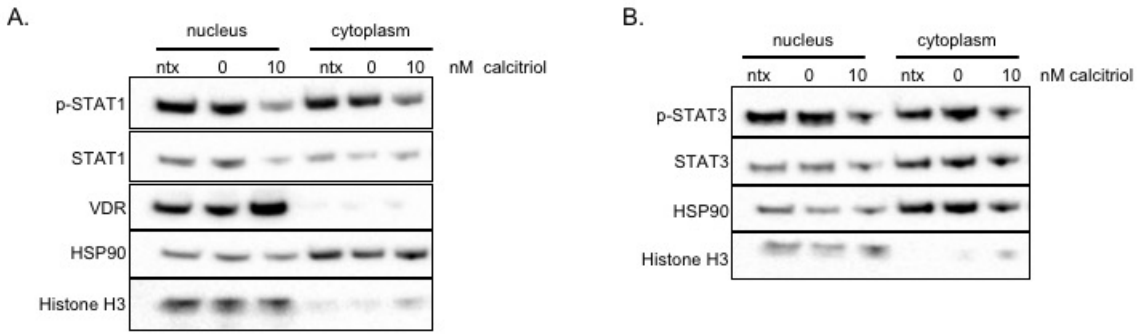

Figure 3.4. p-STAT1 and p-STAT3 are reduced in the nucleus and cytoplasm while VDR is only increased in the nucleus in TL-1 cells following 24 h of calcitriol treatment. TL-1 cells, supplemented with IL-2, were treated with 10 or 100 nM of calcitriol or ethanol, the vehicle control, for 24 h before subcellular fractionation was performed. HSP90 and Histone H3 were used as cytoplasmic and nuclear markers, respectively. Representative western blots are shown for (**A**) p-STAT1, STAT1, and VDR and (**B**) p-STAT3, and STAT3. n=3 biological replicates performed by P Kulling, K Olson, and R Rainbow. Published in Olson KC et al. Cancer Biol Ther. (2017)

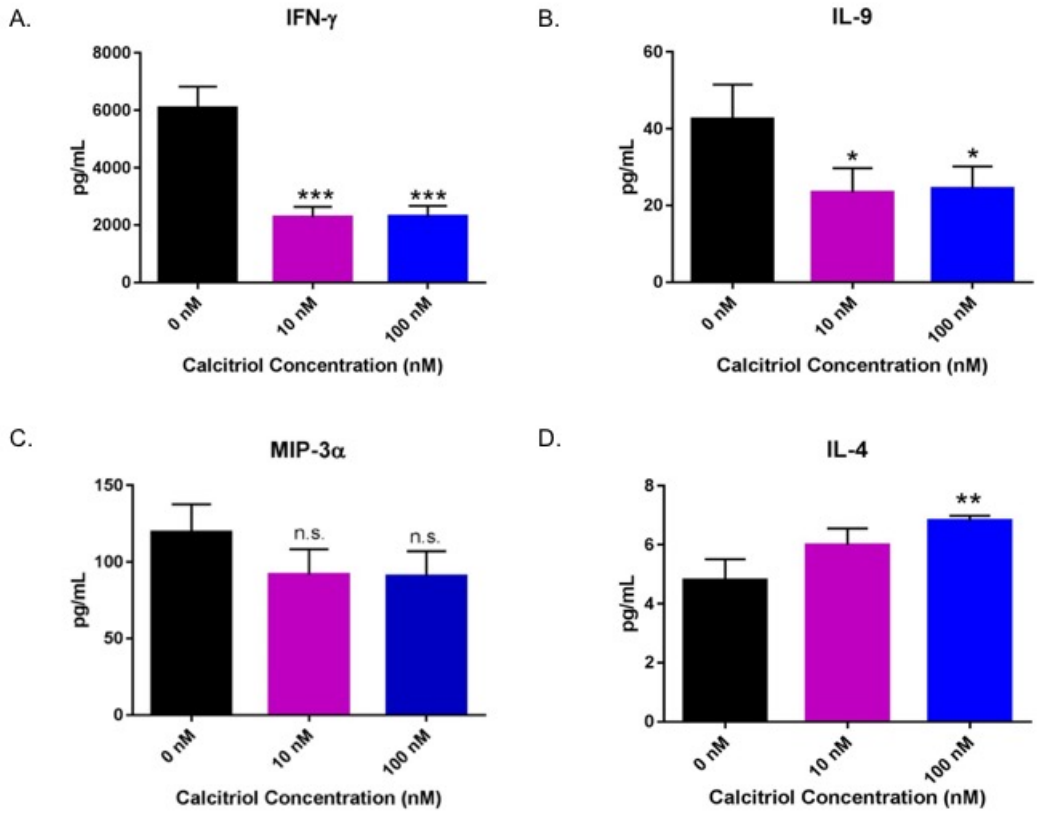

Figure 3.5. Calcitriol reduces pro-inflammatory cytokine secretion and promotes anti-inflammatory cytokine production in TL-1 cells. TL-1 cells, supplemented with IL-2, were treated with 10 or 100 nM of calcitriol or ethanol, the vehicle control, for 24 h before conditioned media was collected and cytokine analysis was performed using the Luminex platform. (**A**) IFN-γ, (**B**) IL-9, (**C**) MIP-3α, and (**D**) IL-4 levels were assessed. A student's T test was performed to assess statistical significance. *= $p<0.05$, **= $p<0.01$, ***= $p<0.005$, ****= $p<0.001$ n=3 biological replicates (+/-Stdev).

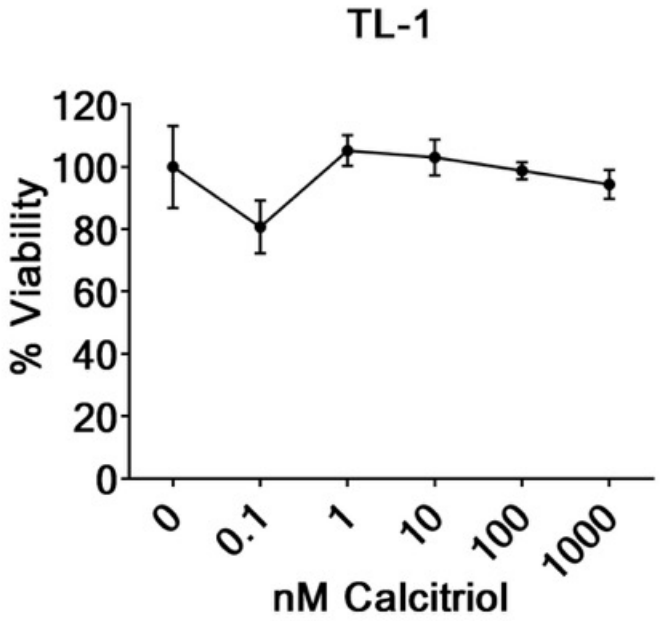

Figure 3.6. Calcitriol does not affect viability of TL-1 cells. TL-1 cells, supplemented with IL-2, were treated with increasing dosages of calcitriol (0.1-100 nM) or ethanol (the vehicle control) for 24 h before viability was assessed using MTS assay. Results were normalized to ethanol treatment. n=3 technical replicates (+/-Stdev). Published in Olson KC et al. Cancer Biol Ther. (2017)

Chapter 4

Calcitriol-mediated reduction in IFN-γ output in T cell large granular lymphocytic leukemia requires vitamin D receptor upregulation

Adapted from: **Kulling PM,** Olson KC, Olson TL, Hamele CE, Carter KN, Feith DJ, and Loughran TP Jr. Calcitriol-mediated reduction in IFN-gamma output in T cell large granular lymphocytic leukemia requires vitamin D receptor upregulation. J Steroid Biochem Mol Biol. 2017. doi: 10.1016/j.jsbmb.2017.07.009. PubMed PMID: 28736298.

4.1. Acknowledgments, Funding, & Abbreviations

Acknowledgements: We thank Alex Wendling and Matt Schmachtenberg for support with blood processing to obtain PBMCs and Holly Davis and Andrea Hines for LGLL Registry support. We also thank Shubha Dighe for technical guidance with siRNA, Dr. Su-Fern Tan for guidance on cycloheximide experiments, and Kabir Ahluwalia for technical support. We thank Mike Solga in the UVA Flow Cytometry Core for performing Luminex Assays. We wish to acknowledge and extend a special thanks to the T-LGLL patient who inspired the original project and to all the patients enrolled in our registry for their continued support.

Funding: This work was funded by the National Cancer Institute of the National Institute of Health under award number R01CA098472, the Bess Family Charitable Fund (to TPL), the LGL Leukemia Foundation (to TPL), the Immunology Training Grants T32AI007496 (to PMK), the UVA Wagner Fellowship (to PMK), and the University of Virginia Cancer Center National Cancer Institute P30-CA044579-23. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Abbreviations: interferon gamma (IFN-γ), interleukin (IL), Janus kinase (JAK), T cell large granular lymphocyte (T-LGL), T cell large granular lymphocytic leukemia (T-LGLL), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), peripheral blood mononuclear cell (PBMC), signal transducer and activator of transcription (STAT), vitamin D receptor (VDR), suppressors of cytokine signaling (SOCS), protein inhibitor of activated STATs (PIAS)

4.2. Highlights

- IFN-γ levels and p-STAT1 are reduced by calcitriol within 4 h in TL-1 cell line.
- IFN-γ is transcriptionally suppressed by calcitriol and correlates with VDR upregulation.
- Reduction in p-STAT1 by calcitriol does not require VDR upregulation.
- Calcitriol inhibits IFN-γ and p-STAT1 through independent mechanisms.
- In the absence of calcitriol, VDR levels correlate with IFN-γ production in TL-1 cells.
- Calcitriol reduces IFN-γ to a similar amount regardless of basal VDR levels.

4.3. Introduction

Our recent findings demonstrated the inhibitory effects of calcitriol, the active form of vitamin D, on the JAK-STAT pathway in primary T-LGLL patient samples and the TL-1 cell line. To summarize, calcitriol treatment significantly decreased IFN-γ output as well as p-STAT1 levels in TL-1 cells (**Chapter 3**). VDR was also markedly increased in TL-1 cells and primary T-LGLL cells compared to naïve CD8+ T cells, with T-LGLL patient PBMCs exhibiting varying degrees of VDR protein levels. However, the requirements for and mechanism behind the observed reduction in p-STAT1 and IFN-γ is unclear. As T-LGLL patients have significantly elevated IFN-γ serum levels, our ultimate goal is to reduce this inflammatory output, in an effort to improve symptomology.

In canonical IFN-γ-STAT1 signaling, IFN-γ binds to the IFN-γ receptor (213-215). The receptor then interacts with JAK1 and JAK2, causing phosphorylation of the receptor by the JAKs (213-215). This allows recruitment and activation of STAT1 through phosphorylation at tyrosine residue 701 (Y701) (214, 216, 217). Thus, IFN-γ activates STAT1 (215, 217, 218), allowing phosphorylated STAT1 monomers to dimerize and move into the nucleus to promote transcription of STAT1 gene targets. Activated STAT1 is also a transcription factor for IFN-γ (219-221), promoting IFN-γ production, until negative regulation occurs through induction of SOCS1. However, this negative regulator, SOCS1, can be decreased in cancers (222), including T-LGLL (40), allowing a continual production of IFN-γ. Thus, there are three main hypotheses for how calcitriol inhibits the IFN-γ-STAT1 pathway: 1) Calcitriol inhibits IFN-γ production, preventing IFN-γ from activating STAT1 2) Calcitriol inhibits STAT1 activation thereby blocking transcription of IFN-γ or 3) Calcitriol inhibits STAT1 and IFN-γ through independent mechanisms.

The vast majority of T-LGLL patients exhibit high levels of VDR protein (**Chapter 3**). This is promising for responsiveness to calcitriol. However, the level of VDR protein varied among patients and one patient did not have any detectable VDR protein. Although we observed upregulation of VDR protein in TL-1 cells and the majority of T-LGLL patient cells, it is unclear whether engagement of VDR is necessary for the effects of calcitriol on STAT1 and IFN-γ. Typically, calcitriol exerts effects through binding to the vitamin D receptor (VDR). This binding induces VDR protein upregulation and a VDR conformational change allowing VDR to modulate transcription of genes (104, 223). Thus, if p-STAT1 and IFN-γ reduction require VDR-mediated transcription of IFN-γ-STAT1 pathway regulators or inhibition of transcription of targets, such as IFN-γ in the case of Jurkat T cells (180), VDR engagement and upregulation is likely necessary. Moreover transcription, translation, protein stabilization, or altered activity of positive or negative regulators may play a role in calcitriol-mediated effects. The main negative regulator of IFN-γ-STAT1 signaling is SOCS1, which binds to the IFNGR and activation loop of JAK2 (89). This blocks further recruitment and activation of the STATs (89). Although SOCS1 is more commonly involved in IFN-γ-STAT1 signaling, SOCS3 can also inhibit JAK2 (224). Moreover, PIAS1 directly inhibits activated STAT1 proteins (199), providing an additional level of regulation. Calcitriol could be acting on any of these pathway components, although this is a relatively unexplored area of study.

We hypothesized that calcitriol decreases p-STAT1 levels, causing decreased IFN-γ transcription. This, in turn, would decrease IFN-γ protein secretion and IFN-γ receptor activation as well as the associated STAT1 phosphorylation, breaking an autocrine loop (219) due to decreased negative regulation. To test this proposed

mechanism in the current study, we utilized genetic and pharmacological manipulation of the vitamin D pathway in the TL-1 cell line. We then confirmed our findings in primary PBMCs isolated from patients with T-LGLL. We found that although IFN-γ and p-STAT1 were decreased in the same temporal manner, the proteins were regulated independently of each other, with IFN-γ reduction correlating with VDR upregulation. Furthermore, basal VDR levels positively correlated with IFN-γ while calcitriol treatment reduced IFN-γ regardless of basal VDR levels. Taken together, vitamin D supplementation or targeting VDR may serve as new therapeutic options for reducing IFN-γ production and STAT1 activation in T-LGLL.

4.4. Results

Calcitriol treatment of T-LGLL cells reduces IFN-γ at the protein and mRNA levels

We previously found that calcitriol significantly reduced IFN-γ levels in conditioned media collected 24 h after calcitriol treatment (74); thus we aimed to better understand the mechanism for IFN-γ reduction. Such inhibition could conceivably occur through changes in transcription, translation, or secretion. A time course was carried out to determine the kinetics of IFN-γ protein reduction. TL-1 cells were treated with calcitriol or vehicle control and protein was harvested over the next 24 h. Immunoblot results were normalized to percent change from vehicle control to better visualize the calcitriol-induced changes. Intracellular IFN-γ protein levels were consistently decreased from 4 h until 24 h (**Figure 4.1A, Figure 4.1B**). The reduction in secreted IFN-γ levels was detected at our earliest measured time point, 6 h, and further decreased until 24 h (**Figure 4.1B**). This suggested that IFN-γ synthesis was inhibited first, prompting us to investigate whether this regulation originates at the transcript level. TL-1 cells were treated with calcitriol or vehicle control for 4 or 24 h, followed by qPCR detection of IFN-γ gene expression. Calcitriol treatment reduced IFN-γ mRNA levels by 52% and 40% at 4 h and 24 h, respectively, (**Figure 4.1C**) thereby demonstrating that calcitriol suppresses IFN-γ transcripts. This finding was recapitulated in PBMCs from T-LGLL patients treated with calcitriol for 24 h (**Figure 4.1C**).

Calcitriol-mediated p-STAT1 reduction correlates with IFN-γ.

We hypothesized that IFN-γ inhibition was due to a decrease in p-STAT1 transcriptional activity as we previously found that calcitriol reduces p-STAT1 levels at 24 h (**Chapter 3**). To address this, we investigated the temporal reduction of p-STAT1 in

TL-1 cells treated with calcitriol and compared this to the IFN-γ reduction. Calcitriol reduced p-STAT1:STAT1 in a similar trend as IFN-γ reduction in TL-1 cells (**Figure 4.2A, Figure 2B)**. Total STAT1 protein levels were relatively unaltered over this timeframe (**Figure 4.2A, Figure 4.2C**) and STAT1 protein half-life was not affected by calcitriol (**Figure 4.2D, Figure 4.2E**). STAT1 transcripts were decreased by less than 20% after calcitriol treatment for 4 or 24 h in the TL-1 cell line but decreased by roughly 50% on average in PBMCs from T-LGLL patients (**Figure 4.2F**), potentially suggesting that STAT1 is more sensitive to reduction by calcitriol in T-LGLL PBMCs. Taken together with the unaltered STAT1 protein half-life, it is likely that decreased STAT1 transcripts result from calcitriol-mediated inhibition of STAT1 activation as STAT1 induces transcription of itself when activated.

Moreover, as TL-1 cells are supplemented with IL-2 for survival, we wanted to determine whether calcitriol acts through an IL-2 dependent mechanism in the cell line. To test this, we treated TL-1 cells with calcitriol or ethanol at the same time as IL-2 or IL-15 supplementation. We selected IL-15 as TL-1 cells require either IL-2 or IL-15 for survival. IL-2 and IL-15, which are hypothesized to play a role in T-LGLL pathogenesis, share a common β and γ receptor chain (46). Both cytokines activate STAT1, prompting us to determine whether calcitriol-reduction of p-STAT1 is specific for IL-2 signaling. We found that calcitriol reduced p-STAT1 in both IL-2 and IL-15 treated cells, with no significant difference in observed reduction between the two cytokine conditions (**Figure 4.3A, Figure 4.3B**). VDR upregulation was also comparable between the IL-2 or IL-15 supplemented cells (**Figure 4.3A, Figure 4.3C**). These data indicate that calcitriol does not act through an IL-2-dependent mechanism as IL-2 and IL-15-driven p-STAT1 content is suppressed upon calcitriol treatment.

Protein levels of positive and negative regulators of STAT1 do not correlate with reduction in p-STAT1 following calcitriol treatment

We next investigated whether the reduction in p-STAT1 was related to changes in negative or positive regulators of STAT1 in TL-1 cells (**Figure 4.4A**). JAK2 protein levels were measured as JAK2 is involved in both IL-2 and IFN-γ-mediated signaling. We observed a significant reduction in IFN-γ with calcitriol treatment when TL-1 cells are supplemented with IL-2 or IL-15; changes observed with STAT1 activation could be a result of perturbations in IFN-γ signaling or IL-2/IL-15 signaling due to calcitriol treatment. There was no noticeable change in JAK2 protein levels following calcitriol treatment (**Figure 4.4A, Figure 4.4B**). In regards to negative regulators, protein levels of SOCS1 (**Figure 4.4C**), SOCS3 (**Figure 4.4D**), or PIAS3 (**Figure 4.4E**) were not correlated with calcitriol-mediated reduction in p-STAT1. Thus, stabilization or degradation of negative or positive regulators, respectively, were not likely to be drivers of calcitriol-mediated effects on STAT1.

VDR protein upregulation follows calcitriol treatment in a similar temporal manner as IFN-γ and p-STAT1 reduction

Our previous work showed 24 h calcitriol treatment upregulates VDR in normal donor activated CD8+ T cells, TL-1 cells, and T-LGLL cells, but not normal donor naïve T cells (74). In this study, we aimed to determine the timing of VDR upregulation and how it relates to p-STAT1 and IFN-γ reduction in TL-1 cells. Following calcitriol treatment, VDR protein levels gradually increased until they reached a maximum at 4 h and maintained this level for 24 h after treatment (**Figure 4.5A, 4.5B**). The initial increase in VDR protein level was not due to extension of VDR half-life (**Figure 4.5C,**

4.5D). VDR transcript levels were relatively unchanged at 4 and 24 h following calcitriol treatment of TL-1 cells and nearly all primary T-LGLL PBMCs followed the same trend at 24 h (**Figure 4.5E**). Taken together with the inability of VDR protein levels to be increased when protein synthesis was blocked (**Figure 4.5C, Figure 4.5D**), VDR upregulation is most likely due to changes in translational regulation. In summary, increased VDR proteins levels showed temporal correlation with the reduction in IFN-γ and p-STAT1, leading us to next determine whether this upregulation is necessary for the decreased IFN-γ and p-STAT1 levels.

Upregulation of VDR is correlated with the reduction in IFN-γ levels but not p-STAT1 content

To determine whether VDR upregulation is necessary to suppress IFN-γ and p-STAT1 levels, we utilized EB1089, a potent calcitriol analog, and 25(OH)D_3, the inactive circulating form of vitamin D. EB1089 strongly engages the VDR and induces a conformational change that promotes transcription of VDR targets (225) while 25(OH)D_3 weakly interacts with the VDR (226). Treatment with 25(OH)D_3 did not upregulate VDR protein (**Figure 4.6A, 4.6B**) but decreased p-STAT1 (**Figure 4.6A, Figure 4.6C**) in a manner comparable to calcitriol. Conversely, EB1089 increased VDR (**Figure 4.6D, 4.6E**) and also decreased p-STAT1 (**Figure 4.6D, 4.6F**), matching our previous observations with calcitriol. Both EB1089 and calcitriol significantly reduced IFN-γ production on the protein ($P<0.0001$) and mRNA transcript level compared to 25(OH)D_3 (**Figure 4.6G, Figure 4.7B**). CYP24A1 mRNA levels, which serve as a readout of VDR transcriptional activity, support the conclusion that 25(OH)D_3 exhibits limited engagement of VDR and that TL-1 cells have very minimal capacity to convert this analogue to the active form at 24 h **(Figure 4.7A)** (227). Therefore, VDR upregulation is

correlated with the decreases in IFN-γ but not p-STAT1, indicating that IFN-γ and p-STAT1 are not regulated by the same mechanism following calcitriol treatment.

VDR levels correlate with IFN-γ production

We previously observed that T-LGLL patient PBMCs have varying amounts of VDR protein (74); therefore, we investigated the relationship between VDR protein levels and IFN-γ production. Using varying dosages of VDR siRNA to reduce VDR mRNA (**Figure 4.8A**) and protein levels (**Figure 4.8B, Figure 4.8C**), we determined that TL-1 cell viability was not decreased upon greater than 80% knockdown of VDR (**Figure 4.9A, 4.9B**). VDR was upregulated in all groups upon calcitriol treatment (**Figure 4.9C, 4.9D**), showing that calcitriol upregulates VDR regardless of baseline VDR levels. VDR protein levels correlated with VDR mRNA levels following VDR knockdown, and VDR mRNA was not stabilized by calcitriol treatment of cells with VDR knockdown (**Figure 4.8**). In the absence of supplemented calcitriol, VDR levels and IFN-γ levels exhibited a clear trend, with IFN-γ production being higher in TL-1 cells with higher basal VDR levels and decreasing IFN-γ as knockdown efficacy increased (**Figure 4.9D, 4.9E**). Calcitriol reduced secreted IFN-γ regardless of initial knockdown status, bringing the amount of IFN-γ to relatively the same level (**Figure 4.9D, 4.9E**). In the absence of calcitriol, this relationship between VDR levels and cytokine production was unique for IFN-γ as basal VDR levels did not correlate with IL-10 levels (**Figure 4.9F**), a cytokine that has been documented to be reduced by calcitriol treatment in other disease models (228).

4.5. Discussion

In this study, we demonstrated that calcitriol treatment of TL-1 cells and T-LGLL patient PBMCs rapidly suppressed IFN-γ mRNA and protein levels with inhibition maintained for at least 24 h (**Figure 4.1, Figure 4.10**). In TL-1 cells, p-STAT1 was inhibited in a similar temporal manner and was not due to reduced STAT1 protein (**Figure 4.2**), alterations in positive or negative regulator protein levels (**Figure 4.4**), or the reduction in IFN-γ (**Figure 4.6**). p-STAT1 was reduced with IL-2 or IL-15 cytokine supplementation, demonstrating a non-IL-2-specific mechanism (**Figure 4.3**). We also found that the kinetics of VDR upregulation were similar to the reduction in IFN-γ and p-STAT1 and were not due to extension of VDR protein half-life or increase in VDR transcript levels (**Figure 4.5**). This is contrary to previous studies that implicated VDR stabilization and protection from proteasome-mediated degradation as the mode of VDR increase following calcitriol treatment (135, 229). Rather, our data showed that in TL-1 cells, VDR upregulation was dependent on protein synthesis, as cycloheximide blocked the upregulation of VDR and calcitriol did not increase VDR transcript levels (**Figure 4.5**).

Treatment of the TL-1 cell line with either EB1089 or 25(OH)D_3 established that VDR upregulation is necessary for calcitriol effects on IFN-γ but not p-STAT1 (**Figure 4.6**). Reduced IFN-γ transcript levels (**Figure 4.1**) were observed in the same timeframe as the upregulation of VDR (**Figure 4.5**). This suggests that VDR transcriptionally regulates IFN-γ as demonstrated in Jurkat T cells where VDR transcriptionally inhibits IFN-γ through binding to the IFN-γ promoter (180). The requirement of VDR upregulation for transcriptional regulation of targets has been established in normal B cells. Weak upregulation of VDR prevented the establishment of the vitamin D-responsive element

(VDRE)-reactive nuclear protein complexes and the transcription of VDR targets (230). In activated T cells, greater upregulation of VDR levels correlated with a larger inhibition of IFN-γ production (133), matching our findings. As p-STAT1 was reduced without a change in total protein levels and regardless of VDR upregulation in TL-1 (**Figure 4.6**), the mechanism behind the p-STAT1 decrease is most likely not transcriptional. Moreover, protein level changes in JAK2, PIAS1, SOCS1 or SOCS3 were not correlated with p-STAT1 reduction (**Figure 4.4**). This suggests that increased protein levels of positive or negative regulators are not responsible for the observed decrease in p-STAT1.

Current research efforts in T-LGLL focus on inhibiting STAT signaling. However, based on our findings (**Figure 4.6**), the use of STAT inhibitors, particularly those specific for STAT1, may not reduce IFN-γ. In addition to the elevated IFN-γ serum levels in patients, IFN-γ plays a critical role in diseases that co-occur with T-LGLL, including aplastic anemia (79) and systemic lupus erythematosus (SLE) (80). Therefore, patients with co-occurring T-LGLL and aplastic anemia or SLE could benefit from therapy that specifically targets IFN-γ production such as calcitriol treatment. As there appears to be a portion of IFN-γ production that is independent of calcitriol regulation (**Figure 4.9**), combinatorial treatments of calcitriol and T-LGLL treatments such as cyclosporine A could be considered. Experimental models have shown that cyclosporine A reduces IFN-γ expression in PBMCs (231-236) and patient samples (237, 238). Additional studies are necessary to determine whether calcitriol and cyclosporine A may be an effective combinatorial treatment for reducing IFN-γ particularly in the T-LGLL patient population with co-occurring diseases driven by IFN-γ.

Many hematological malignancies highly express VDR, including B cell Hodgkin's lymphoma (123) and cutaneous T cell leukemia (120), suggesting a functional role for VDR in cancer cells. To explore the importance of VDR in T-LGLL, we utilized VDR siRNA to modulate VDR levels in order to replicate the varying levels found in patient cells (**Chapter 3**). We found that VDR protein levels in the absence of calcitriol correlated with IFN-γ production, while calcitriol reduced IFN-γ to the same level regardless of initial VDR level (**Figure 4.9**). These experiments present three major findings. First, in the absence of supplemented calcitriol, VDR levels could potentially serve as biomarkers for IFN-γ production. Second, T-LGLL patients may experience a reduction in IFN-γ regardless of initial VDR protein levels. Third, there is an unidentified mechanism for IFN-γ production that cannot be reduced by calcitriol. This could be a result of a transcription factor of IFN-γ that is not inhibited by calcitriol. To better understand IFN-γ transcriptional regulation, we characterize the role of STAT1, STAT3, and STAT5b in IFN-γ transcription in **Chapter 5**.

Serum levels of $25(OH)D_3$ are used to determined vitamin D sufficiency yet this compound exhibits limited biological effects. $25(OH)D_3$ has been shown to be ineffective in reducing IFN-γ secretion in phytohemagglutinin-activated PBMCs (239), matching our finding in TL-1 (**Figure 4.6, Figure 4.7**). Given that $25(OH)D_3$ is unable to reduce IFN-γ in TL-1 cells, it is critical to determine the conversion of $25(OH)D_3$ to calcitriol in T-LGLL patients by healthy immune cells and T-LGLL cells. Normal activated T cells are able to convert $25(OH)D_3$ to calcitriol (135), but it is unknown whether T-LGLL patients possess single nucleotide polymorphisms (SNPs) in essential vitamin D related enzymes and regulators that could affect this process. Thus, it will be important to assess SNP profiles and vitamin D levels in T-LGLL patient serum in order to advance this field. Future

directions relating to vitamin D metabolisms and SNPs are addressed further in **Chapter 6**.

In summary, this study increases our understanding of the mechanism behind calcitriol-mediated decreases in IFN-γ in T-LGLL. Calcitriol suppresses IFN-γ transcription within 4 h, which correlates with VDR upregulation. In the absence of supplemented calcitriol, and thus absence of conformational change and calcitriol-mediated gene transcription, VDR levels correlate with IFN-γ production. Further studies are required to determine the relationship between IFN-γ and disease state. Future clinical studies are necessary to evaluate the ability of calcitriol supplementation to reduce IFN-γ production in T-LGLL patients. Thus, the vitamin D pathway offers multiple opportunities for clinical utility in T-LGLL.

4.6. Figures & Tables

Patient #	Sex	Age	TCR	CD3+CD8+ purity (%)	ANC	STAT3 Mutation Status	Treatment
1	F	68	αβ	30*	2459	Y640F	None
2	M	73	αβ	81	1800	WT	None
3	F	29	77% αβ, 7% γδ	48	460	WT	None
4	M	52	αβ	93	1200	Y640F	None, previously on methotrexate/ cyclophosphamide
5	F	69	αβ	42	2500	WT	None, previously on cyclosporine/ methotrexate/Neupogen

Table 4.1. T-LGLL Patient Clinical Data Summary. Fresh PBMCs were obtained from five T-LGLL patients for use in qPCR studies. Inclusion criteria are described in the materials and methods section. Relevant clinical information is included in this table. *=CD3+CD8+CD7- instead of CD3+CD8+. *Published in Kulling et al, JSBMB (2017).*

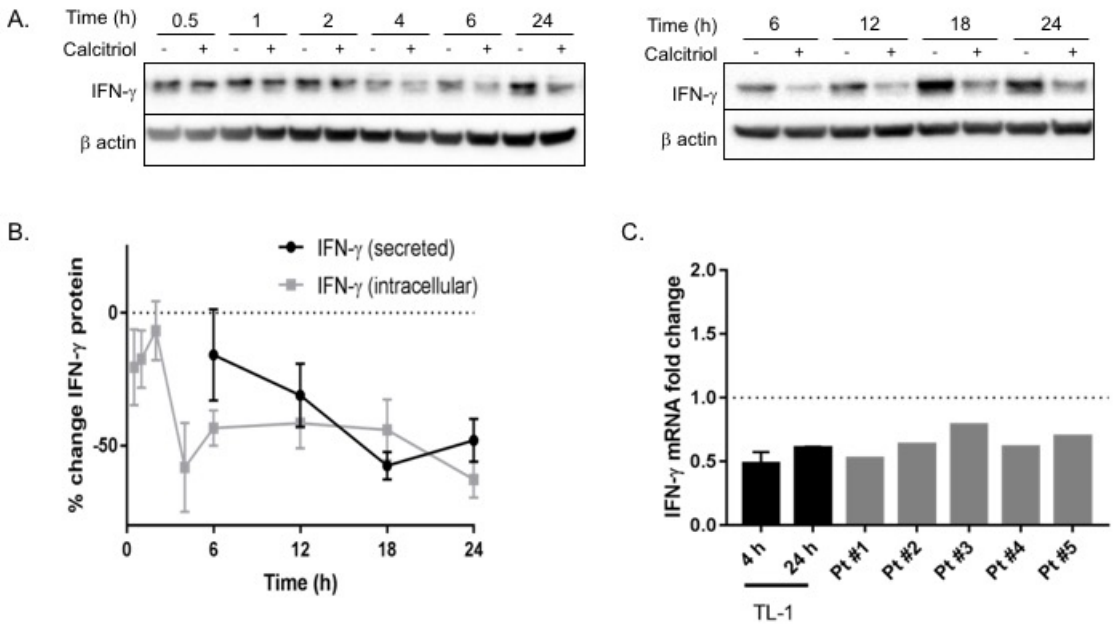

Figure 4.1. IFN-γ is reduced on the mRNA and protein levels following calcitriol treatment. **A**. Western blot analysis was performed on TL-1 lysates harvested at time points following calcitriol or vehicle treatment. Representative western blots are shown, with β actin used as a loading control. **B**. IFN-γ band intensity from part A was normalized to β actin and then expressed relative to the ethanol control to illustrate changes in protein levels as a result of calcitriol treatment (n=3-7, +/- SEM). Supernatant was collected from cells treated as in A and sent for Luminex analysis of secreted IFN-γ (n=3, +/- SEM). **C**. TL-1 cells were treated with calcitriol for 4 or 24 h and IFN-γ transcript levels were assayed by qPCR. Primary T-LGLL patient PBMCs were treated with calcitriol for 24 h. Results were normalized to housekeeping gene UBC and then to the ethanol control (for TL-1, n=3, +/- SEM). *Published in Kulling et al, JSBMB (2017).*

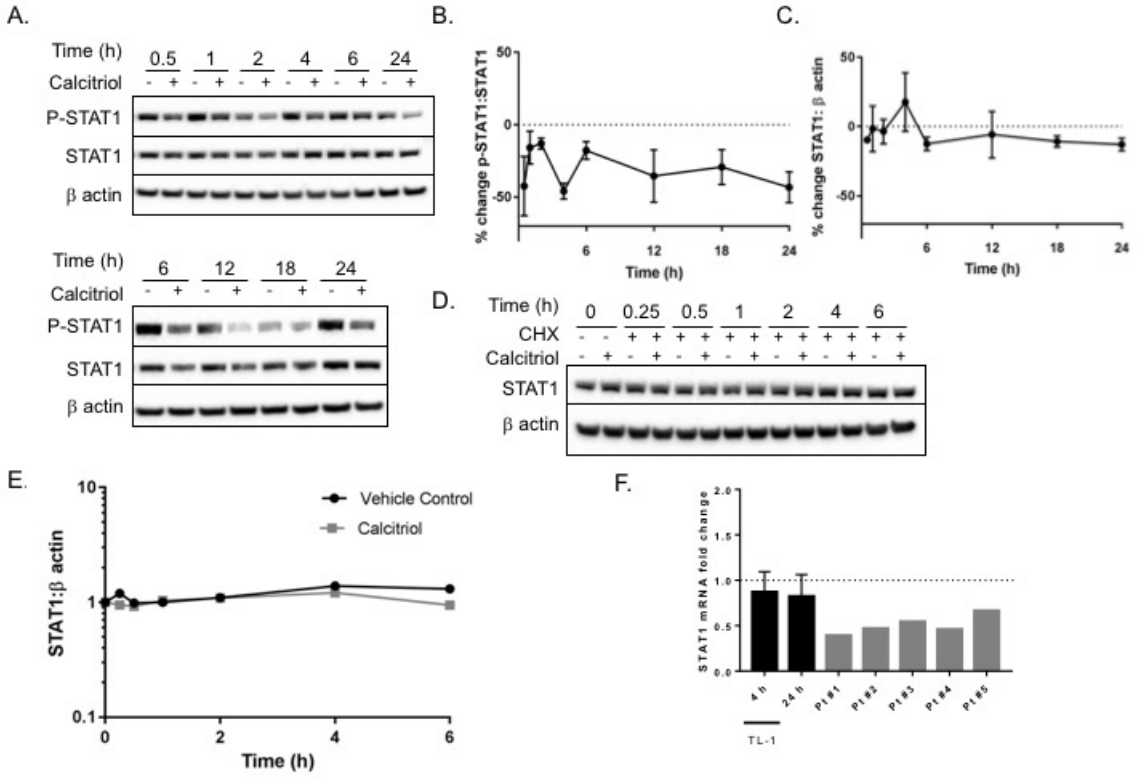

Figure 4.2. p-STAT1 reduction correlates with IFN-γ inhibition and is independent of total STAT1 protein levels. A. Western blot analysis was performed on TL-1 protein lysates harvested at time points following calcitriol or vehicle treatment. Representative western blots are shown, with β actin used as a loading control. **B**. p-STAT1 (Y701) or **C**. STAT1 results were normalized to total STAT1 or β actin, respectively, and then further normalized to the vehicle control to illustrate changes in protein levels as a result of calcitriol treatment (n=3-7, +/- SEM). **D**. TL-1 cells were pretreated with calcitriol or ethanol for 1 h and then treated with cycloheximide (10 μg/mL) to inhibit protein synthesis. Lysates were prepared and western blot analysis was performed at the indicated time points for the designated proteins. **E**. Quantification of D, normalizing STAT1 to β actin. **F**. TL-1 cells were treated with calcitriol for 4 or 24 h and STAT1 transcript levels were assayed by qPCR. Primary T-LGLL patient PBMCs were treated with calcitriol for 24 h. Results were normalized to the housekeeping gene UBC and then

to the ethanol control (for TL-1, n=3, +/- SEM). *Published in Kulling et al, JSBMB (2017)*

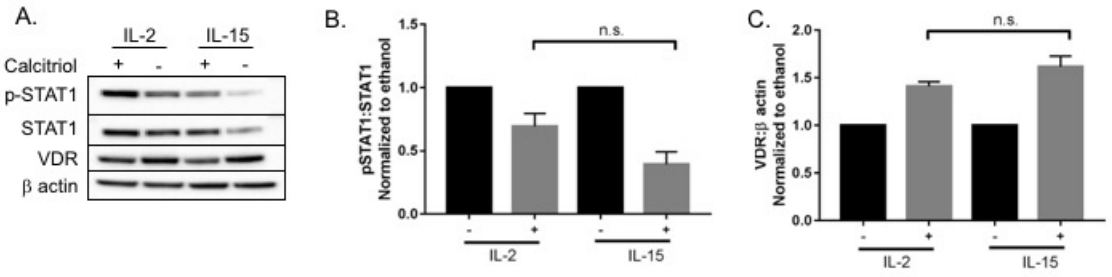

Figure 4.3. Calcitriol reduces IL-2 and IL-15-induced STAT1 phosphorylation. TL-1 cells were deprived from IL-2 for 10 h before IL-2 or IL-15 was added for 24 h. Cells received calcitriol or ethanol at the time of cytokine stimulation. Protein lysates were harvested after 24 h and western blots were run. **A**. Representative western blot for p-STAT1, STAT1, VDR and β actin is shown. Quantifications were performed for (**B**) p-STAT1:STAT1 and (**C**) VDR: β actin. All quantifications were normalized to ethanol. A student's T test were performed to determine differences between the effect of calcitriol on IL-2 compared to IL-15 treated cells. N=3 biological replicates, +/- SEM.

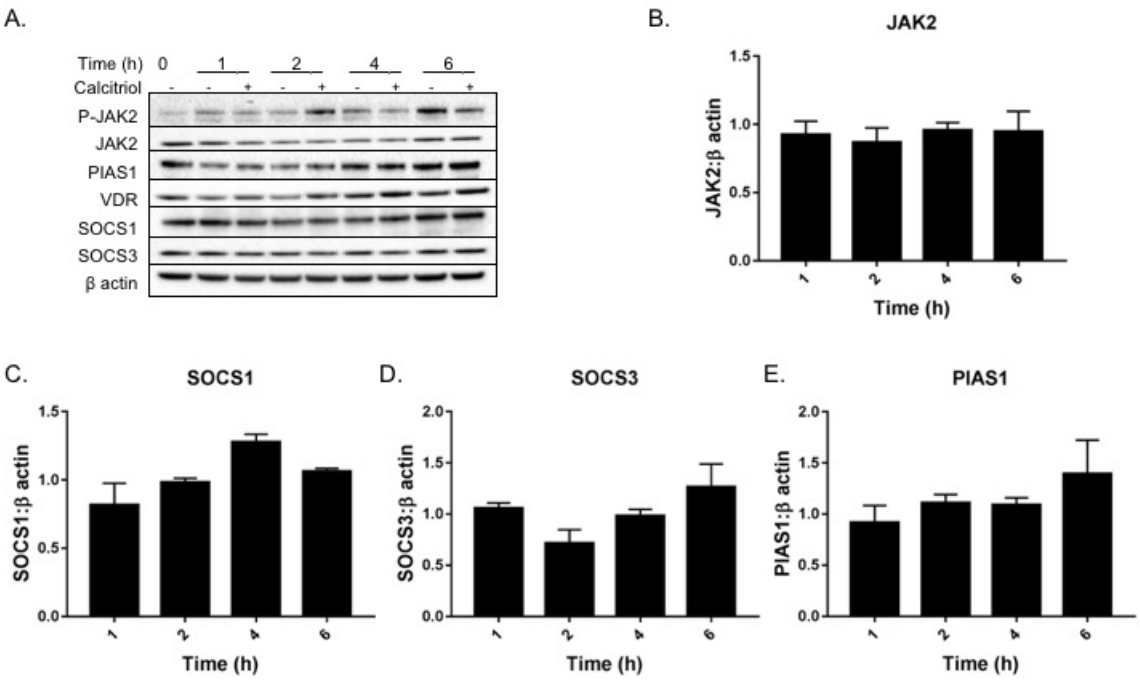

Figure 4.4. Alterations in protein levels of JAK-STAT regulators are not responsible for decreases in p-STAT1 or IFN-γ. Western blot analysis was performed on TL-1 protein lysates harvested at time points following calcitriol or vehicle treatment. **A**. Representative western blots are shown, with β actin used as a loading control. Quantification of (**B**) JAK2, (**C**) SOCS1, (**D**) SOCS3, and (**E**) PIAS1 are shown normalized to β actin and then ethanol treatment. n=3 biological replicates, +/- SEM

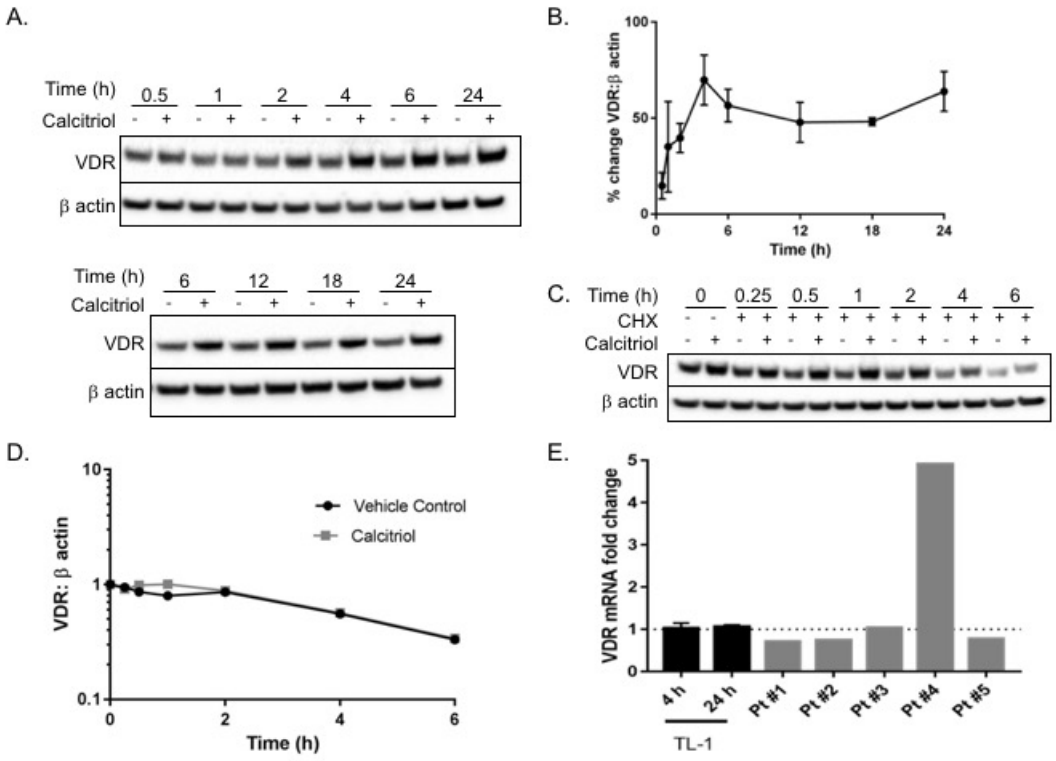

Figure 4.5. VDR protein levels increase following calcitriol treatment. Western blot analysis was performed on TL-1 protein lysates harvested at time points following treatment with calcitriol or vehicle control and probed for the VDR. **A**. Representative western blots are shown. **B**. Quantification of western blots from A normalized to β actin (n=3-7, +/- SEM). **C**. TL-1 cells were pretreated with calcitriol or ethanol for 1 h and then treated with cycloheximide (10 μg/mL) to inhibit protein synthesis. Lysates were prepared at the indicated time points and western blot analysis was performed. **D**. Quantification of C, normalizing VDR to β actin. **E**. TL-1 cells were treated with calcitriol for 4 or 24 h and VDR transcript levels were assayed by qPCR. Primary T-LGLL patient PBMCs were treated with calcitriol for 24 h. Results were normalized to the housekeeping gene UBC and then to the ethanol control (for TL-1, n=3, +/- SEM).

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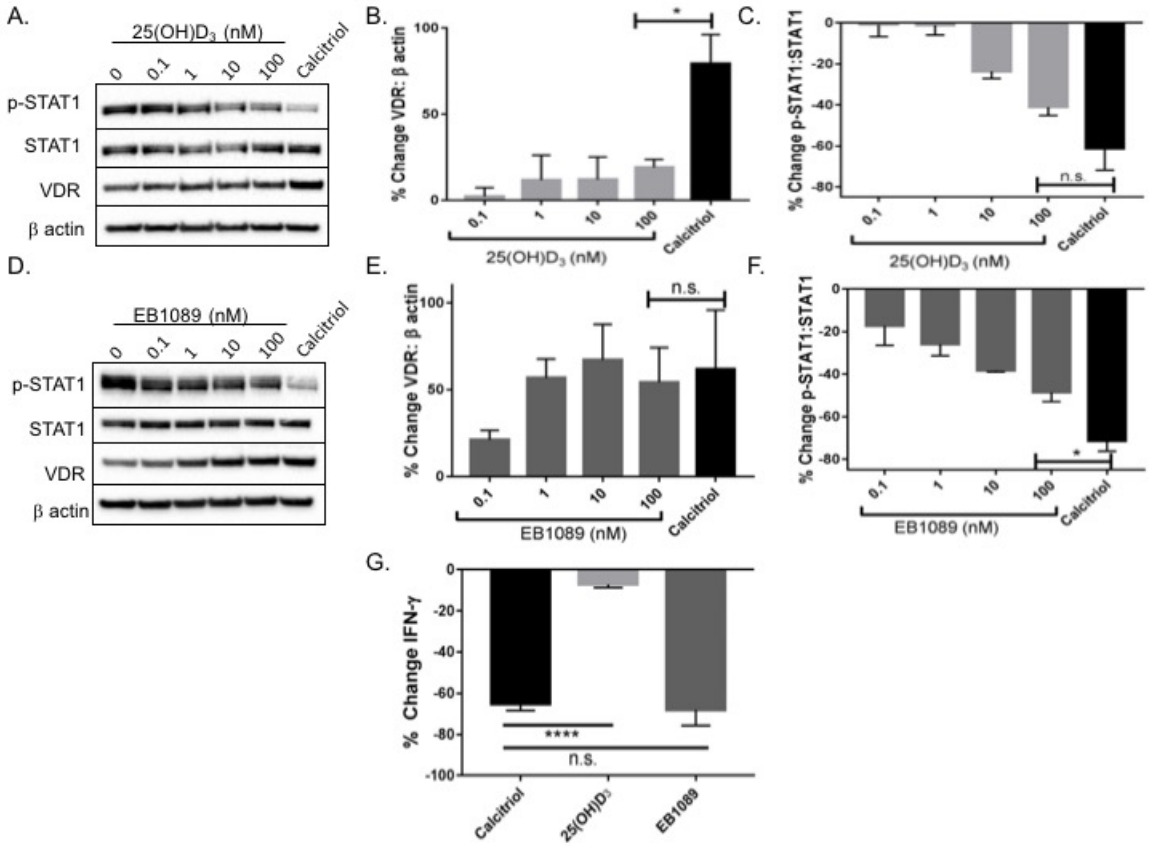

Figure 4.6. **VDR upregulation is necessary for reduction in IFN-γ but not p-STAT1 after treatment with vitamin D or analogs.** TL-1 cells were treated with increasing dosages of the inactive form of vitamin D, 25(OH)D_3 (**A-C**), or the high affinity calcitriol analog EB1089 (**D-F**), as well as vehicle control or calcitriol for comparison. **A, D.** Protein lysates were prepared and western blot analysis was performed (n=3, +/- SEM). Representative western blots are shown. Protein levels were normalized to (**B, E**) β actin for VDR or (**C, F**) STAT1 for p-STAT1, and then further normalized to the vehicle control to illustrate changes as a result of calcitriol treatment. **G**. Conditioned media was collected from the 100 nM calcitriol, 25(OH)D_3, and EB1089 samples after 24 h and submitted for Luminex cytokine analysis (n.s.=not significant, *p<0.05, ****p<0.0001).

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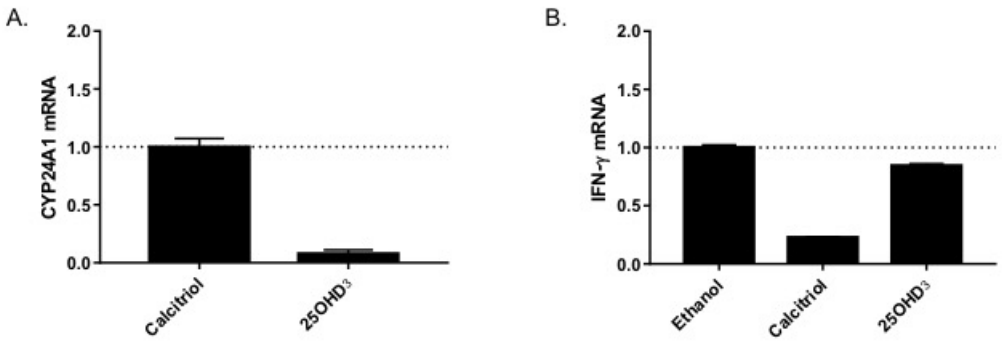


Figure 4.7. 25(OH)D_3 does not alter CYP24A1 or IFN-γ gene expression to the same levels as calcitriol. TL-1 cells were treated with 100 nM of 25(OH)D_3, calcitriol, or ethanol vehicle for 24 h and (**A**) CYP24A1 and (**B**) IFN-γ transcript levels were assessed using qPCR. Results were normalized to the housekeeping gene UBC. CYP24A1 transcript levels were undetectable for ethanol treated cells and thus the transcript levels for 25(OH)D_3 were normalized relative to calcitriol. For IFN-γ, transcript levels were normalized to ethanol treated cells. (n=3 technical replicates, +/- SEM). *Published in Kulling et al, JSBMB (2017)*

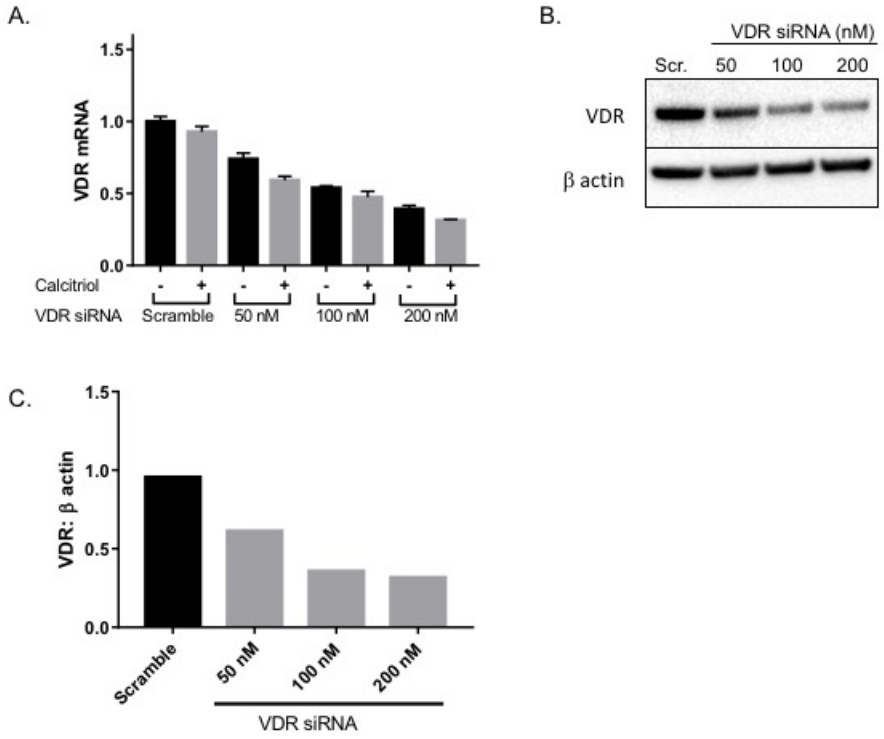


Figure 4.8. VDR mRNA levels correlate with VDR protein levels in VDR knockdown cells. TL-1 cells were treated with VDR or scrambled siRNA for 48 h. Cells were then plated for 24 h with calcitriol or ethanol vehicle, followed by protein harvest or mRNA extraction. **A.** VDR transcript levels normalized to UBC and ethanol treatment **B**. Western blot of VDR knockdown status 24 h following calcitriol or ethanol treatment. **C**. Quantification of VDR protein levels in B, normalized to β actin. (n=3 technical replicates, +/- SEM). *Published in Kulling et al, JSBMB (2017)*

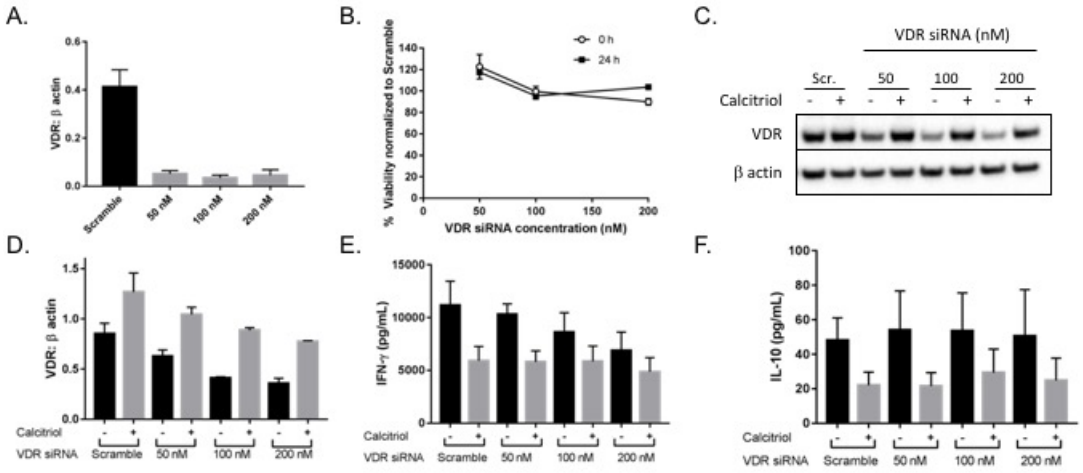


Figure 4.9. VDR levels correlate with IFN-γ production. TL-1 cells were treated with VDR or scrambled siRNA for 48 h. Cells were then harvested for western blot analysis of knockdown efficiency (**A**) and plated for 24 h viability assay to assess survival with knockdown (**B**). An additional aliquot was re-plated with calcitriol or ethanol for 24 h, followed by protein harvest. **C**. Representative western blot of VDR knockdown status 24 h following calcitriol or ethanol treatment. **D**. Quantification of VDR protein levels in C, normalized to β actin (n=3, +/-SEM). **E-F**. Conditioned media was subjected to cytokine analysis to quantify (**E**) IFN-γ or (**F**) IL-10 production following calcitriol treatment and VDR knockdown (n=3, +/-SEM). *Published in Kulling et al, JSBMB (2017)*

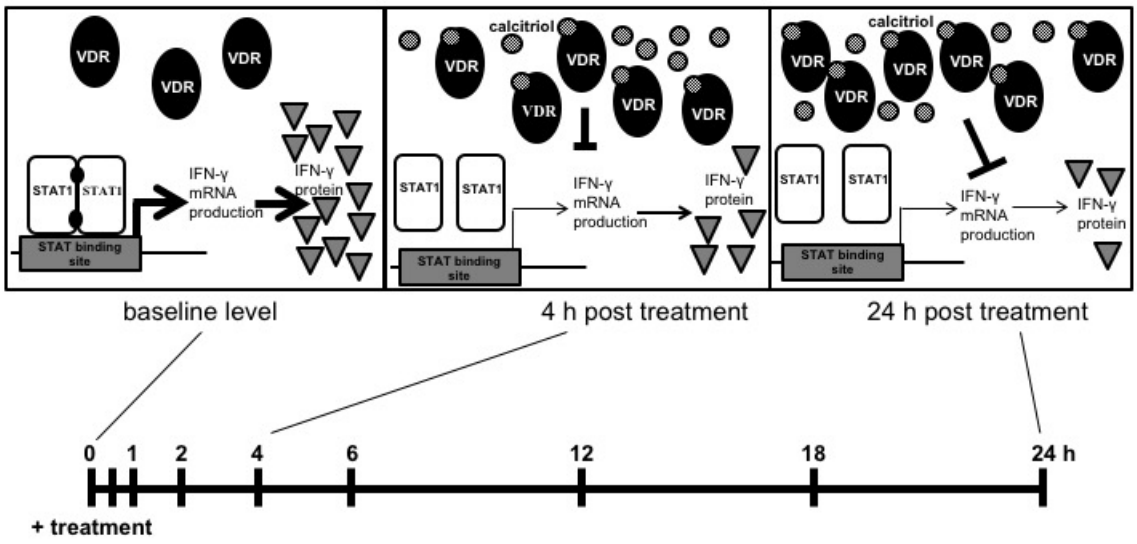


Figure 4.10. Model of calcitriol-mediated reduction in p-STAT1 and IFN-γ in T-LGLL. In TL-1 cells supplemented with IL-2, there is a detectable level of VDR and high baseline STAT1 Y701 phosphorylation and IFN-γ production. Within 4 h of calcitriol treatment of TL-1, p-STAT1 and IFN-γ are reduced while VDR is increased. This effect is sustained at 24 h and the final outcome is decreased IFN-γ production that correlates with increased VDR protein. p-STAT1 levels are not dependent on the VDR increase or IFN-γ reduction, showing that p-STAT1 and IFN-γ decreases are mediated by different mechanisms. *Published in Kulling et al, JSBMB (2017)*

Chapter 5

Dysregulation of the IFN-γ-STAT1 signaling pathway in a cell line model of large granular lymphocyte leukemia

Adapted from: **Kulling PM**, Olson KC, Hamele CE, Toro MF, Tan S-F, Feith DJ, et al. (2018) Dysregulation of the IFN-γ-STAT1 signaling pathway in a cell line model of large granular lymphocyte leukemia. PLoS ONE 13(2): e0193429. https://doi.org/10.1371/journal.pone.0193429

5.1. Acknowledgments, Funding, and Abbreviations

Acknowledgments: We thank Alex Wendling and Joanne Lannigan in the UVA Flow Cytometry Core facility for guidance and assistance with flow cytometry experiments. We also thank Mike Solga in the UVA Flow Cytometry Core for performing Luminex Assays and Mark Conaway for assistance with statistical analysis.

Funding: This work was funded by the National Cancer Institute of the National Institute of Health under award numbers R01CA098472 and R01CA178393, the Bess Family Charitable Fund (to TPL), a generous anonymous donor (to TPL), the LGL Leukemia Foundation (to TPL), Immunology Training Grant T32AI007496 (to PMK), UVA Wagner Fellowship (to PMK), and the University of Virginia Cancer Center National Cancer Institute P30-CA044579. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Abbreviations: interferon gamma (IFN-γ), interferon beta (IFN-β), interleukin (IL), Janus kinase (JAK), T cell large granular lymphocyte (T-LGL), T cell large granular lymphocyte leukemia (T-LGLL), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), peripheral blood mononuclear cell (PBMC), signal transducer and activator of transcription (STAT), interferon gamma receptor (IFNGR), interferon regulatory factor 1 (IRF-1), suppressors of cytokine signaling (SOCS), ubiquitin C (UBC), interferon alpha receptor (IFNAR)

5.2. Highlights

- TL-1 cells have a significantly lower mRNA and protein expression of IFNGR1 and IFNGR2 compared to Jurkat T cells.
- TL-1 cells are unresponsive to IFN-γ as demonstrated by the lack of induction of p-JAK2, p-STAT1, and IFN-γ-inducible gene targets.
- The defect in cytokine signaling is unique to IFN-γ as TL-1 cells robustly respond to IFN-β.
- STAT5b promotes transcription of IFN-γ in TL-1 cells and Jurkat T cells.
- p-STAT1 positively correlates with p-STAT3 while STAT5b suppresses STAT1 and STAT3 activation.

5.3. Introduction

The Janus Kinase (JAK)-Signal Transducers and Activators of Transcription (STAT) pathway is commonly dysregulated in cancers, leading to an upregulation of pro-survival pathways and inflammatory cytokine secretion, including IFN-γ. IFN-γ, a type II interferon (75), is associated with worse symptomology and disease progression in multiple diseases when produced in excess (77, 82). IFN-γ directly binds to the IFN-γ receptor (IFNGR), leading to phosphorylation of JAK1, JAK2, and the IFNGR (75, 76, 87, 88). This promotes recruitment and docking of STAT1, allowing activation of STAT1 through phosphorylation of tyrosine residue 701 (p-STAT1) (89). p-STAT1 then forms a homodimer and moves into the nucleus to promote transcription of genes with gamma interferon activation site (GAS) elements including IRF-1 and suppressors of cytokine signaling 1 (SOCS1) (76, 89). SOCS1, a negative regulator of IFN-γ signaling, binds to the IFNGR and JAK2 to prevent further activation of the pathway (89). SOCS1 also participates in cross talk with other pathways, including IL-2-mediated signaling, transcription of IFN-γ (240). Thus, in healthy cells, IFN-γ signaling is tightly controlled through transcription of negative regulators.

The IFNGR is composed of two subunits, IFNGR1 and IFNGR2, with both subunits required for IFN-γ signaling. IFNGR1 directly interacts with the IFN-γ ligand while IFNGR2 is necessary for signal transduction (75). Although most cell types moderately and constitutively express IFNGR1, IFNGR2 is responsive to external stimuli and is critically important for downstream IFN-γ-mediated signaling (76, 95). Higher IFNGR2 expression promotes faster STAT1 phosphorylation and subsequent IRF-1 transcription (95). Interestingly, IFNGR1 and IFNGR2 levels can be reduced, with profound impacts on downstream signaling. Although the IFNGR is constitutively

expressed on most cell types (75, 76), healthy IFN-γ producing cells, such as T helper cells 1 (Th1 cells) or T cell receptor (TCR) engaged T cells, exhibit decreased receptor expression during critical times to prevent apoptosis or induction of negative regulators (90, 94, 95, 241, 242). In contrast to this well-controlled process, IFNGR downregulation can occur in diseased cells (91, 92, 96, 243, 244), rendering the cells less responsive or completely unresponsive to IFN-γ signaling. This is particularly problematic as decreased responsiveness prevents IFN-γ-induced apoptosis of malignant cells. In fact, elegant murine models demonstrate that IFNGR1-/- mice exhibit more rapid ovarian tumor progression (245) and the reduction in IFNGR has been proposed as a mechanism for evading tumor surveillance (96).

In T cell large granular lymphocyte leukemia (T-LGLL), there is substantial evidence for dysregulation of IFN-γ-mediated signaling. T-LGLL patient serum has significantly elevated IFN-γ compared to healthy controls (40) and T-LGLL patient peripheral blood mononuclear cells (PBMCs) exhibit constitutively activated STAT1 (56), suggesting hyperactivation of the IFN-γ signaling pathway. However, SOCS1 (40) and SOCS3 (57) gene transcripts are significantly reduced in T-LGLL PBMCs, demonstrating a lack of negative regulation. Based on our recent finding, TL-1 cells appear to exhibit a disconnect between STAT1 signaling and IFN-γ signaling as STAT1 is pharmacologically reduced without impacting IFN-γ levels. As STAT1 is a known inducer of IFN-γ transcription, the lack of IFN-γ reduction with STAT1 inhibition is rather surprising. Moreover, calcitriol inhibited STAT1 and IFN-γ independently, and by inhibiting IFN-γ, STAT1 was not further reduced compared to STAT1 inhibition alone with $25(OH)D_3$, suggesting that IFN-γ does not activate STAT1 in T-LGLL. Taken together, we

hypothesized that the IFN-γ-STAT1 pathway is dysregulated in T-LGLL.

Here we further characterize the IFN-γ-STAT1 signaling pathway from IFN-γ-induced activation events through the regulation of IFN-γ production in TL-1 cells, a cell line model of T-LGLL. TL-1 cells, like normal T cells, require IL-2 for proliferation. As IL-2 also drives IFN-γ production (246, 247), we investigated the regulation of IFN-γ production in the context of IL-2 supplementation. Using genetic and pharmacological manipulation of the pathway, we found that TL-1 cells exhibit reduced IFNGR levels compared to Jurkat T cells, an IFN-γ responsive cell line, and were unresponsive to IFN-γ. Moreover, in IL-2-induced signaling, we correlated STAT1 activation with STAT3 protein levels and identified STAT5b as a positive regulator of IFN-γ production and inhibitor of STAT1 in TL-1 cells. Taken together, we propose a novel mechanism that prevents induction of IFN-γ-signaling negative regulators in TL-1 cells. Our findings may have therapeutic implications for T-LGLL based on characterization of the inter-relationship between STAT activation.

5.4. Results

TL-1 cells exhibit decreased IFNGR compared to Jurkat T cells

Because both IFNGR1 and IFNGR2 are necessary for IFN-γ signaling, we chose to quantify IFNGR1 (**Figure 5.1A**) and IFNGR2 (**Figure 5.1B**) cell surface expression in TL-1 cells using flow cytometry. We utilized Jurkat T cells as a positive control cell line known to express IFNGR1 and IFNGR2. Based on median fluorescent intensity, TL-1 cells exhibited 36% ($p<0.0001$) and 46% ($p<0.0001$) less IFNGR1 and IFNGR2, respectively, compared to Jurkat T cells (**Figure 5.1C**). Due to the very low median fluorescent intensity of IFNGR2 in TL-1, we next measured IFNGR1 and IFNGR2 transcript levels to account for potential background. IFNGR1 and IFNGR2 transcripts were 57% ($p=0.0005$) and 92% ($p<0.0001$) lower in TL-1 cells compared to Jurkat T cells (**Figure 5.1D**), further demonstrating the reduction in IFNGR. Thus, we hypothesized that TL-1 cells would be less responsive or completely unresponsive to IFN-γ treatment compared to Jurkat T cells as a result of lower levels of the IFNGR1 and IFNGR2.

JAK2 is not phosphorylated in response to IFN-γ treatment in TL-1 cells

To assess whether the lower levels of IFNGR affect downstream kinase activation, we measured JAK2 phosphorylation following IFN-γ treatment in TL-1 cells compared to Jurkat T cells, our positive control. As expected, IFN-γ activated JAK2 within 0.5 h with effects lasting at least 2 h in Jurkat T cells (**Figure 5.2A**). However, in TL-1 cells p-JAK2 was not induced with IFN-γ (**Figure 5.2B**), demonstrating an IFN-γ unresponsive state in TL-1 cells. IL-2 was selected as a positive control cytokine for induction of JAK2, STAT1, STAT3, and STAT5 in TL-1 cells (**Figure 5.2B, 5.3B, and 5.4B**). We have previously demonstrated that the TL-1 cell line is responsive to IL-2 (74,

248). In the present manuscript, we demonstrate that the cytokine unresponsiveness appeared to be unique for IFN-γ and not an inherent defect in JAK2 activation as p-JAK2 was induced in TL-1 cells in response to IL-2 (**Figure 5.2B).** Since JAK2 is required for IFN-γ-induced STAT1 activation (76, 87), we determined whether the JAK2-STAT1 interaction is intact in TL-1 cells. We utilized ruxolitinib, a JAK1 and JAK2 specific inhibitor, and measured IL-2-induced STAT1 phosphorylation. JAK1/JAK2 activity is required for activation of STAT1 as p-STAT1 was completely ablated with ruxolitinib (**Figure 5.2C**). p-STAT3 and p-STAT5 were also reduced (**Figure 5.2C**). Therefore, JAK2 is phosphorylated and activates downstream STATs in response to IL-2 treatment, but not IFN-γ, in TL-1 cells.

IFN-γ does not activate STAT1 or induce transcription of IFN-γ-inducible gene targets in TL-1 cells

As p-JAK2 was not induced by IFN-γ in TL-1 cells, we hypothesized that STAT1 would not be activated as a result of IFN-γ treatment. Following IFN-γ treatment, Jurkat T cells exhibited robust STAT1 activation at 0.5 h, with activation lasting at least 2 h (**Figure 5.3A**). On the contrary, p-STAT1 was undetectable in TL-1 cells at any time point following IFN-γ treatment but was detectable following IL-2 treatment, again demonstrating an IFN-γ specific defect (**Figure 5.3B**). Given the inability to detect p-STAT1 in IFN-γ treated TL-1 cells, we next assessed whether STAT1-mediated-IFN-γ-inducible gene targets would be upregulated in response to IFN-γ treatment. TL-1 and Jurkat T cells were treated with IFN-γ for 6 h and fold change in transcript levels of target genes was measured and compared. IRF1, SOCS1, and STAT1 mRNA levels were increased by 2.7- ($p<0.005$), 3.5- ($p<0.01$), and 9.2-fold ($p<0.01$), respectively, in Jurkat

T cells treated with IFN-γ compared to the vehicle control (**Figure 5.3C**). In contrast, IFN-γ treatment of TL-1 cells did not induce transcription of IRF1, SOCS1, or STAT1 (**Figure 5.3C**). Moreover, neither STAT3 nor STAT5 activation was detected following IFN-γ treatment, while IL-2 robustly induced STAT3 and STAT5 phosphorylation (**Figure 5.4**), further demonstrating a specific lack of IFN-γ signaling. These findings further demonstrate the IFN-γ unresponsive state of TL-1 cells.

TL-1 cells are responsive to IFN-β, suggesting a specific IFN-γ-mediated signaling defect independent of type I interferon signaling

There is mounting support for crosstalk between type I and type II interferon pathways. There are several different type I interferons including IFN-α, IFN-β, IFN-ω, and IFN-κ, while only IFN-γ comprises the type II interferons (75). Unlike IFN-γ, which signals through its unique receptor, all type I interferons signal through the IFN-α receptor 1 (IFNAR1) and IFN-α receptor 2 (IFNAR2) (75). Although signaling by type I and type I interferons occurs through different receptors, lack of IFNAR can lead to a diminished IFN-γ signaling pathway, implicating IFNAR in regulation of IFN-γ responsiveness (249). To address whether the lack of STAT1 induction in TL-1 cells is specific to IFN-γ or a result of diminished IFNAR-mediated signaling, we assessed the ability of TL-1 cells to respond to IFN-β. TL-1 cells were treated with 5000 U/mL of IFN-β for up to 2 h before induction of p-STAT1 was measured, with Jurkat T cells serving as our positive control for IFN-β responsiveness (**Figure 5.5A**). IFN-β robustly induced p-STAT1 in TL-1 cells within 0.5 h (**Figure 5.5B**). Treatment of Jurkat T cells and TL-1 cells with IFN-β led to a significant increase in STAT1 gene transcription ($p<0.05$) but not IFN-γ (**Figure 5.5C**). Therefore, IFNAR signaling is intact and functional in TL-1 cells

despite the lack of IFN-γ responsiveness.

STAT5b promotes transcription of IFN-γ

The significantly elevated IFN-γ production by T-LGLL cells coupled with the decreased levels of SOCS1 suggests that there is a lack of negative regulation in T-LGLL (40). Our results demonstrate that IFN-γ treatment does not induce the IFN-γ negative regulator, SOCS1 (**Figure 5.3C**). Thus, it is key that we identify IFN-γ transcription factors that may be targeted to reduce the production of IFN-γ and prevent further damage to healthy cells. Since STAT1, STAT3, and STAT5 have been shown to promote transcription of IFN-γ (182, 183, 250-252) and play a role in T-LGLL pathogenesis (56, 162), we chose to focus on these transcription factors in the context of IL-2 supplementation. STAT1 (**Figure 5.6A**), STAT3 (**Figure 5.6B**), or STAT5b (**Figure 5.6C**) siRNA was transfected into TL-1 cells to knockdown their respective protein levels. STAT1 and STAT3 protein knockdown did not affect IFN-γ protein (**Figure 5.6D**) or transcript levels (**Figure 5.6E**). However, STAT5b knockdown resulted in a 19% decrease in secreted IFN-γ protein ($p=0.055$) (**Figure 5.6D**) and 28% decrease in IFN-γ transcript ($p<0.005$) (**Figure 5.6E**) 48 h after STAT5b siRNA treatment, implicating STAT5b in the regulation of IFN-γ. STAT5b knockdown also reduced IFN-γ mRNA content by 34% in Jurkat T cells (**Figure 5.7**), demonstrating that this relationship is conserved across cell lines.

Because IFN-γ and IL-10 classically work in opposition (253), we next investigated the effects of the STAT knockdowns on IL-10 production in TL-1 cells. As expected, IFN-γ and IL-10 production were inversely related with a nearly 2-fold increase

in IL-10 in STAT5b knockdown cells ($p<0.05$) (**Figure 5.6F**) while IFN-γ was decreased (**Figure 5.6E**). STAT3 knockdown cells exhibited a 39% decrease in IL-10 ($p<0.005$) (**Figure 5.6F**), matching the established role of STAT3 in regulating IL-10 production (254), while STAT1 knockdown cells showed no significant change in IL-10 production. These changes in cytokine production were not associated with any dramatic differences in cell viability (**Figure 5.6G**). As STAT5b alters cytokine production, we investigated whether STAT5b knockdown changed IFNGR1 or IFNGR2 mRNA levels. IFNGR1 was significantly ($p<0.05$) reduced while IFNGR2 was not altered in TL-1 cells following STAT5b knockdown (**Figure 5.8**). Taken together, STAT5b is required for optimal production of IFN-γ and IFNGR1 mRNA in TL-1 cells.

Relationship between STATs 1, 3, and 5b and their activation states

Given the role of STAT1, STAT3, and STAT5 in T-LGLL pathogenesis, we investigated the relationship between STAT1, STAT3, and STAT5 activation. This would allow us to better understand how inhibiting the STATs individually, in an effort to target cytokine production and induce apoptosis, would impact STAT signaling in T-LGLL. We next determined whether selected STAT knockdowns produced compensatory changes in other STATs. Knockdown of STAT1 did not impact phosphorylated or total protein levels of STAT3 or STAT5 (**Figure 5.9A and 5.9B).** STAT3 knockdown led to a significant reduction in p-STAT1 ($p<0.005$), with phosphorylated and total protein level of STAT5 unchanged (**Figure 5.9C and 5.9D**). However, when STAT5b was knocked down, there was a significant increase in total STAT1 ($p<0.05$) as well as a notable increase in p-STAT1 and p-STAT3 (**Figure 5.9E and 5.9F**), demonstrating a suppressive effect of STAT5b. Taken together, our findings suggest STAT5b inhibits STAT1 while STAT3 promotes STAT1 activation.

5.5. Discussion

Our results show that TL-1 cells are nonresponsive to IFN-γ-mediated signaling. Here we demonstrate that the TL-1 cell line model of T-LGLL exhibits minimal protein and mRNA levels of both IFNGR1 and IFNGR2 (**Figure 5.1**). Moreover, downstream IFN-γ-mediated signaling is deficient, with a lack of induction of p-JAK2 (**Figure 5.2**), p-STAT1 (**Figure 5.3**), or IFN-γ-inducible gene targets including SOCS1, IRF1, and STAT1 (**Figure 5.3**). The low expression of the IFNGR may provide a mechanistic basis for the known lack of IFN-γ-induced negative regulation yet high levels of IFN-γ production in T-LGLL (40). The impairment of SOCS1 induction would prevent the negative feedback regulation of IL-2-mediated IFN-γ production, which is observed as elevated IFN-γ levels in primary T-LGLL patient samples and the TL-1 cell line.

For our experiments, we utilized Jurkat T cells as an IFN-γ responsive cell line. However, Jurkat T cells are considered slow responders to IFN-γ due to established lower IFNGR2 levels compared to other IFN-γ responsive cell lines (95, 241). Thus, our findings of greater observed reduction in IFNGR1 and IFNGR2 in TL-1 cells, relative to Jurkat T cells, are even more remarkable. Such low receptor expression renders TL-1 cells unresponsive to IFN-γ treatment (**Figures 5.2 and 5.3**). Decreased expression of IFNGR can be due to receptor internalization, degradation, or suppressed transcription of the receptor subunits (92, 244). Based on our mRNA data (**Figure 5.1**), we found that TL-1 cells have fewer IFNGR transcripts, resulting in less IFNGR protein, compared to Jurkat T cells. Moreover, our results suggest that STAT5b may play a role in regulating IFNGR1 mRNA levels but not IFNGR2 mRNA levels (**Figure 5.8**).

Furthermore, we demonstrate that the lack of IFN-γ responsiveness is not due to defects in the type I interferon signaling pathway (**Figure 5.5**). Deficiency in the type I interferon receptors IFNAR1 and IFNAR2 has been shown to dampen IFN-γ signaling (249). However, we found that TL-1 cells robustly induce p-STAT1 in response to IFN-β treatment (**Figure 5.5**). These results demonstrate three concepts. First, a deficiency in IFN-β signaling is not responsible for the unresponsiveness of TL-1 cells to IFN-γ. Second, IFN-β signaling and IL-2 signaling (**Figures 5.2, 5.3, 5.4, and 5.5**) are both intact, which supports a specific IFN-γ signaling defect in TL-1 cells. Third, the deficiency in IFN-γ signaling does not impact the ability of TL-1 cells to respond to IFN-β. To the best of our knowledge, this is the first study to evaluate type I interferon STAT signaling in LGLL.

The JAK-STAT pathway is commonly dysregulated in malignancies leading to an increase in inflammatory cytokine production and a decrease in induction of pro-apoptotic pathways (255). In T-LGLL, STAT1 and STAT3 are constitutively activated (56). Approximately 40% of T-LGLL patients have activating somatic mutations in *STAT3* (162), and a small percentage of T-LGLL patients have activating *STAT5b* somatic mutations (39). As STAT1, STAT3, and STAT5b have been identified as important transcription factors in T-LGLL pathogenesis (36, 39, 56, 162), we investigated the role of these transcription factors in IL-2-driven IFN-γ production. Our studies with specific siRNA-mediated targeting of STAT proteins suggest that STAT5b, rather than STAT1 and STAT3, drives IFN-γ production in TL-1 cells under IL-2-stimulated conditions (**Figure 5.6**). We confirmed that STAT5b regulates IFN-γ levels in Jurkat T cells (**Figure 5.7**) and other studies also indicate a role of STAT5 in IFN-γ regulation (182, 183, 250, 251). Specifically, when supplemented with IL-2, as done with the TL-1 cell line, STAT5

has been shown to drive IFN-γ production (250, 252) and when STAT5 is inhibited pharmacologically, IFN-γ decreases (256).

The IL-2-mediated production of IFN-γ by STAT5b can be explained through the previously observed decreased SOCS1 levels. SOCS1, although classically associated with IFN-γ-mediated signaling, acts on several other cytokine-mediated pathways, including those mediated by IL-2 (240). SOCS1 associates with IL-2Rβ, leading to an inhibition of IL-2-induced STAT5 activation (240). Thus, the lack of SOCS1 would allow STAT5b to continue to produce IFN-γ without negative regulation in the presence of IL-2, which is in agreement with our observations. Other transcription factors that are known to regulate IFN-γ in CD8+ T cells, such as T-bet (257, 258), Eomes (258), and STAT4 (182, 258), may also be considered for future studies to more completely define the regulators of IFN-γ production in T-LGLL and assess the effects of SOCS1 deficiency on their regulation.

In addition to the role of STAT5b in promoting IFN-γ production, STAT5b significantly represses STAT1 protein levels and moderately decreases STAT3 activation in TL-1 cells (**Figure 5.9**). This matches the literature as STAT3 and STAT5 have been documented to have opposing roles in malignancies and development (259, 260), with competitive DNA binding to the same site (254, 261-263), leading to displacement of STAT3 (261). STAT1 and STAT5 have also been shown to have an inverse relationship. STAT1 gain of function in natural killer cells leads to impaired STAT5 activation (264), suggesting antagonistic regulation between these two STATs.

Contrary to its inverse relationship with STAT5b, p-STAT1 positively correlates

with p-STAT3 in the TL-1 cell line (**Figure 5.9**). Although STAT1 and STAT3 are commonly associated with opposing cellular roles (50, 265, 266), STAT3 gene expression can enhance STAT1 activation (267) and STAT3 can form heterodimers with STAT1 (268). In TL-1 cells, STAT1 and STAT3 are simultaneously activated in response to IL-2 and are dually reduced in response to vitamin D (74), suggesting a cooperative relationship between the two STATs. Moreover, in T-LGLL specifically, STAT1 and STAT3 are both constitutively activated and form heterodimers in patient leukemic cells (56). The mechanism behind STAT3-mediated STAT1 activation in T-LGLL is currently unclear, although likely explanations include production of activating cytokines or the documented STAT1-STAT3 heterodimer formation in T-LGLL cells (56).

In this current study, we evaluated the IFN-γ signaling pathway and IL-2-induced production of IFN-γ in the TL-1 cell line (**Figure 5.10**). The main finding is that TL-1 cells are unresponsive to IFN-γ supplementation most likely due to decreased IFNGR expression. Furthermore, the lack of IFN-γ-mediated SOCS1 expression allows IL-2 to drive STAT5b-mediated production of IFN-γ without negative regulation. Although receptor-targeting antibodies are gaining traction in cancer research, our results suggest that IFNGR targeting would be ineffective in inhibiting IFN-γ-induced signaling, given that these cells do not have adequate IFNGR levels. To inhibit excessive IFN-γ production in TL-1 cells, the positive regulators of IFN-γ, including JAKs, STATs, and other cytokines that drive its expression could be targeted. Additionally, the TL-1 cell line offers a novel model for better understanding diseases that exhibit similar unresponsiveness to IFN-γ as a result of decreased IFNGR levels (91, 92, 96, 243, 244). Several studies have demonstrated lower IFNGR levels in primary cells in multiple diseases (91, 92, 96, 243, 244). However, the use of primary cells poses limitations for determining long term

effects or allowing manipulation of the IFN-γ pathway or related pathways. Through utilization of the TL-1 cell line, potential cross talk with other critical signaling pathways, such as IL-2-mediating JAK-STAT signaling, could be elucidated. Such studies might validate novel therapeutic targets for such IFN-γ non-responsive diseases.

5.6. Figures & Tables

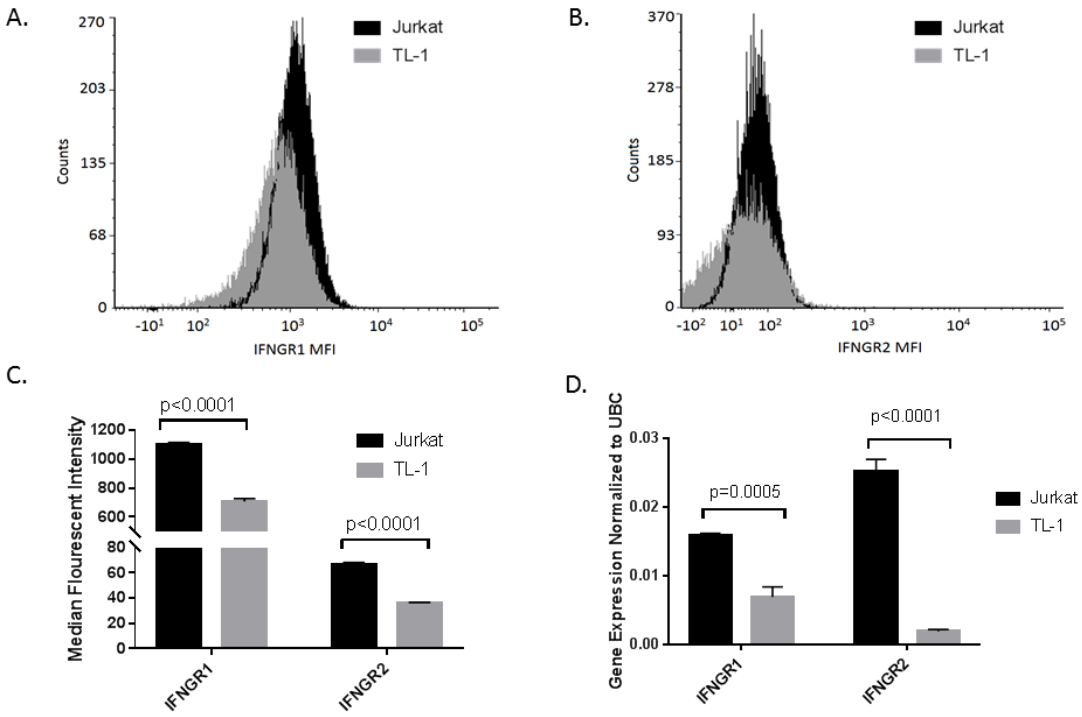


Figure 5.1. TL-1 cells exhibit lower expression of the IFNGR1 and IFNGR2 compared to Jurkat T cells. The surface expression of the IFNGR1 and IFNGR2 was determined using flow cytometry in the TL-1 cell line compared to Jurkat T cells, an IFN-γ responsive cell line. Representative flow cytometry histograms of (A) IFNGR1 and (B) IFNGR2 and (C) median fluorescence intensity (MFI) of IFNGR1 and IFNGR2 in TL-1 and Jurkat T cells are shown. (D) IFNGR1 and IFNGR2 transcripts were quantified using qPCR. Results were normalized to ubiquitin C (UBC), a housekeeping gene. Student's T test was used to determine significance of TL-1 cells compared to Jurkat T cells. All data are presented as mean +/- Stdev (n=3 biological replicates). *Published in Kulling et al., PLoS ONE (2018).*

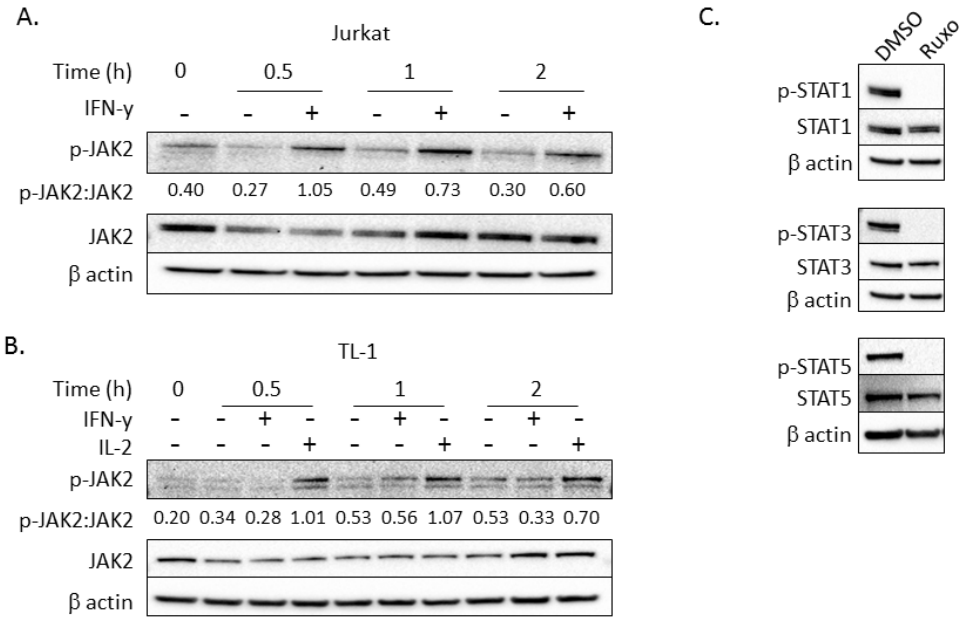


Figure 5.2. JAK2 is unresponsive to IFN-γ in TL-1 cells. (**A**) Jurkat T cells or (**B**) TL-1 cells were treated with 10 ng/mL IFN-γ or water (vehicle control) for the indicated time. TL-1 cells were also treated with IL-2 as a positive control for induction of p-JAK2. p-JAK2, total JAK2, and β actin were measured using western blot. The ratio of p-JAK2:JAK2 is shown for each condition. (**C**) TL-1 cells were pre-treated with 5 μM ruxolitinib (Ruxo) or DMSO for 2 h prior to the addition of IL-2. Protein lysates were created 1 h after the addition of IL-2. Western blots were probed for p-STAT1, total STAT1, and β actin. p-STAT3, total STAT3, p-STAT5 and total STAT5 were used as controls for the functionality of the JAK inhibitors. *Published in Kulling et al., PLoS ONE (2018).*

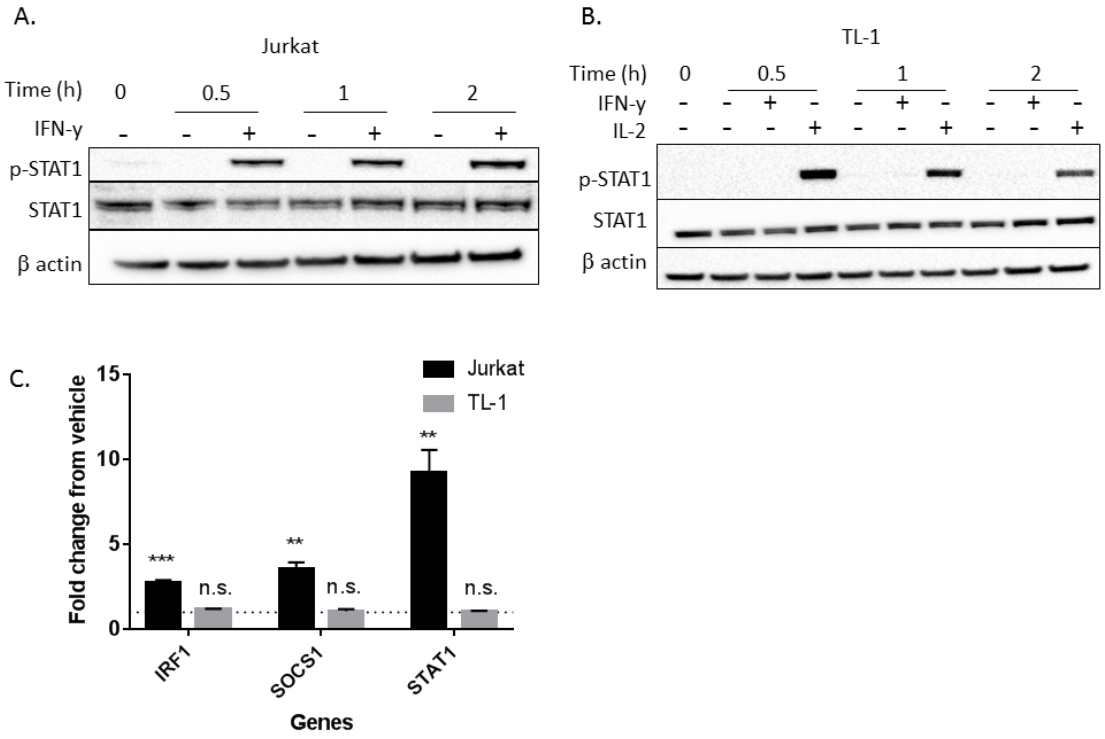


Figure 5.3. IFN-γ does not activate STAT1 or induce transcription of IFN-γ-regulated genes in TL-1 cells. (**A**) Jurkat T cells or (**B**) TL-1 cells were treated with 10 ng/mL IFN-γ or water (vehicle control) for the indicated time. TL-1 cells were also treated with 200 U/mL IL-2 as a positive control for induction of p-STAT1. p-STAT1, total STAT1, and β actin were measured using western blot. (**C**) Jurkat or TL-1 cells were treated with 10 ng/mL IFN-γ or water for 6 h prior to RNA extraction. Induction of IRF-1, SOCS1, and STAT1 transcripts was quantified using qPCR. Results were normalized to UBC and then to the water control to demonstrate fold change. Student's T test was used to determine significance compared to vehicle control. *= $p<0.05$, **= $p<0.01$, ***=$p<0.005$, ****= $p<0.001$. Data are presented as mean +/- Stdev (n=3 biological replicates). *Published in Kulling et al., PLoS ONE (2018).*

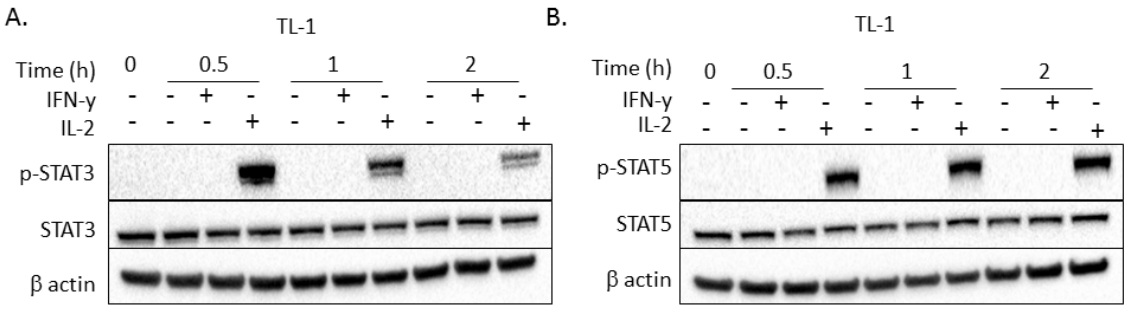


Figure 5.4. STAT3 and STAT5 phosphorylation is induced by IL-2, but not IFN-γ, in TL-1 cells. IL-2-starved TL-1 cells were treated with 10 ng/mL IFN-γ, 200 U/mL IL-2 (positive control), or water (vehicle control) for the indicated time. (**A**) p-STAT3 and total STAT3 or (**B**) p-STAT5 and total STAT5 were measured using western blot. β actin was used as a loading control. *Published in Kulling et al., PLoS ONE (2018).*

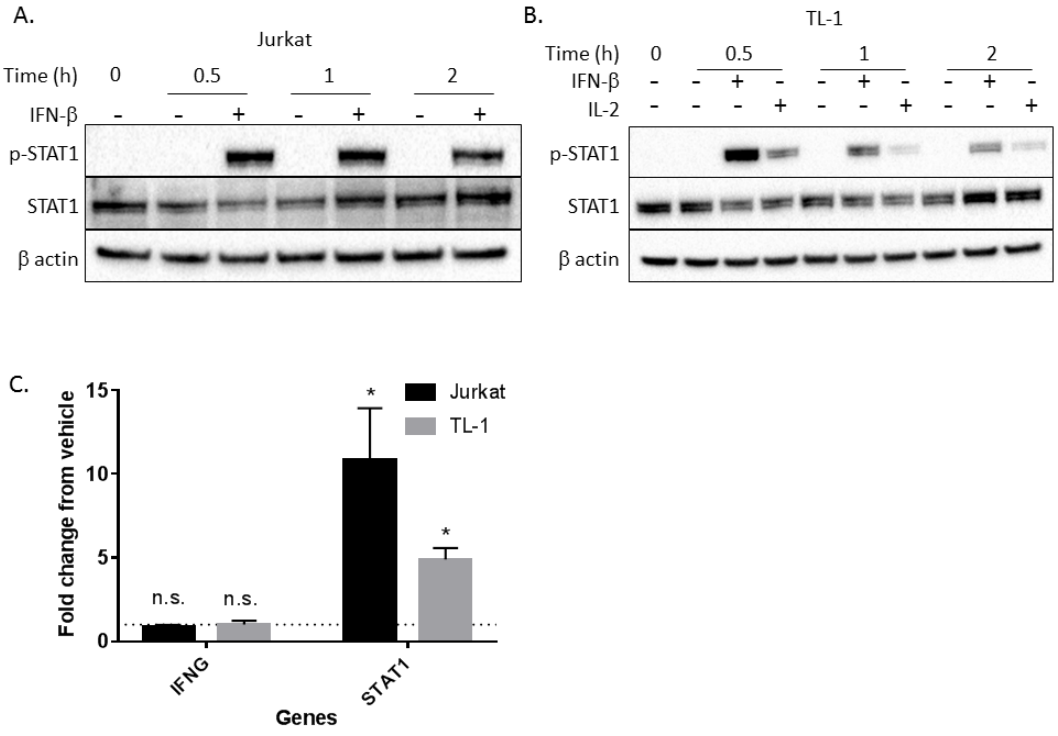


Figure 5.5. TL-1 cells are responsive to IFN-β, supporting a type II interferon-specific signaling defect. (**A**) Jurkat T cells or (**B**) IL-2-starved TL-1 cells were treated with 5000 U/mL IFN-β or water (vehicle control) for the indicated time. TL-1 cells were also treated with 200 U/mL IL-2 as a positive control for induction of p-STAT1. p-STAT1, total STAT1, and β actin were measured using western blot. (**C**) Jurkat or TL-1 cells were treated with 5000 U/mL IFN-β or water for 6 h prior to RNA extraction. Induction of IFN-γ and STAT1 transcripts was quantified using qPCR. Results were normalized to UBC (a housekeeping gene) and then to the water control to demonstrate fold change. Student's T test was used to determine significance compared to vehicle control. *= $p<0.05$, **= $p<0.01$, ***=$p<0.005$, ****= $p<0.001$. Data are presented as mean +/- Stdev (n=3 biological replicates). *Published in Kulling et al., PLoS ONE (2018).*

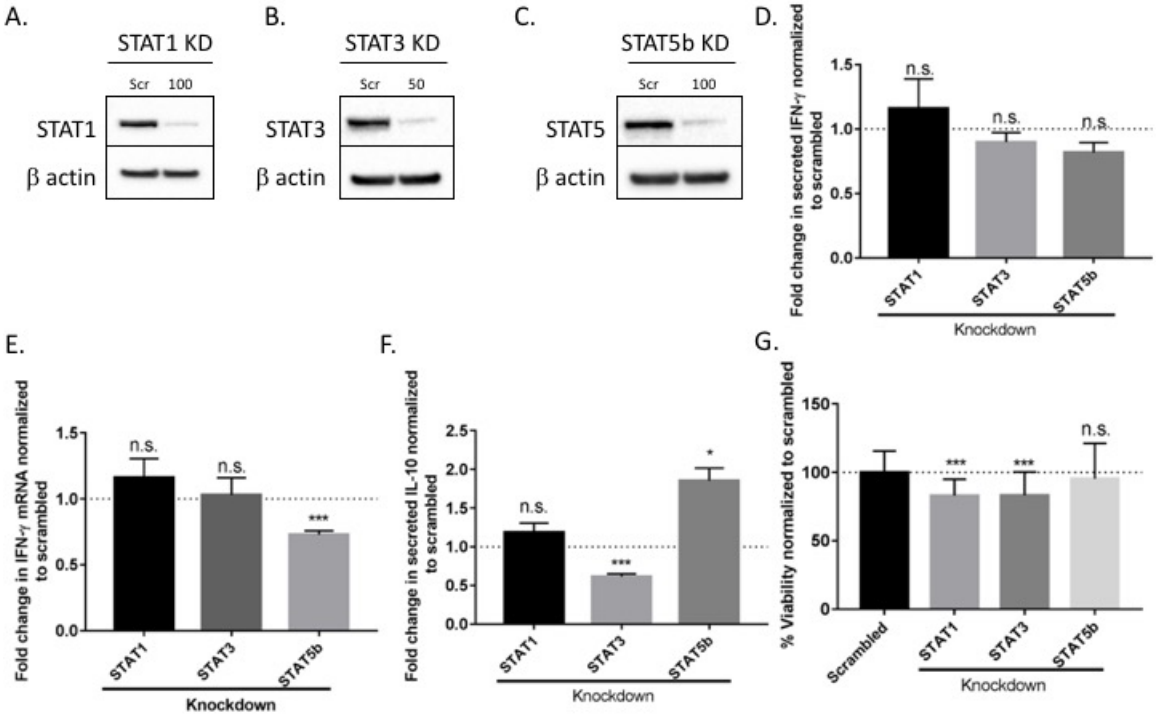


Figure 5.6. STAT5b is required for maximal production of IL-2-induced IFN-γ mRNA in TL-1 cells. (**A**) STAT1, (**B**) STAT3, or (**C**) STAT5b were knocked down using siRNA in TL-1 cells supplemented with IL-2. Protein lysates and RNA were harvested as well as conditioned media collected 48 h after siRNA transfection. Representative western blots of the knockdowns are shown. (**D**) Supernatant was analyzed to determine changes in secreted IFN-γ as a result of each knockdown. (**E**) The effect of knockdown on IFN-γ transcript levels was determined using qPCR. Results were normalized to the UBC gene and further normalized to the scrambled siRNA control. (**F**) Changes in IL-10 production were also assessed in each knockdown group using Luminex. (**G**) Viability following knockdown was measured by MTS assay at 48h. All results were normalized to scrambled siRNA treated cells. Student's T test was used to determine significance compared to scrambled siRNA. *= $p<0.05$, **= $p<0.01$, ***=$p<0.005$, ****= $p<0.001$. Data are presented as mean +/- Stdev (n=3 biological replicates). *Published in Kulling et al., PLoS ONE (2018)*

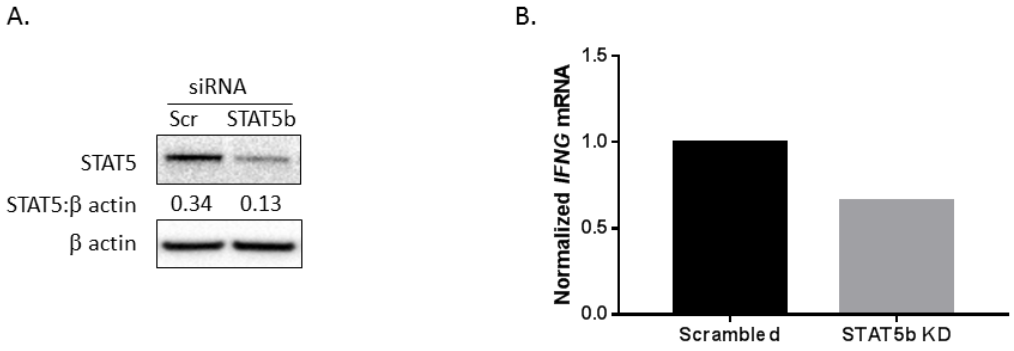


Figure 5.7. STAT5b regulates IFN-γ mRNA content in Jurkat T cells. STAT5b was knocked down using siRNA in Jurkat T cells. Protein lysates and RNA were harvested 48 h after siRNA transfection. (**A**) Western blot quantification of STAT5 protein with β-actin as a loading control. (**B**) The effect of knockdown on IFN-γ transcript levels was determined using qPCR. Results were normalized to UBC, a housekeeping gene, and further normalized to scrambled siRNA. *Published in Kulling et al., PLoS ONE (2018).*

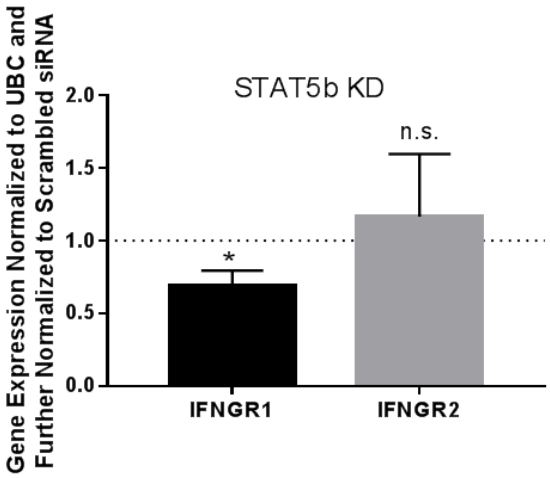


Figure 5.8. STAT5b regulates IFNGR1, but not IFNGR2, in TL-1 cells. STAT5b was knocked down using siRNA in TL-1 cells supplemented with IL-2. Protein lysates and RNA were harvested 48 h after siRNA transfection. Representative western blots of the knockdowns can be found in Fig. 5.6C. The effect of knockdown on IFNGR1 and IFNGR2 transcript levels were determined using qPCR. Results were normalized to UBC, a housekeeping gene, and further normalized to the scrambled siRNA control. Student's T test was used to determine significance compared to scrambled siRNA. *= $p<0.05$, **= $p<0.01$, ***=$p<0.005$, ****= $p<0.001$. Data are presented as mean +/- Stdev (n=3 biological replicates). *Published in Kulling et al., PLoS ONE (2018).*

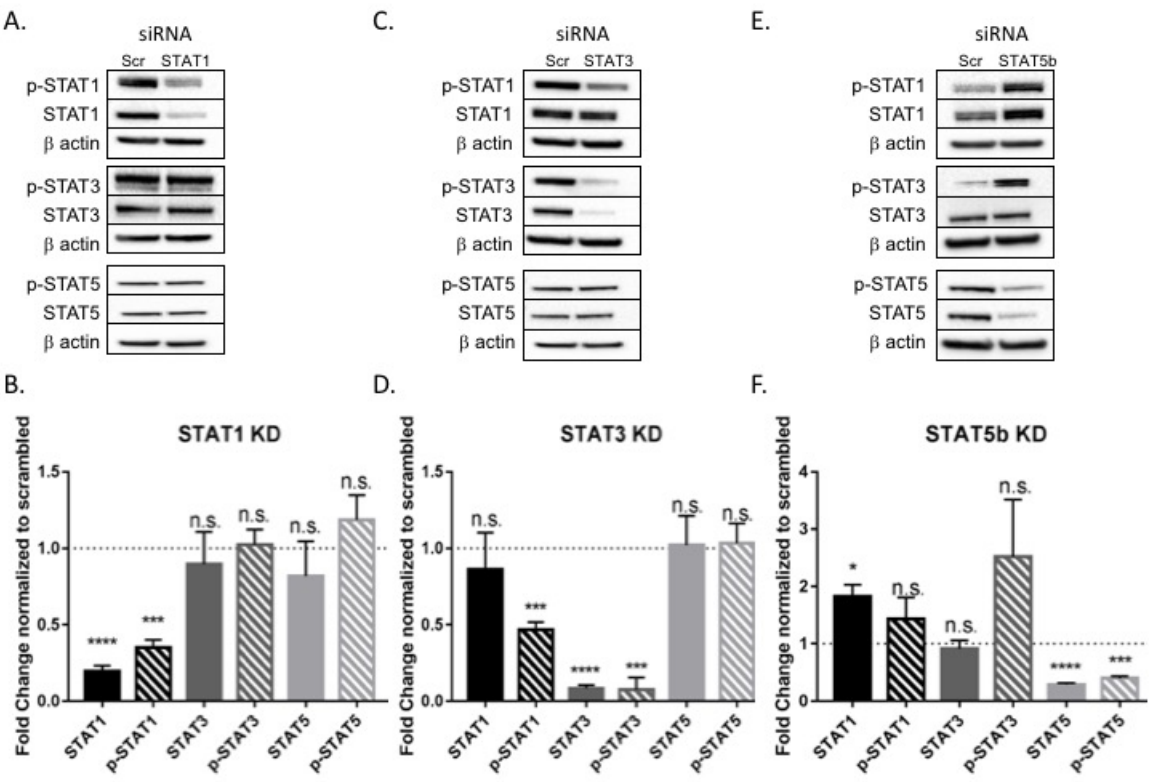


Figure 5.9. Relationship between STATs 1, 3, and 5b and their activation states. (**A, B**) STAT1, (**C, D**) STAT3, and (**E, F**) STAT5b were knocked down using siRNA in IL-2 supplemented TL-1 cells. Protein lysates were harvested 48 h after siRNA transfection. Blots were probed for p-STAT1, STAT1, p-STAT3, STAT3, p-STAT5, STAT5, and β actin. p-STAT proteins were normalized to their respective total STAT proteins. STAT1, STAT3, and STAT5 were normalized to β actin. Representative western blots are shown (**A, C, E**) as well as quantification of experimental replicates (**B, D, F**). Student's T test was used to determine significance compared to scrambled siRNA. *= $p<0.05$, **= $p<0.01$, ***=$p<0.005$, ****= $p<0.001$. Data are presented as mean +/- Stdev (n=3 biological replicates). *Published in Kulling et al., PLoS ONE (2018).*

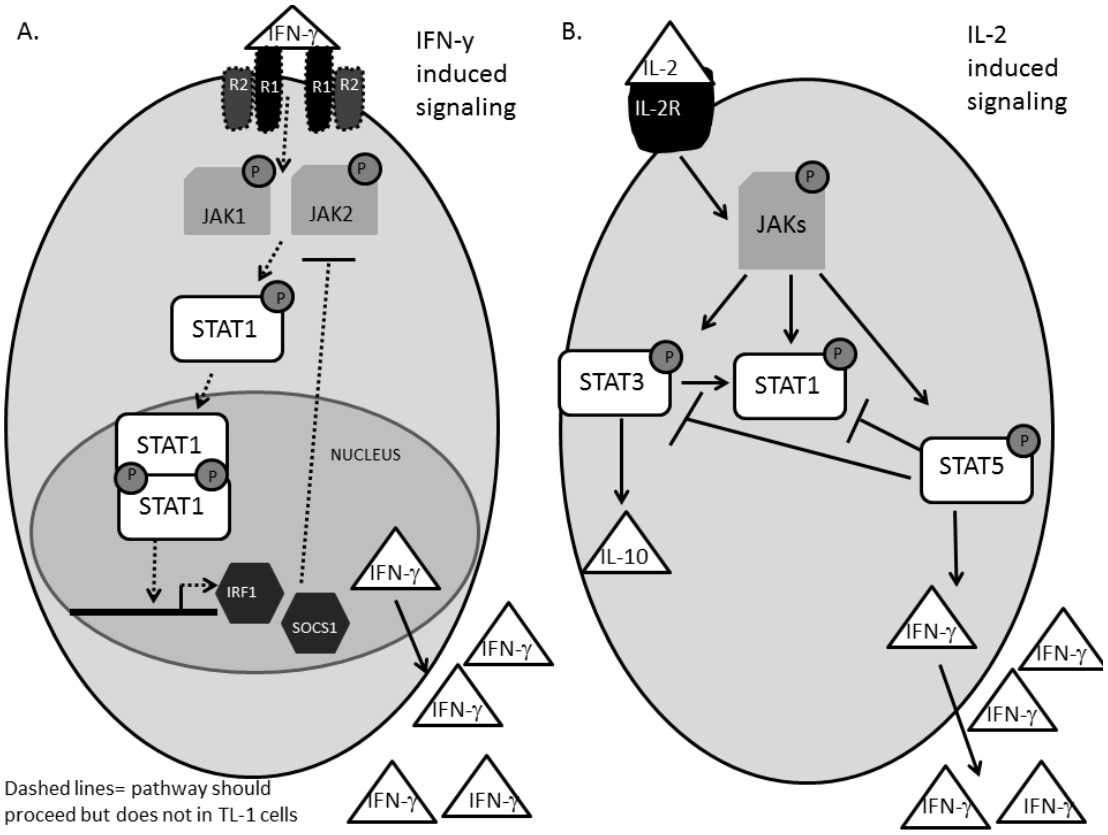


Figure 5.10. Working model for JAK-STAT signaling pathway in TL-1 cells. (**A**) Based on our findings, TL-1 cells have a decreased expression of IFNGR1 and IFNGR2, rendering the cells unresponsive to IFN-γ-induced signaling. This allows uncontrolled production of IL-2 induced IFN-γ production due to lack of induction of negative feedback regulators. (**B**) However, TL-1 cells are responsive to IL-2 leading to activation of STAT1, STAT3, and STAT5. STAT1 activation positively correlates with STAT3 while the activation of these proteins is enhanced upon knockdown of STAT5b. STAT5b and STAT3 promote transcription of IFN-γ and IL-10, respectively. *Published in Kulling et al., PLoS ONE (2018).*

Chapter 6

Future Directions, Ongoing Experiments, and Overall Conclusions

6.1. Abbreviations

Abbreviations: interferon gamma (IFN-γ), interleukin (IL), Janus kinase (JAK), T cell large granular lymphocyte (T-LGL), T cell large granular lymphocytic leukemia (T-LGLL), peripheral blood mononuclear cell (PBMC), signal transducer and activator of transcription (STAT), vitamin D receptor (VDR), suppressors of cytokine signaling (SOCS), myelodysplastic syndrome (MDS), hypomyelodysplastic syndrome (hypo-MDS), B cell chronic lymphocytic leukemia (B-CLL), IL-15 receptor α (IL-15Rα), single nucleotide polymorphism (SNP), cyclosporine A (CsA), methotrexate (MTX), natural killer cell LGLL (NK-LGLL), rheumatoid arthritis (RA), Fischer 344 rats (F344), IFN-γ receptor (IFNGR), Src homology 2 domain-containing protein tyrosine phosphatases 2 (SHP-2), IFN-α receptor 1 (IFNAR1), activating protein 2α (AP-2α), human T cell leukemia/lymphoma virus (HTLV), human immunodeficiency virus (HIV), B cell receptor (BCR), T cell receptor (TCR)

6.2. Future Directions—Vitamin D as a Potential Therapy for T-LGLL (Chapters 3 & 4)

6.2.1. What are the possible effects of calcitriol treatment *in vivo*?

We found that calcitriol significantly reduced IFN-γ production in TL-1 cells and T-LGLL PBMCs (**Chapter 3 & 4**). Although these results are promising, our experiments were performed *in vitro*. By using cell culture, we were able to pharmacologically and genetically manipulate the cells to elucidate the mechanism of calcitriol (**Chapter 4**). However, our ultimate goal is to move from the *in vitro* system to *in vivo* through a clinical trial, if future studies support this. There is no animal model for T-LGLL, but calcitriol has very minimal toxicity and has been well tolerated in the clinic for other diseases (269). There are numerous successful clinical trials examining the use of calcitriol, its analogs, or precursors in an array of autoimmune diseases and cancers (105, 206, 269-271). We would predict that we would observe a decrease in overall IFN-γ serum levels, as seen in a trial for dialysis patients (206), with calcitriol treatment and an improvement in symptoms and disease state in T-LGLL patients.

Calcitriol acts directly on T-LGLL cells, but it may also exert effects on non-malignant PBMCs. We assessed the anti-inflammatory actions of calcitriol on the leukemic cells and normal T cells themselves, but did not investigate the likely positive impact of calcitriol on other PBMCs. One study found that 43% of T-LGLL patients presented with at least one form of B cell dyscrasia (272). Hypogammablobulinemia (272), aplastic anemia, and myelodysplastic syndrome (MDS), in particular, are associated with both LGLL and decreased B cell function. In hypomyelodysplastic syndrome (hypo-MDS), a form of MDS, B cell progenitors are reduced even more so

than in aplastic anemia, a similar disease state (273). This reduction in B cell progenitors is hypothesized to result from an inhibition of B cell proliferation due to increased cytotoxic LGL cells (273). B cell reduction is also prominently observed in pediatric MDS cases with GATA-2 deficiency (274). Another study found that patients with idiopathic aplastic anemia exhibited hypogammaglobulinemia and their B cells were unable to produce immunoglobulin upon stimulation with pokeweed mitogen (275).

There is also support for abnormal LGL cell populations directly reducing functionality of B cells, which may explain the decreased function of B cells in diseases that can co-occur with LGLL. For instance, growth-factor induced normal B cell proliferation was inhibited by co-culture with LGL cells from B cell chronic lymphocytic leukemia (B-CLL) (276). B-CLL is associated with diseases that can co-occur with LGLL, including aplastic anemia and pure red cell aplasia, as well as LGL lymphocytosis, suggesting a role of abnormal LGL cells in B cell dysfunction (276). Additionally, leukemic LGL cells isolated from a rat model of NK-LGLL inhibited B cell function *in vitro* as evidenced by decreased plaque formation (277). In fact, there was a leukemic LGL cell dose-dependent inhibition of plaque formation by the splenic B cells (277), directly implicating LGL cells in suppressing B cell function.

With LGL cells being the main producers of IFN-γ, these LGL-associated B cell diseases likely result from the overactivation of the leukemic or abnormal T cells and subsequent increase in IFN-γ production. IFN-γ inhibits proliferation (83), reduces antigen processing (176), and induces apoptosis in B cells (83). Moreover, IFN-γ induces aplastic anemia in mice through inhibition of myeloid progenitor differentiation and B cells (79). Thus, as calcitriol inhibits IFN-γ, LGL-suppressed B cells should regain normal

function in LGLL patients with these B cell disorders.

On the other hand, another subset of patients exhibits hyperactivated B cells. Documented B cell dysfunctions that can occur with T-LGLL include chronic lymphocytic leukemia (272), monoclonal gammopathy of unknown significance, monoclonal B cell lymphocytosis (278), follicular lymphoma (279), polyclonal hypergammaglobulinemia (272, 280), and B cell neoplasms (12). Calcitriol acts directly on chronically activated B cells to inhibit the production of immunoglobulin, potentially by reducing IL-2 receptor levels (145). This would address the occurrence of hypergammaglobulinemia. For hyperproliferative B cells, as in the case of B cell lymphocytosis, calcitriol treatment inhibits proliferation (126, 143) and promotes apoptosis of activated B cells (126), reducing the number of activated circulating B cells.

In addition to the effects of calcitriol on modulating B cell function, calcitriol acts on PBMCs to further reduce activation of T cells, which is desirable for chronic activation of T cells, including T-LGLL. Calcitriol prevents B cells from activating T cells through reduction in co-stimulatory molecule expression in B cells (147). Calcitriol also decreases T cell proliferation (103) through inhibition of maturation, activation, differentiation, and survival of dendritic cells, leading to a suppression of T cell activation and proliferation (148). Thus, calcitriol acts on both B cells and dendritic cells to reduce T cell cytokine production and proliferation (147).

IFN-γ can also alter the responsiveness of cells to other cytokine stimulation through modulation of cytokine receptor expression. For instance, IFN-γ can induce production of IL-15 receptor α (IL-15Rα). IL-15Rα is elevated in T-LGLL and IL-15-

mediated signaling promotes proliferation and survival of T-LGLL cells (61). Therefore, calcitriol-mediated inhibition of IFN-γ production may reduce IL-15 signaling through downregulation of IL-15Rα in T-LGLL. Taken together with the potential inhibition of B cell and dendritic cell-mediated activation of T-LGLL cells, calcitriol may be able to even further reduce the activation state of T-LGLL cells *in vivo*.

IFN-γ is directly implicated in the development of aplastic anemia, a disease that co-occurs with T-LGLL (79, 86). Single nucleotide polymorphisms (SNPs) in the IFN-γ gene were found in patients with aplastic anemia, leading to an increased production of IFN-γ (281). The elevated level of IFN-γ may also explain the occurrence of neutropenia in T-LGLL patients as IFN-γ is known to upregulate FasL and promote Fas-mediated apoptosis of immature myeloid cells, leading to neutropenia (13). Therefore, in theory, the IFN-γ reduction observed with calcitriol treatment should improve the B cell disorders and other co-occurring diseases, such as aplastic anemia and neutropenia, resulting from elevated IFN-γ production. Thus, calcitriol may prove to be more potent in the human body than in culture systems due to its positive direct and indirect effects on non-malignant PBMCs in addition to its antitumor effects on cancer cells.

6.2.2. What are considerations for further evaluation of calcitriol for use in the clinic for treatment of T-LGLL?

Appropriate dosage and timing of calcitriol treatment would need to be calculated if calcitriol were used as a treatment. Calcitriol doses are generally administered daily or weekly for greater than 3 months in clinical trials (206, 271) compared to our 24 h time points. Due to the relatively short survival of isolated PBMCs and the TL-1 cell line if deprived from IL-2, long term studies were not explored in our studies. It would be

beneficial to examine the effects of calcitriol on activated patient PBMCs and repeated calcitriol treatment co-administered with IL-2 treatment. It is possible that continual inhibition of STAT1 and STAT3 activation as a result of prolonged calcitriol treatment would lead to apoptosis even though we did not observe apoptosis at 24 h following treatment. This would be supported by previous studies demonstrating that complete inhibition of STAT1 and STAT3 using a potent JAK inhibitor induced apoptosis of T-LGLL patient cells (56). We did see a significant reduction of STAT1 and STAT3 phosphorylation following calcitriol treatment, but it was never completely ablated in our experimental conditions (**Chapter 3**). Even with an efficient STAT1 and STAT3 knockdown using targeted siRNA (**Chapter 5**), viability was only measured 48 h after knockdown. It is likely that viability may be more greatly impacted with repeated dosages of calcitriol and longer treatment courses, which would happen in clinical trial use, compared to the shorter treatments in our cell line experiments.

One important future study is to measure the serum levels of $25(OH)D_3$ in T-LGLL patients. This will be essential to better understand the vitamin D status in T-LGLL patients. Although it is likely that T-LGLL patients are vitamin D deficient considering the high rate of vitamin D deficiency in hematological malignancies, there have been no published studies on the prevalence of vitamin D deficiency in the T-LGLL patient population. We have noted that several patients have previously had their vitamin D levels measured and were found to be deficient (unpublished data). However, we plan to quantify $25(OH)D_3$ levels in a large cohort of T-LGLL patients to assess the prevalence of vitamin D deficiency. Although this will not inform us of whether deficiency is a cause or effect of T-LGLL, we will use the LGL Leukemia Registry at UVA to immediately select banked patient serum samples. The LGL Leukemia Registry is national database and

repository for primary patient specimens. Blood and tissue samples are collected in clinic or sent from clinics around the country for use in research studies. Tissue samples include bone marrow biopsy, liver biopsy, splenectomy, and saliva sample. Blood draws are typically arranged during scheduled office visits, with blood samples sent from remote physician offices. As Dr. Loughran is the leading expert in T-LGLL, patients from around the country schedule appointments with Dr. Loughran and we are fortunate to be able to obtain samples from these patients directly from the UVA clinic on the same day as the blood draw occurs. Samples are used for research purposes, including evaluation of novel therapies and characterization of signaling pathways (**Chapter 3 & Chapter 4**). We are able to access patient medical records for obtaining relevant medical history including treatment course, STAT3 mutational status, co-occurring diseases, age, and sex. We currently have over 1,200 LGLL patients enrolled in this registry program.

Utilization of the LGL Leukemia Registry at UVA will allow us to examine differences between cytokine production, symptomology, disease progression, response to treatment, additional disease states, and STAT mutational status. This information may allow us to find correlations between vitamin D deficiency and disease. If we discover that T-LGLL patients are deficient in $25(OH)D_3$, our findings would encourage routine laboratory tests for $25(OH)D_3$ levels in patients. Once deficiency is diagnosed, patients may be recommended appropriate vitamin D supplementation at the discretion of their primary care physician. As vitamin D deficiency is associated with development of many other autoimmune diseases and malignancies, identifying patients with insufficient levels of vitamin D could aide in the prevention of additional disorders or malignancies.

Over-the-counter vitamin D supplements are commonly consumed by the general population in cases when a vitamin D deficiency is discovered. Communication from the LGLL population to Dr. Loughran as well as medical chart review have indicated that many LGLL patients take over-the-counter vitamin D tablets even without knowledge of their current vitamin D levels. As we plan to continue our vitamin D studies, we (Kulling, Hines, Shemo) are in the process of creating a patient survey that will collect data regarding supplementation use. We will ask questions regarding exposure to sunlight, occupation, and dietary intake that is high in vitamin D (ex. fish). After collecting the responses, we will work to incorporate this information into our patient database and registry. Through this endeavor we can identify which patients are taking vitamin D supplementation or consume high quantities of vitamin D rich foods. This will allow us to include or exclude patients for future *in vitro* vitamin D experiments or clinical trials. As vitamin D has been demonstrated to work synergistically with other anti-cancer agents, it is critical that we determine which patients are taking vitamin D and what dosage so that we can account for any synergistic effects that may be happening.

Even if T-LGLL patients are not deficient in $25(OH)D_3$, T-LGLL patients may possess mutations or SNPs in the vitamin D pathway, preventing conversion of $25(OH)D_3$ to adequate levels of calcitriol, which we found to be necessary for reducing IFN-γ levels. SNPs in the vitamin D pathway have not previously been explored in T-LGLL, but have been well documented in other malignancies. Two key enzymes that are often dysregulated in vitamin D metabolism are CYP27B1 and CYP24A1. CYP24A1 SNPs, rs2762939 and rs2181874, are associated with non-Hodgkin lymphoma (122) and non-small cell lung cancer (282), respectively. A SNP, rs118204009, in CYP27B1

has been reported for multiple sclerosis, with this mutation resulting in a loss of function of CY27B1 (283). In vitamin D metabolism, CYP27B1 metabolizes 25(OH)D_3 to calcitriol, which is later hydroxylated by CYP24A1, reducing its biological activity (284). Loss of CY27B1 activity prevents the conversion of 25(OH)D_3 to calcitriol while increased activity of CYP24A1 breaks down calcitriol into its less active form more quickly. If SNPs in these enzymes are present in T-LGLL patients, vitamin D metabolism could be dysregulated. For example, if 25(OH)D_3 levels are sufficient, available calcitriol may be dramatically reduced as a result of decreased CYP27B1 expression or increased CYP24A1 activity. There are several options to circumvent the dysregulated expression or activity of these enzymes. In regards to reduction of CYP27B1 activity, calcitriol can be supplemented in place of vitamin D_3 found over the counter in pharmacies. By using calcitriol, CYP27B1 does not need to convert 25(OH)D_3 to calcitriol as calcitriol is already available, avoiding the necessity of a functional CYP27B1. Therefore, knowledge of SNPs in key vitamin D pathway enzymes in T-LGLL patients could help tailor an effective vitamin D supplementation regimen.

Another method of achieving the effects of calcitriol despite dysregulated metabolic enzymes is the use of calcitriol analogs. Calcitriol analogs, including EB1089, are commonly used in clinical trials in place of calcitriol as analogs are typically more potent, more stable, and are not prone to inducing hypercalcemia in patients (284). The mechanism of action of these analogs vary, but like calcitriol, do not require CYP27B1 activity to convert to an active state. Moreover, due to the higher potency of calcitriol analogs, anti-cancer effects may be achieved at a lower dosage and shorter time frame, potentially reducing the effects of increased CYP24A1 activity.

Regardless of vitamin D metabolic enzyme status, the use of calcitriol analogs is beneficial and has been approved for use in the clinic for other diseases. Although calcitriol is effective in the clinic, hypercalcemia is common, preventing continued high dose use. Therefore, calcitriol analogs have been evaluated for anti-cancer effects while avoiding or reducing hypercalcemia (269). We showed that EB1089 significantly reduced IFN-γ and p-STAT1 in the TL-1 cell line (**Chapter 4**). Based on our results, we plan to evaluate the effects of EB1089 and other calcitriol analogs using T-LGLL patient cells. These experiments would provide us with rationale for pursuing analogs in the clinic in the future.

6.2.3. Can we combine calcitriol with existing T-LGLL therapies for synergistic effect?

In addition to evaluating analog use, we are interested in understanding whether calcitriol, or its analogs, could be used in combination with existing T-LGLL therapies to induce a synergistic or additive anti-cancer effect. Although calcitriol significantly reduced STAT1 activation and IFN-γ production (**Chapters 3 & 4**), calcitriol was unable to completely ablate either process alone. However, cyclosporine A (CsA), which is a commonly used T-LGLL therapy, has been shown to inhibit IFN-γ-induced activation of STAT1 (218) and production of IFN-γ (231, 234). There have been a few studies regarding combinatorial treatments of CsA and calcitriol with contradictory results. One study found that CsA inhibited the vitamin D pathway (285) while another study demonstrated that calcitriol or its analogs further reduced CsA-mediated inhibition of proliferation in T cells from patients with active ulcerative colitis (286). This synergistic effect allowed the dosage of CsA to be lowered, reducing the toxic side effects of this drug (286). Given that malignant T cells from T-LGLL leukemia, like ulcerative colitis, are

activated, we hypothesize that CsA and calcitriol may act synergistically to reduce IFN-γ or STAT activation in T-LGLL and should be investigated further.

Another combinatorial treatment option could be methotrexate (MTX) and calcitriol. Calcipotriol, a derivative of calcitriol, is commonly used to treat psoriasis (287). One study found that combining calcipotriol and MTX allowed for a lower dose of MTX to be used and the time to relapse of psoriasis was delayed with this combination (287). However, to the best of our knowledge, there have not been any published studies regarding the combined effects of MTX and calcitriol on modulating the JAK-STAT pathway. This is rather surprising as MTX was identified as an inhibitor of JAK-STAT signaling, specifically inhibiting JAK2, STAT3, and STAT5 phosphorylation (288). By using MTX and calcitriol, STAT1, STAT3, and STAT5 should be inhibited, with perhaps a greater reduction in STAT3 than seen with calcitriol alone. The further reduction in STAT3 may induce apoptosis in T-LGLL cells as previously seen with complete ablation of STAT3 phosphorylation pharmacologically (56). With STAT1, STAT3, and STAT5 activation impeded, we would likely avoid the negative effects of inhibiting STAT1/STAT3 or STAT5 alone. This is important as we found that inhibiting STAT5 activation increased STAT1 and STAT3 (**Chapter 5**). As all three of these STATs are implicated in the pathogenesis of T-LGLL, we would ideally target all three. This would prevent the production of inflammatory cytokines and transcription of pro-survival gene targets, possibly leading to sensitization of T-LGLL cells to apoptosis and reduction in inflammation. Although the literature on IFN-γ regulation by MTX is contradictory (289, 290), calcitriol, in combination with MTX, could be used to further reduce IFN-γ production through inhibition of STAT5 activation. Therefore, we propose future investigation into the combinatorial use of MTX and calcitriol for treatment of T-LGLL.

6.2.4 Does calcitriol work for other LGL disease states, including T-LGLL with RA and NK-LGLL?

LGLL is a diverse disease affecting T or NK cells. For our current studies, we evaluated the effects of vitamin D on indolent T-LGLL alone (**Chapters 3 & 4**) as indolent T-LGLL comprises over 85% of all LGLL cases. Our findings using the TL-1 cell line and T-LGLL patient cells prompted us to evaluate calcitriol for use in aggressive natural killer cell LGLL (NK-LGLL). To address this question, we treated the aggressive NK-LGLL cell line, NKL, as well as primary NK-LGLL patient samples with calcitriol or EB1089. We found that EB1089 significantly increased VDR protein levels while decreasing p-STAT1 and p-STAT3 in NKL cells, matching our findings in TL-1 cells. Contrary to the significant decrease in IFN-γ by calcitriol in TL-1 cells, we found that EB1089 significantly reduced both IL-10 and IFN-γ production in NKL cells while calcitriol treatment did not significantly alter these cytokines. Both TL-1 cells and NKL cells respond to EB1089 and neither cell line experienced a change in viability with calcitriol treatment (*K Olson, P Kulling, et al. Manuscript in preparation*). Our results demonstrate that calcitriol and EB1089 inhibits STAT activation and cytokine production in both NK-LGLL and T-LGLL cells. We plan to submit these results as a manuscript in Spring 2018.

With the knowledge of potential mechanisms and effects of calcitriol in both T-LGLL and NK-LGLL, future directions include the evaluation of the use of calcitriol for treatment of aggressive T-LGLL and NK-LGLL. We were unable to collect patient data on these populations as the occurrence of aggressive LGLL is rare and adequate patient sample size collection would be difficult. There are only several documented cases of aggressive T-LGLL (291). However, aggressive NK-LGLL occurs at a higher rate (albeit

still very rare) and is well documented in the Asian population (292). This aggressive disease is rapidly progressing, predominantly affects a young patient population, and can be fatal within weeks often from systemic complications including organ failure (292). There are no effective treatments (292), making novel therapeutic discoveries a priority.

Unlike indolent or aggressive T-LGLL, which do not have an animal model, aggressive NK-LGLL has an established rat model. Spontaneous development of NK-LGLL is the major cause of death in aging Fischer 344 (F344) rats (293, 294), occurring in 26.5% of male and 17.3% of female rats usually over the age of 18 months (295). The rate of occurrence can be modulated through environment changes, including supplementation with corn oil (295). F344 rats exhibit weight loss, enlarged spleen, and death within 2-6 weeks of clinical manifestations (293, 295). The factors contributing the development of LGLL in F344 rats are unknown, although it is likely related to genetics resulting from aging rather than a viral factor (295). F344 rats pose a unique advantage for *in vivo* work as these rats are immunocompetent and isogenic compared to animal models that are xenografts in an immunocompromised host. This rat model has previously been used for evaluating NK-LGLL drug therapies including nanoliposomal ceramide (296) and leukemic NK-LGLL cell lines have been derived from F344 rats to allow for manipulation of signaling and other *in vitro* work (297, 298). The use of this rat model would allow us to determine the effect of calcitriol on symptoms, LGL cells, and other circulating PBMCs. We can utilize this model to examine long term effects of calcitriol and experiment with dosing regimens. We can also validate mechanisms or pathways using the NKL cell line. As there are fewer studies on aggressive NK-LGLL drug development, future studies with calcitriol could support a novel treatment with

minimal predicted side effects.

In the present study, we did not investigate the effects of calcitriol on T-LGLL co-occurring with RA. As 30-40% of T-LGLL patients have co-occurring RA, it is critical that we investigate the effects of calcitriol on T-LGLL cells from patients with RA. To begin assessing the effect of calcitriol on cells from T-LGLL patients with RA, we need to perform *in vitro* experiments to determine if cells from T-LGLL patients with RA experience the same degree of STAT deactivation and IFN-γ reduction as cells from T-LGLL patients without RA. This is important to consider as response of T-LGLL cells may be affected by RA-specific cytokines or signaling pathways. The ability of T-LGLL cells from T-LGLL patients with RA to respond to calcitriol may prompt further investigation in the clinic. Vitamin D deficiency is associated with RA disease (299, 300), with 64.5% and 35.5% of RA patients exhibiting vitamin D insufficiency and deficiency, respectively, in one study. Another study found that 76.3% of RA patients had vitamin D deficiency (301). Serum vitamin D levels also inversely correlating with stiffness, inflammatory markers, pain, swollen joints (300), disease activity score (300, 301), and severity of disease (300-302). Although there are contradictory findings depending on the analog used and patient population, vitamin D treatment appears to alleviate many symptoms of RA. Supplementation with high doses of vitamin D analogs, specifically alphacalcidol, was correlated with disease improvement in 89% of RA patients, with 45% of patients experiencing complete remission and no side effects documented (303). Another study found that calcitriol supplementation improved pain-related symptoms in early RA patients compared to the placebo control (304). Based on previous vitamin D treatment in RA clinical trials, we would hypothesize that T-LGLL patients with RA would respond favorably to calcitriol, with additional mechanisms and cellular roles potentially

at play regarding IFN-γ and STAT signaling in T-LGLL cells. Moreover, based on the high rate of vitamin D deficiency in the RA patient population, T-LGLL patients with RA should be included in our future serum vitamin D studies.

6.2.5. Can other natural therapeutics be used to treat T-LGLL?

After publishing our vitamin D findings, we were contacted by multiple T-LGLL patients who are interested in the use of other natural therapeutics, in combination with existing T-LGLL therapies, for management of symptoms. As immunosuppressant therapies often induce toxic effects and are not curative, many patients are excited by supplements that have anti-cancer properties without the toxic effects. Although vitamin D was the first to be proposed by our patients and has the largest body of literature supporting its use, there are several other compounds potentially worth investigating in the future. The most intriguing compound is curcumin, which has had contradictory reports regarding efficacy but has been demonstrated to inhibit the JAK-STAT signaling pathway *in vitro* (305). Curcumin, the active component of turmeric, has been shown to suppress IFN-γ production, STAT1 activation and transcription of gene targets, and JAK1 activation in colonocytes (306). Curcumin also reduced STAT1, STAT3, JAK1, and JAK2 activation in microglia through activation of Src homology 2 domain-containing protein tyrosine phosphatases 2 (SHP-2) (307). In addition to its effects on IFN-γ production, curcumin reduces sensitivity to IFN-γ through internationalization, lysosomal fusion, and degradation of IFN-γ receptor 1 (IFNGR1) (306).

Although curcumin has promising anti-inflammatory effects, curcumin has low bioavailability, which is attributed to the metabolism and poor solubility of curcumin (305, 308). To circumvent these effects, alternative delivery methods, including nanomaterial

drug carriers, and creation of analogs are currently being investigated and may offer an advantage for use in the clinic (305, 308). If the bioavailability of curcumin can be increased, we would be interested in determining the effects of curcumin on the JAK-STAT pathway and IFN-γ production in T-LGLL, in combination with calcitriol and immunosuppressant therapies and as a solo agent, pending safety evaluation of curcumin.

6.3. Future Directions: IFN-γ Signaling (Chapter 5)

6.3.1. Do IFNGR levels in T-LGLL cells correlate with disease state?

Our work regarding the characterization of IFN-γ-mediated signaling in T-LGLL was performed entirely in the TL-1 cell line, compared to the Jurkat T cell line, our positive control. This was primarily due to the difficulty of isolating pure leukemic cells from T-LGLL patients in clinic and performing FACS before the cell viability or cytokine profile were impacted, which can affect IFNGR levels. It is critical that the isolated cells are leukemic cells, and not the heterogeneous PBMC population, as normal T and B cells have detectable levels of IFNGR1 and IFNGR2, likely confounding our results. Moreover, the cell number required for conducting time courses and flow cytometry was not achievable during the time of our experiments. As a result of these constraints, we were unable to confirm these findings in primary T-LGLL patient cells. However, the reduction in IFNGR1 mRNA, compared to normal donor cells, has previously been confirmed in one T-LGLL patient in a small study (309). Despite the isolation obstacles, we propose future measurement of IFNGR1 and IFNGR2 levels in pure populations of freshly isolated primary T-LGLL leukemic cells. Through quantification of IFNGR levels in primary pure T-LGLL patient cells, we may be able to make correlations between

disease state and presence and functionality of the IFNGRs. If lower IFNGR levels are found in T-LGLL patient cells, as in TL-1 cells, IFNGR levels could be used as additional markers for T-LGLL cells.

Furthermore, we should investigate the relationship between IFNGR and IFN-γ levels. Likely, if cells are not responding to IFN-γ, negative regulation is not induced allowing higher IFN-γ levels, as seen in TL-1 cells. As LGL cells are the main source of IFN-γ (310), downregulation of IFN-γ negative regulators in LGL cells would greatly increase IFN-γ as the production of this inflammatory cytokine would not be controlled. Thus, lower IFNGR levels on T-LGLL cells will likely coincide with higher IFN-γ levels, which are associated with many of the T-LGLL symptoms and co-occurring autoimmune diseases. This hypothesis is supported by research indicating that lower IFNGR1 levels were correlated with greater metastasis in prostate cancer (311) and poorly differentiated breast cancer cells (312). If we find a population of T-LGLL with lower IFNGR levels and higher IFN-γ levels, these patients could benefit from IFN-γ reducing agents, such as cyclosporine A and calcitriol. Similar to the studies of STAT3 mutational status and response to MTX, a STAT3 inhibitor, we suggest future studies investigating IFNGR levels and response to cyclosporine A and calcitriol, in combination and as solo agents.

We are also interested in assessing the prevalence of SNPs in the IFNGR1 and IFNGR2 genes in TL-1 cells and T-LGLL patient cells. This information may further explain the complete unresponsiveness of TL-1 cells to IFN-γ-mediated signaling as SNPs in these genes have been associated with other diseases. Two damaging missense SNPs in IFNGR1 (V14M and Y397C) are associated with a partial deficiency

in IFNGR1 and reduced responsiveness to IFN-γ signaling (313). These SNPs were also significantly associated with a higher risk for atopic dermatitis complicated by eczema herpeticum (313). Other SNPs in IFNGR1 are associated with a significantly higher risk for development of tuberculosis (314), colorectal cancer (315), or early gastric carcinoma (316). Based on SNPs in IFNGR1 in other diseases, it would be worthwhile to determine the presence of SNPs and relationship to disease state in the T-LGLL patient population.

6.3.2. What is the mechanism behind the observed reduction in IFNGR levels in TL-1 cells?

We found that IFNGR1 and IFNGR2 were decreased on the mRNA and surface protein levels in TL-1 cells (**Chapter 5**). However, we did not determine the mechanism behind this decrease. Given that both the protein level and mRNA content are decreased in TL-1, IFNGR protein internalization and degradation are not likely mechanisms. One possibility, stemming from our observations, is that IFNGR mRNA is degraded, leading to a reduction in both mRNA content and protein levels. Another potential mechanism is that transcription of IFNGR is inhibited by another protein, such as EZH2, a histone methyltransferase. EZH2 is a commonly dysregulated protein in cancers, but has previously not been explored in T-LGLL. EZH2, through a MYC-dependent manner, suppresses transcription of IFNGR1 in prostate cancer cells, leading to a reduction in functional IFNGR1 protein (311). As with our results, this decrease in IFNGR1 levels lead to a reduction in JAK2 and STAT1 activation as well as IFN-γ downstream gene targets in response to IFN-γ (311). EZH2 binds directly to the promoter region of IFNGR1, blocking its transcription (311). On the contrary, the IFN-α receptor 1 (IFNAR1) gene was not inhibited by EZH2, showing a specific IFNGR1 inhibition (311). This matches our findings in TL-1 cells as IFNAR-mediated signaling

was intact (**Chapter 5**). In addition to EZH2, activating protein 2α (AP-2α) can bind to the IFNGR1 promoter, with its expression negatively correlated with IFNGR1 transcripts (312). Overexpression of AP-2α decreased IFN-γ signaling as evidenced by a marked reduction in p-STAT1, providing a plausible mechanism for IFN-γ unresponsiveness observed in cancer cells (312).

Other cytokines may be responsible for suppressing IFNGR1 levels. IFN-β, a type I interferon, reduces IFNGR1 mRNA transcripts in macrophages (317). At the IFNGR1 promoter, activated RNA polymerase II and acetylated histone H3 and H4 are reduced and Egr3 and Bab1 are recruited following IFN-β treatment (317). The abundance of IFN-β in T-LGLL has not previously been investigated. However, TL-1 cells are responsive to IFN-β stimulation (**Chapter 5**), which could inhibit the transcription of IFNGR1. Future studies should investigate the role of EZH2, AP-2α, and IFN-β in IFN-γ signaling in T-LGLL. We can address role of these proteins and cytokines in TL-1 cells through siRNA-mediated knockdown of EZH2, AP-2α, and IFN-β. Following reduction in each of these individual proteins, we would measure IFNGR1 levels on the mRNA and protein levels to determine whether these proteins play a suppressive role on IFNGR1 levels. If IFNGR1 levels increase following knockdowns, we could then stimulate with IFN-γ and measure p-JAK2, p-STAT1, and IFN-γ-inducible gene transcription to see if recovery of IFNGR1 rescues IFN-γ signaling.

6.3.3. Is the IFN-γ unresponsiveness unique to TL-1 cells?

We found that TL-1 cells were unresponsive to IFN-γ stimulation as demonstrated by lack of p-JAK2, p-STAT1, and STAT1-mediated gene induction

(**Chapter 5**). This is likely due to the reduction in IFNGR levels (**Chapter 5**). However, we were curious as to whether NKL cells, the cell line model of NK-LGLL, were responsive to IFN-γ or whether this unresponsiveness was common to LGLL in general. We have begun experiments to investigate this question and our preliminary data suggests that NKL cells are not responsive to IFN-γ (*K Olson, P Kulling, et al, unpublished data*), matching our observations in TL-1 cells. We plan to continue these experiments and compare our results to TL-1 cells.

Although TL-1 and NKL cells are the only defined cell line models for T-LGLL and NK-LGLL, respectively, there are two additional patient-derived cell line models for T-LGLL and NK-LGLL combinations. These cell lines are derived from a patient with atypical disease states and thus are not representative of pure T-LGLL or NK-LGLL. However, these cell lines serve as additional models to evaluate whether a mechanism is common in multiple rare forms of LGLL. The MOTN-1 cell line was derived from a patient with T-LGLL and has the unusual markers of CD4+, CD3-, CD8-, TCRαβ-, and TCRγδ- (309), which is unlike the vast majority of T-LGLL cells that are CD4-, CD3+, CD8+, and TCRαβ or TCRγδ+. Moreover, this cell line has characteristically NK cell-associated markers, creating a T/NK LGLL fusion cell line (318). MOTN-1 represents the chronic phase of disease while the PLT-2 cell line, derived from the same patient, represents progression to the terminal aggressive disease state (319). Thus, using both MOTN-1 and PLT-2 would allow us to compare the differences in IFN-γ signaling in indolent and aggressive disease states as these cell lines are known to have differential gene expression (320). The aggressive NK-LGLL cell line, KHYG-1, provides the ability to determine response in a malignancy displaying unusual NK cellular markers, including CD8+, and possessing a p53 point mutation (321). Another unique NK-LGLL-related cell

line, NK-YS, was derived from a patient with Epstein-Barr virus-associated nasal angiocentric NK cell lymphoma/leukemia (322). Further studies will investigate whether the IFN-γ unresponsiveness is unique to TL-1 and NKL cell lines or conserved across LGLL using these additional cell lines.

6.3.4. What other cytokines and STATs should be targeted in future therapies?

One goal of T-LGLL therapy development is to target constitutively activated pathways in T-LGLL. Our results demonstrate that TL-1 cells have decreased IFNGR1 and IFNGR2 levels. This suggests that using IFNGR neutralizing antibodies would not decrease STAT activation as these cells are not responsive to IFN-γ. Instead, STAT1, STAT3, and STAT5b should be targeted, in combination, to reduce inflammatory cytokine production, including IFN-γ, and pro-survival gene transcription. We found that STAT1 phosphorylation positively correlated with STAT3 phosphorylation while both STAT1 negatively correlated with STAT5 in TL-1 cells (**Chapter 5**). As all three STATs are implicated in T-LGLL development and progression, it is critical to target all three therapeutically to prevent compensation or upregulation of STATs in response to targeting solely one. Our experiments were 48 h following induction of temporary STAT knockdown so we were unable to assess the long-term effects of each individual knockdown in terms of survival, downstream gene targets, and cytokine production. However, based on our results, STAT1, STAT3, and STAT5b should be targeted in combination and explored further. This is particularly important as current T-LGLL focus on targeting STAT3 activation alone, which would not reduce STAT5b activation or transcription of IFN-γ. In addition, our findings show that STAT3 targeting alone or in combination with STAT1 through STAT3 siRNA or use of calcitriol, respectively, does not reduce cell viability within 48 h. Although longer time courses would be helpful in

identifying the downstream effects of STAT3 targeting, our results suggest that phosphorylation of the STATs implicated in LGLL are related and should be targeting together to avoid upregulation of another LGLL-promoting STAT. Therefore, it would be beneficial to explore combinatorial targeting of STAT1, STAT3, and STAT5b for use in the clinic. This can be accomplished by using both calcitriol, which targets STAT1 and STAT3, and a STAT5b inhibitor.

In addition to targeting the STATs, the production and response to other pro-inflammatory cytokines should be explored. Trans-signaling of IL-6 has previously been shown to play a role in T-LGLL cell activation. Neutralization of IL-6 receptor or IL-6 cytokine inhibited p-STAT3 levels and survival of T-LGLL patient PBMCs (57). IL-15 is another cytokine implicated in T-LGLL pathogenesis. IL-15Rα was found to be upregulated in T-LGLL patient leukemic and normal PBMCs (61). Moreover, when treated with exogenous IL-15, some T-LGLL patient cells proliferated more than normal donors, exhibiting a higher sensitivity to IL-15-mediated signaling (61). Given its role in T-LGLL, IL-15 receptor blocking antibodies have been developed and evaluated in preclinical and Phase I clinical trials (72, 73). Although toxicity was not observed, leukemic count was not affected by the antibody (72). This is hypothesized to be due to the inability of the antibody to inhibit *cis* presentation of IL-15 (73). As IL-15 and IL-2 signaling through a shared receptor chain, efforts are ongoing to target this signaling pathway. A clinical trial has been initiated for use of BNZ-1 as a therapeutic for LGLL. BNZ-1 is a peptide antagonist that interacts with the shared gamma chain receptor of IL-2 and IL-15, thereby preventing IL-2 and IL-15 signaling. *In vitro* work performed by another graduate student in the Loughran lab, Tiffany Wang, showed that BNZ-1 reduced viability of TL-1 cells through induction of apoptosis. The clinical trial enrollment

for this compound has begun and is anticipated to be completed by the end of 2018.

We are in the process of identifying additional cytokines that may play a role in T-LGLL pathogenesis. Our lab has previously conducted cytokine studies in T-LGLL patients (40). However, we are in the process of conducting a large study regarding cytokine involvement in both T-LGLL and NK-LGLL patients compared to healthy donors. This endeavor will answer two main questions: 1) What cytokines are differentially expressed in LGLL patients compared to normal donors? 2) Are the cytokine profiles between T-LGLL and NK-LGLL similar or different? Our findings could implicate the involvement of additional cytokines and cytokine-related pathways in LGLL disease. Through identification of important cytokine pathways, we can assess roles of transcription factors that promote or inhibit the signaling pathways, offering potential new therapeutic targets. Moreover, as T-LGLL and NK-LGLL patients are prescribed the same treatments, if different cytokines are upregulated or downregulated between the two disease forms, novel therapies may be developed that specifically target T-LGLL or NK-LGLL. This work is currently ongoing, with an anticipated completion of summer 2018 (*P Kulling/K Olson co-first, et al*).

6.3.5. What is causing the initial increase in IFN-γ production?

Overall, T-LGLL patients have significantly elevated IFN-γ levels compared to normal donors (40, 61). Therefore, we aimed to pharmacologically target this pathway and characterize the dysregulation of the IFN-γ-signaling pathway. Our results demonstrate that calcitriol effectively inhibits IFN-γ production through a VDR-dependent mechanism (**Chapter 4**). Moreover, TL-1 cells are unresponsive to IFN-γ stimulation likely due to the low levels of IFNGR1 and IFNGR2 (**Chapter 5**). However, the origin of

the initiation of IFN-γ production is unknown.

As discussed previously, there is ample support for the involvement of a retroviral agent in LGLL pathogenesis (26, 27, 30), which would provide a plausible explanation for the initiation of IFN-γ production. We are actively characterizing this viral component through multiple techniques. Building on previous data from Dr. Susan Nyland, I continued to screen LGLL patients for seroreactivity against peptides derived from human T cell leukemia/lymphoma virus (HTLV) and human immunodeficiency virus (HIV). In one of our studies, I found that 45% (65/144), 13% (18/144), and 42% (61/144) of LGLL patients exhibited high reactivity, low reactivity, or were not reactive, respectively, to an envelope region of HTLV, designated BA21 (**Figure 6.1**). LGLL patients also showed reactivity to a gag HIV peptide, designated GISP2, with 27% (39/144), 16% (23/144), and 57% (82/144) of LGLL patients categorized as having high reactivity, low reactivity, or were not reactive, respectively (**Figure 6.2**). Overall, LGLL patients had a significantly higher level of IgG to HIV and HTLV peptides compared to normal donors. Although some patients had elevated IgG for both HTLV and HIV, this was not found in all patients (**Table 6.1**), suggesting that seroreactivity was not due to a general IgG increase. Furthermore, in a previous study, I found that total IgG levels were not correlated with seroreactivity level (*unpublished data*). Taken together, roughly half of LGLL patients exhibit elevated seroreactivity to BA21 (58%) and GISP2 (43%) compared to normal donors, further supporting the role of a viral agent in LGLL pathogenesis.

Based on our seroreactivity data, we plan to select patient samples for future to potentially correlate seroreactivity to molecular characteristics, sex, age, and disease

states (**Figure 6.3**). One of our ongoing projects involves TCR sequencing 100 T-LGLL patient samples. We are in the process of selecting 50 patients with high seroreactivity and 50 non-reactive patient samples for TCR sequencing. We have also recently submitted 5 highly reactive patient samples, 5 non-reactive patient samples, and 5 normal donor samples for exosome analysis by our collaborators. Through this collaboration, proteomics will be utilized to see if there are differences in protein expression in patients with and without viral seroreactivity compared to normal donors. Through this process, we may identify altered proteins that are involved in viral infection or are viral proteins. This may provide insight into how viral exposure affects cellular processes and further indicate viral infection. We also plan to compare exome sequencing of T-LGLL patient with and without seroreactivity to BA21.

I have also analyzed seroreactivity of a separate cohort of nearly 100 additional patient samples for use in a project investigating the correlation between seroreactivity and clonal populations of B cells. To accomplish this, we utilized B cell receptor (BCR) sequencing of T-LGLL patient cells. Based on my seroreactivity data, I chose 24 T-LGLL patients, half of which were highly reactive to HTLV peptides, and submitted their cells for BCR sequencing. I also selected these patients based on total IgG and IgM levels as immunoglobulin disorders, including hypergammaglobulinemia, are found in T-LGLL. By sequencing the BCR, we can determine whether the B cell compartment is skewed, potentially suggesting a viral component. We are in the process of analyzing these data and plan to submit the manuscript by the end of 2018 (*T Olson, P Kulling, et al*).

Through characterization of the viral component of T-LGLL, we may be able to determine the initial cause of IFN-γ production. By knowing how the virus interacts with

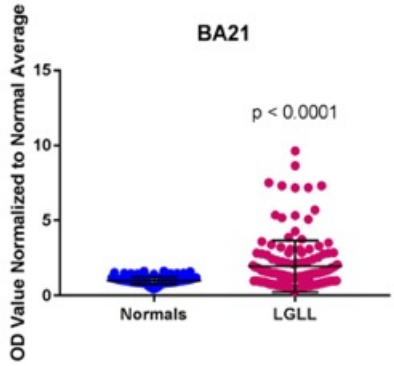
the cells, we could develop new therapies or treatments to control IFN-γ production and target the virus through inhibition of replication, for example.

6.4. Conclusions

In summary, my thesis has laid the groundwork to show that calcitriol effectively reduces inflammatory signaling in T-LGLL. This is accomplished through the independent reduction in p-STAT1 and IFN-γ, via a VDR-independent and VDR-dependent mechanism, respectively. As the majority of T-LGLL patient cells have high levels of VDR protein, calcitriol is a promising inhibitor of IFN-γ and should be investigated further for clinical use to combat the lack of negative regulation of the IFN-γ signaling pathway. The unresponsiveness of TL-1 cells likely stems from the low levels of IFNGR1 and IFNGR2, leading to the inability of IFN-γ to activate JAK2, STAT1, or induce transcription of IFN-γ gene targets. Given this dysfunctional pathway, therapies should target the excess production of IFN-γ, supporting further investigation into the use of calcitriol. Our results demonstrate the fundamental mechanisms for calcitriol-mediated effects, but even greater effects may be observed in human clinical trials as calcitriol exerts positive effects on normal PBMCs and overall health in other disease models. This thesis is the first study to evaluate calcitriol for use in T-LGLL. With its efficacy and minimal toxic effects in other clinical studies, calcitriol may offer an effective specific targeting approach for inflammation in T-LGLL and associated autoimmune diseases.

6.5. Figures & Tables

A.

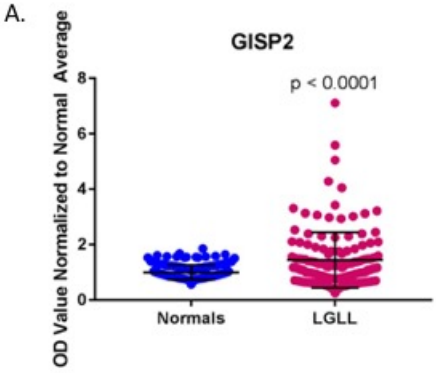


B.

Level of seroreactivity	# of patients	% of patients
High	65	45
Low	18	13
Non-reactive	61	42

Figure 6.1. T-LGLL patient seroreactivity to a peptide derived from HTLV, BA21. (**A**) T-LGLL patient serum (n=144) were compared to normal donor serum for IgG against an envelope region of HTLV using ELISA. Values are expressed in OD normalized to the values of normal donors to standardize across plates. Each dot represents a patient sample. A Student's T test was used to determine significance. (**B**) level of seroreactivity of patient samples compared to normal donors. The following values determined the level of reactivity in regards to standard deviation from average normal donor OD: Non-reactive samples were <1 standard deviation, low reactivity samples were between 1-2 standard deviations, and high seroreactivity samples were >2 standard deviations above the average OD of the normal.

A.



B.

Level of seroreactivity	# of patients	% of patients
High	39	27
Low	23	16
Non-reactive	82	57

Figure 6.2. T-LGLL patient seroreactivity to a peptide derived from HIV, GISP2. (**A**) T-LGLL patient serum (n=144) were compared to normal donor serum for IgG against an envelope region of HIV using ELISA. Values are expressed in OD normalized to the values of normal donors to standardize across plates. Each dot represents a patient sample. A Student's T test was used to determine significance. (**B**) Level of seroreactivity of patient samples compared to normal donors. The following values determined the level of reactivity in regards to standard deviation from average normal donor OD: Non-reactive samples were <1 standard deviation, low reactivity samples were between 1-2 standard deviations, and high seroreactivity samples were >2 standard deviations above the average OD of the normal.

A.

High Reactivity	% (out of BA21 High)
Both	58.5%

B.

High or Low Reactivity	% (out of BA21 High or Low)
Both	69.9%

Table 6.1. The majority of T-LGLL patient serum that is seroreactive to the HTLV peptide (BA21) are also seroreactive to HIV peptide (GISP2). Samples were selected based on (**A**) high seroreactivity or (**B**) high or low seroreactivity to BA21. Of those samples, dual reactivity, either (**A**) high or (**B**) high or low, to GISP2 was determined. Percentage of patients with specified seroreactivity to BA21.0 and GISP2 is shown to demonstrate any shared seroreactivity for each patient.

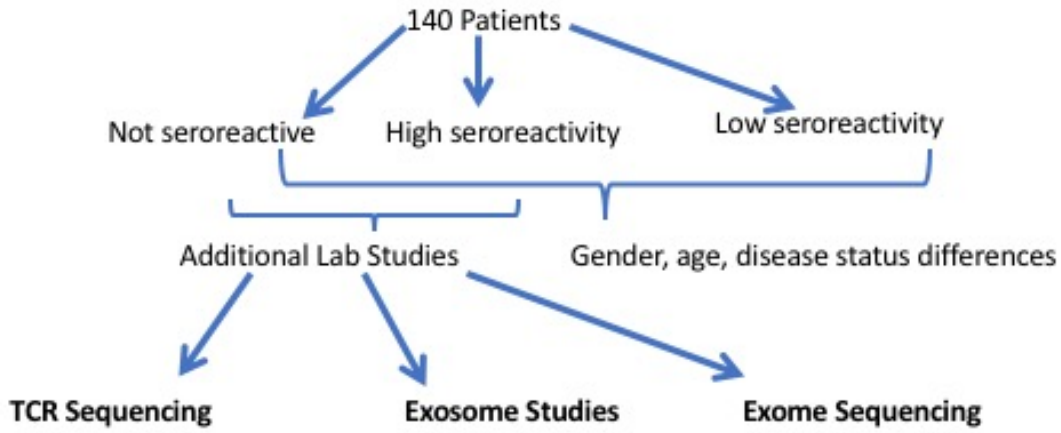


Figure 6.3. Schematic showing the ongoing experiments based on determined seroreactivity in the cohort of 140 T-LGLL. Serum from 140 patients were analyzed for seroreactivity to BA21 and GISP2. Based on their reactivity profile, samples were selected for future studies including TCR sequencing, exosome studies, and exome sequencing. The overall goal of these studies is to further characterize the viral agent in order to better understand LGLL pathogenesis.

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