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SUPPRESSION OF ANTI-TUMOR IMMUNITY IN CHRONIC LYMPHOCYTIC LEUKEMIA VIA INTERLEUKIN-10 PRODUCTION

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SUPPRESSION OF ANTI-TUMOR IMMUNITY IN CHRONIC LYMPHOCYTIC LEUKEMIA VIA INTERLEUKIN-10 PRODUCTION

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By

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2017

ABSTRACT OF DISSERTATION

SUPPRESSION OF ANTI-TUMOR IMMUNITY IN CHRONIC LYMPHOCYTIC LEUKEMIA VIA INTERLEUKIN-10 PRODUCTION

The most common human leukemia is B-cell chronic lymphocytic leukemia (B-CLL), which is characterized by a progressive accumulation of abnormal B-lymphocytes in blood, bone marrow and secondary lymphoid organs. Typically disease progression is slow, but as the number of leukemic cells increases, they interfere with the production of other important blood cells, causing the patients to be in an immunosuppressive state. To study the basis of this immunoregulation, we used cells from the transgenic Eμ-*TCL1* mouse, which spontaneously develop B-CLL due to a B-cell specific expression of the oncogene, TCL1. Previously we showed that Eμ-*TCL1* CLL cells constitutively produce an anti-inflammatory cytokine, IL-10. Here we studied the role of IL-10 in CLL cell survival *in vitro* and the development of CLL *in vivo.* We found that neutralization of IL-10 using anti-IL-10 antibodies or blocking the IL-10 receptor (IL-10R) using anti-IL-10R antibodies did not affect the survival of CLL cells *in vitro*. On the other hand, adoptively transferred Eμ-*TCL1* cells grew at a slower rate in IL-10R KO mice vs. wild type (WT) mice. There was a significant reduction in CLL cell engraftment in the spleen, bone marrow, peritoneal cavity and liver of the IL-10R KO compared to WT mice. Further studies revealed that IL-10 could be playing a role in the tumor microenvironment possibly by affecting anti-tumor immunity. This was seen by a reduction in the activation of $CD8^+$ T cells as well as a significantly lower production of IFN-γ by $CD4^+$ T cells purified from CLL-injected WT mice compared to those purified from CLL-injected IL-10R KO mice. Also CLL-primed IL-10R null T cells were more effective than those from similarly CLL-primed wild type mice in controlling CLL growth in immunodeficient recipient mice. These studies demonstrate that CLL cells suppress host anti-tumor immunity via IL-10 production. This led us to investigate possible mechanisms by which IL-10 is produced. We found a novel role of B-cell receptor (BCR) signaling pathway in constitutive IL-10 secretion. Inhibition of Src or Syk family kinases reduces the constitutive IL-10 production by Eμ-*TCL1* cells in a dose dependent manner. We identified the transcription factor Sp1 as a novel regulator of IL-10 production by CLL cells and that it is regulated by BCR signaling via the Syk/MAPK pathway.

KEYWORDS: Chronic Lymphocytic Leukemia, Interleukin-10, Anti-tumor Immunity, B Cell Receptor Signaling, Specific Protein 1

Sara Samir Alhakeem
Student's Signature

Date

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I dedicate this dissertation to my amazing and supporting parents

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CHAPTER 1

Introduction

B-cell malignancies represent a diverse collection of diseases, including non-Hodgkin's lymphomas (NHLs) and Hodgkin's lymphoma [1]. NHLs are a heterogeneous group of more than 30 cancers that include chronic lymphocytic leukemia (CLL), which mainly affects B lymphocytes [1]. In the United States, B cell lymphomas represent 80-85% of all NHL cases, with 15-20% being T-cell lymphomas [1]. Regarding these B-cell lymphomas, diffuse large B-cell lymphoma (DLBCL) has the highest incidence of 30%, followed by follicular lymphoma (20%) [1]. NHL and leukemia are the seventh and eleventh most common neoplasms, respectively, in the US, with CLL accounting for about one third of all leukemia cases [1, 2].

B cell Chronic lymphocytic leukemia is a clinically heterogeneous disease originating from B lymphocytes that may differ in activation or maturation state [3]. Although CLL is rare in some countries, such as Japan and Korea, it is the most common adult leukemia in the Western world [4]. CLL is a disease of the elderly but has also been seen in younger adults [2]. CLL cells originate from clonal expansion of mature B cells expressing the T-cell marker CD5 [3]. The disease is defined by abnormal lymphocytes produced in the bone marrow that have defects in apoptosis, or other cell death mechanisms, leading to the accumulation of small, mature-appearing neoplastic lymphocytes in the blood, bone marrow and secondary lymphoid tissues, resulting in lymphocytosis,

leukemia cell infiltration of the marrow, lymphadenopathy and splenomegaly [3, 4]. Leukemic cell accumulation occurs because of survival signals delivered to the cells from the external environment through a number of receptors (e.g., B-cell receptors and chemokine and cytokine receptors) and their cell-bound and soluble ligands. B cell receptor (BCR) signaling influences the behavior of chronic lymphocytic leukemia, where engagement of surface immunoglobulin by antigen has been shown to be a key driver of CLL cells with outcome influenced by the nature of the cell, the level of stimulation and the microenvironment [5]. CLL patients are subdivided into two groups, those with mutations in the immunoglobulin variable heavy chain (IGVH) and those without mutations in the IGVH [3]. CLL cells that express an unmutated IGHV (U-CLL) originate from a B cell that has not undergone somatic hypermutation in germinal centers [4]. CLL cells with mutated IGHV (M-CLL), arise from a post-germinal center B cell that expresses immunoglobulin, which has undergone somatic hypermutation and, in some cases, also immunoglobulin isotype switching [4]. Patients with CLL cells that express an unmutated IGHV typically have more-aggressive disease than patients with CLL cells that express a mutated IGHV [6]. Other characteristics such as expression of TCL1, ZAP70 and CD38 proteins are broadly associated with poor prognosis [6-9].

(1a) Epidemiology, diagnosis and clinical features of CLL

Chronic lymphocytic leukemia is more common in adults and more common among men than women, particularly Caucasian men (Figure 1.1) [10].

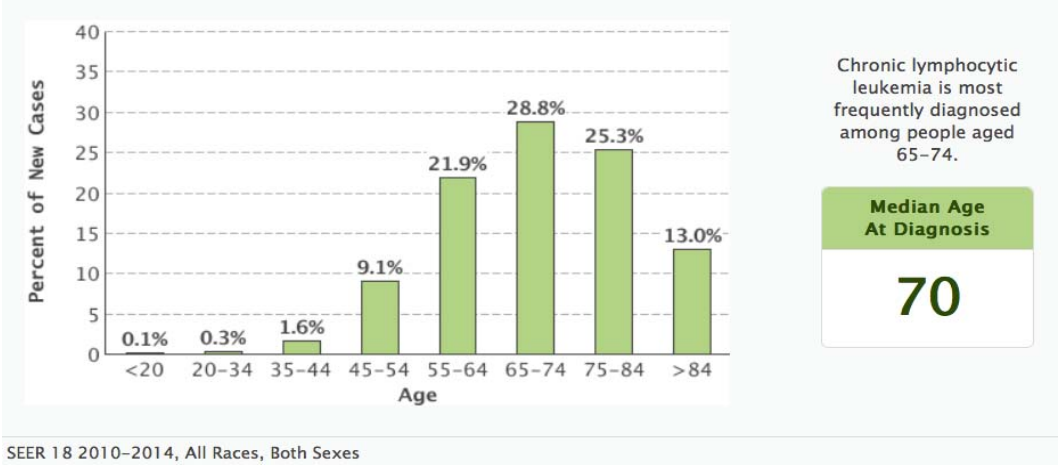


Figure 1.1: Percent of new cases of CLL by age group.

Adapted from SEER Cancer Stat Facts: Chronic Lymphocytic Leukemia. National Cancer Institute.

The number of new cases of CLL was 4.7 per 100,000 men and women per year based on 2010-2014 cases [10]. In 2017, it is estimated that there will be 20,110 new cases of chronic lymphocytic leukemia and an estimated 4,660 people will die of this disease (Figure 1.2) [10].

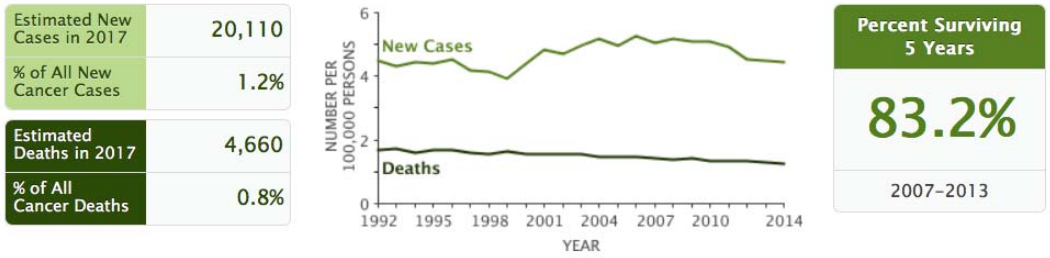


Figure 1.2: Epidemiology of chronic lymphocytic leukemia at a glance.

Adapted from SEER Cancer Stat Facts: Chronic Lymphocytic Leukemia. National Cancer Institute.

Diagnosing CLL is based on differential blood count, flow cytometry of the peripheral blood to determine the immunophenotype of circulating lymphocytes, and examination of the peripheral smear [11]. The National Cancer Institute guidelines require two criteria to be met for the diagnosis of CLL. 1) Absolute B lymphocyte count in the peripheral blood should be ≥5000/μl [5 x 10^9/L], with the appearance of a population of morphologically mature-appearing small lymphocytes [11]; 2) Demonstration of clonality of the circulating B cells by flow cytometry. The majority of the population should express the following pattern of B cell markers: low levels of surface immunoglobulins (CD20 and CD79b), and either kappa or lambda (but not both) light chains, expression of B cell associated antigens (CD19, CD20, and CD23) and expression of the T cell associated antigen CD5 [11]. In addition, although a bone marrow test is normally not required for CLL initial diagnosis or confirmation, it is commonly performed to establish a baseline to measure response to therapy [2, 11]. A baseline bone marrow test for CLL patients often establishes an increase in the number of B lymphocytes and a decrease in the number of normal marrow cells [2]. How the cells are clustred in the bone marrow can also classify them into one of four kinds of CLL cell patterns; nodular, interstitial, mixed or diffused [2].

The majority of CLL patients are asymptomatic at diagnosis and CLL is often found based on routine blood counts. Otherwise, patients may present with swollen lymph nodes, splenomegaly, fatigue, fever, weight loss and night sweats [2]. Many of the signs and symptoms of advanced CLL occur because the leukemic cells replace the normal blood cells. Therefore, patients can suffer from

anemia due to low red blood cell count, bleeding and bruising due to shortage of platelets and an increase in infections due to lower normal white blood count [2]. Moreover, systemic immunosuppression has been found to be associated with a more aggressive CLL disease and secondary cancers such as skin cancer, head and neck cancer, and lymphoblastic leukemia [12]. This immunosuppression increases susceptibility to infection, which is the leading cause of death in CLL patients [13-15]. Patients with CLL are at risk for infection for a variety of reasons. CLL patients have inherent immune defects in humoral, as well as cell-mediated immunity, which are related to the primary disease process. These defects include hypogammaglobulinaemia, abnormalities in T cell subsets and defects in complement activity and neutrophil/monocyte function [13, 16]. In addition, specific immunodeficiencies related to therapies administered to patients can result in additional immunosuppression [13].

(1b) Clinical staging and treatment of CLL

Two clinical staging systems are widely used for classifying CLL patients into three broad prognostic groups [4]. The Rai staging system is more commonly used in the United States, whereas the Binet classification is more commonly used in Europe [4]. The staging systems each recognize the importance of bone marrow function and define late-stage or high-risk disease by the presence of pronounced anemia or thrombocytopenia [4]. Table 1.1 and Table 1.2 summarize both classifications.

Table 1.1: CLL Rai staging system

Risk group	Clinical features	Median life expectancy*
Low risk (Rai stage 0/I)	Lymphocytosis without cytopenia, lymphadenopathy or splenomegaly	13 years
Intermediate risk (Rai stage II)	Lymphocytosis, lymphadenopathy and/or splenomegaly, but without cytopenia	8 years
High risk (Rai stage III/IV)	Lymphocytosis and cytopenia (a haemoglobin level of ≤11 g per dl and/or a platelet count of ≤100,000 cells per µl)	2 years

*These life-expectancy estimates are increasing with the advent of newer therapies.

Kipps, T.J., et al., *Chronic lymphocytic leukaemia.* Nat Rev Dis Primers, 2017. 3: p. 16096. (reporoduced with permission from Nature).

Table 1.2: CLL Binet staging system

Risk group	Clinical features	Median life expectancy*
Low risk (Binet stage A)	Less than three palpably enlarged sites‡ without cytopenia	13 years
Intermediate risk (Binet stage B)	Three or more palpably enlarged sites‡ without cytopenia	8 years
High risk (Binet stage C)	Cytopenia (a haemoglobin level of ≤10 g per dl and/or a platelet count of ≤100,000 cells per µl)	2 years

*These life-expectancy estimates are increasing with the advent of newer therapies. ‡There are five sites of lymphoid organs: cervical, axillary and inguinal nodes, the spleen and the liver.

Kipps, T.J., et al., *Chronic lymphocytic leukaemia.* Nat Rev Dis Primers, 2017. 3: p. 16096. (reporoduced with permission from Nature).

Patients with CLL can manage their disease with their physicians for years with observation, which is referred to as the wait-and-watch method [2]. The watch-and-wait approach is the standard care for patients who are considered low-risk with slow growing disease, minimal changes in their blood counts and no symptoms [2]. Since no treatment to date has made a significant impact on the outcome of patients with early-stage CLL, when to initiate therapy becomes an

important decision. Generally, indications to initiate therapy include pronounced disease related anemia or thrombocytopenia as well as symptoms that are associated with active disease, such as night sweats, fatigue, weight loss and fever with no apparent infection [4]. For patients in need of treatment, a number of factors play a role in the choice of treatment. Briefly, patients with del(17p) or mutated TP53 are treated with therapy that does not require functional TP53, such as ibrutinib (Bruton tyrosine kinase (BTK) inhibitor) [4]. For patients without these mutations, IGHV mutational status can help define the treatment strategy [4]. Patients with unmutated IGHV are often considered for therapy with ibrutinib as CLL cells with unmutated IGHV seem to be more sensitive to inhibitors of BCR signaling than CLL cells with mutated IGHV [4]. Patients with mutated IGVH are good candidates for chemoimmunotherapy as they have excellent outcomes with this regimen, such as fludarabine, cyclophosphamide and rituximab (anti-CD20 antibody), with >50% of patients having a median progression-free survival of >10 years [4]. Patients who develop de novo del(17p) or TP53 mutations or who develop resistance or intolerance to ibrutinib are often considered for therapy with idelalisib (phosphoinositide 3-kinase (PI3K) inhibitor) and rituximab or the B-cell lymphoma 2 (BCL-2) inhibitor venetoclax [4]. Patients who develop resistance or intolerance to inhibitors of BTK, PI3K and/or BCL-2 are considered for clinical trials or alternative agents [4].

(1c) Cellular origin of CLL

A number of cell types have been suggested to give rise to CLL, with no consensus as to the normal counterpart of CLL cells [17]. The debate

surrounding the cellular origin of CLL cells mainly arise from the heterogeneity found in the disease. For example, the use of both unmutated and mutated IGVH genes, which as mentioned earlier distinguishes CLL patient subgroups, gave rise to a 2-cell origin model in which the 2 subgroups of CLL originated from distinct cell types [17]. In support of this theory, B cell receptor signaling has been found to be a promoting factor that could lead to differing cell biology and patient pathology.[17] For instance, since the development of IGVH gene mutations requires BCR crosslinking, then CLL cases that exhibit IGVH gene mutations must have ascended from previously stimulated B cells. Therefore, the CLL cells without IGVH gene mutations would have possibly originated from naïve B cells [5]. But the fact that absence of IGVH gene mutations does not necessary mean the lack of prior antigen stimulation, the cases of U-CLL could simply be derived from antigen-stimulated B cells that didn't accumulate mutations [5]. Chiorazzi *et al*, hypothesize that the lack of mutations in those cases could either be a consequence of the type of antigenic stimulation that the cell received (e.g., T-independent) or a result of the timing of the transformation event (e.g., occurred before a germinal center (GC) founder cell entered a GC) [5].

On an opposing note, other studies utilizing gene expression analysis revealed only a small number of differences between genes expressed in U-CLL and M-CLL, which suggested a one-cell originating model for CLL [18, 19]. In that case, the difference in cellular features and clinical outcomes between U-CLL and M-CLL could be accounted for possibly by additional promoting factors. To

bring together the 2-cell origin model, which is more consistent with BCR findings, with the one-cell model, supported by gene expression data, a new theory was proposed in which both U-CLL and M-CLL derive from marginal zone (MZ) B cell [3, 5]. This unifying theory arose due the similarity between MZ B cells and CLL cells. MZ B cells ($IgM^{high}IgD^{low}$) respond to bacterial polysaccharides in a T cell-independent manner [20]. They can express either mutated or unmutated IGVHs [21]. Also, evidence suggest that MZ B cells may accumulate IGVH gene mutations outside of classical GC, possibly in the MZ itself, which would provide an explanation to a continuous accumulation of such mutations in some CLL cases [5, 17, 22]. However, despite the similarities found between MZ B cells and CLL cells, an MZ origin for CLL still faces some reservations. MZ B cells express surface IgM and IgD as most CLL clones do, however; they are $CD5^{-}CD23^{-}CD22^{+}$, which is a surface phenotype different than that of a CLL cell [20]. It has been shown that up-regulation of CD5 and CD23 could occur upon activation of cells, which could reflect this phenotypic difference [23, 24]. Since comparisons of surface phenotypes of malignant and normal B cells are often used to identify normal counterparts in hematological malignancies, challenges remain in considering MZ B cells as the normal counterpart of CLL.

Another possible cellular origin of CLL is a unique subset of B cells called B-1 cells. B-1 cells unlike MZ B cells express the T cell marker CD5 [25-27]. B-1 cells express IgM, low levels of CD23 and unmutated IGVH genes [28, 29]. Another unique feature of B-1 cells is the secretion of polyreactive and natural

antibodies [28, 29]. Murine B-1 cells are predominantly found in the peritoneal and pleural cavities and only constitute 1-2% of splenic B cells [29, 30]. Unlike conventional B-2 cells, which are produced continuously in the bone marrow, mouse B-1 cells are generated only from hematopoietic stem cells in the fetal liver or in the bone marrow the first few weeks after birth and are subsequently maintained by self-renewal in the periphery [28, 30]. Recently, a specific B-1 cell restricted progenitor ($Lin^-CD45R^{lo/-}CD19^+$ cells) in the bone marrow has been identified, which preferentially reconstituted B-1 cells but not B-2 cells, *in vivo* [31, 32]. Like murine B-1 cells, the antigen specificities of CLL BCRs are more polyreactive, which allows the cells to bind autoantigens and therefore secrete natural autoantibodies [33-35]. Since developing B cells are naturally autoreactive until antigen-driven clonal selection and somatic IGVH mutations during GC reactions [36], U-CLL cases have the greater level of polyreactivity and autoreactivity than M-CLL [37]. However, if M-CLL gene rearrangements are reverted back to the germline sequence, antibodies produced by the cells can display polyreactivity [17, 37, 38]. In support of B-1 cell as the cellular origin of CLL Hayakawa *et al.* was able to demonstrate that early generated B-1 B cells with distinct BCRs can become CLL in aging mice [39]. They identified an unmutated BCR in mouse that is autoreactive with non-muscle myosin IIA (AMyIIA) [39]. B cells with this AMyIIA BCR are generated by BCR signaling during B-1 fetal and neonatal development [39]. These early generated B-1 cells can self-renew, increase during aging and can progress to aggressive CLL in aged mice [39]. Interestingly, BCRs autoreactive to AMyIIA are also commonly

seen in some human CLLs [39]. In a follow up study, they showed that B-1 B cells with other stereotyped BCRs commonly found in mouse CLL can generate CLL [40]. More importantly, the progression to CLL by B-1 B cells is not only a result of their ability to express specific BCRs because CLL did not develop from other B cell subsets even with BCRs identical to the ones on B-1 cells, suggesting that both specific BCRs and B-1 cell environment were important for CLL progression [40].

Regardless of the comparable nature between B-1 and CLL cells, human B cells with characteristics of murine B-1 cells have not been identified. Although human $CD5^+$ B cells exist in the circulation, the majority of these cells do not exhibit features expected of B-1 cells [5, 41]. For example, IgM antibodies produced by $CD5^+$ cells in adult human blood are usually not polyreactive, although coded by unmutated IGHVs [42]. In addition, human $CD5^+$ B cells do not proliferate when stimulated with T-cell independent antigens [17, 43]. However, in a recent study Griffin *et al.* described a $CD20^+CD27^+CD43^+CD70^-$ subset present in adult and human cord blood with functional characteristics that would describe murine B-1 cells [44]. These cells spontaneously secrete IgM, maintain constitutive BCR signaling and are able to drive allogeneic T cell proliferation [44]. Further studies need to be performed to completely understand this cell subset and if it can be identified as the cellular origin of CLL in human patients.

(1d) Immunosuppression and other risks in CLL

Patients with CLL have an increased risk of other medical conditions such as infections, autoimmune diseases, or secondary cancers. Infections in CLL patients have been recognized as a common cause of morbidity and mortality [13-15]. The risk of infection increases with the increase of CLL duration. In addition, the new encouraging therapeutics in CLL often come at the cost of serious opportunistic infections [15]. In early, untreated CLL, patients infection risk is mainly related to hypogammaglobulinemia [15]. Infections by encapsulated bacteria are also common in such a setting [15]. On the other hand, patients with advanced CLL will mostly suffer from neutropenia and defects in cell-mediated immunity. A large variety of pathogens, including *Listeria monocytogenes*, mycobacteria, opportunistic fungi, *Pneumocystis carinii* and herpesviruses are seen in advanced CLL patients [15]. Pathogens seen frequently in CLL patients undergoing different therapeutic regimens are listed in Table 1.3 [15]. In addition, Table 1.4 describes some of the predominant immune defects found in different stages of CLL [15].

Table 1.3: Frequent pathogens seen in chronic lymphocytic leukemia patients undergoing different treatment regimens

		Purine analogues		
Pathogen	**Alkylating agents/sic roids**	**Fludarabine**	**Cladribinc**	**Pcntostatin**
Bacieria	*S pneumoniae*	L *monocytogenesf*	*L monocytogenes*	*S pneumoniae*
	S aureus		*Slaphylococcus* spp	*Pseudomonas* spp
	P aerug'mosa		*Streptococcus* spp	
	H influenzae		*E coli*	
	Legionella spp		*Klebsiella* spp	
	Salmonella spp		*Enterobacter* spp	
			Acinetobacier spp	
			N meningilidis	
			C difficile	
Fungi	C *neoformans*	*Candida* spp	*Candida* spp	*Candida* spp
	H capsulatum	*Aspergiltus* spp	*Aspergitlus* spp	*Aspergillus* spp
	Candida spp			
	Aspergitlus spp			
Viruses	**HSV**	vzv	**HSV**	HSV
	Adenovirus	CMV	**VZV**	VZV
			CMV	
Other		PCP[†]†	PCP	PCP
		Mycobacteria		

[*] CMV = cytomegalovirus; HSV = herpes simplex virus; PCP = *P carinii* pneumonia; VZV = varicella zoster virus.
[†] Especially with coilicosteroids.

Tsiodras, S., Samonis, G., Keating, M.J. & Kontoyiannis, D.P. Infection and immunity in chronic lymphocytic leukemia. Mayo Clin Proc 75, 1039-1054 (2000). (Reproduced with permission from Mayo Clinic Proc.).

Table 1.4: Immune defects found in different stages of chronic lymphocytic leukemia

Immune defect	Early-stage untreated CLL	Late stage CLL	
		Treated using cytotoxic agents	Treated using purine analogues ††
Hypogammaglobulinemia	IgG class and subclasses (IgG3, IgG4); low mucosal IgM/IgA; low serum IgA; qualitative loss of immunoglobulin function	More severe than in early-stage CLL	More severe than in early-stage CLL
Neutropenia and phagocytic-celi defects	—	Neutropenia; lysozyme and myeloperoxidase deficiencies; monocytopenia; defects in granulocyte chemotaxis and chemiluminescence	Often more severe than in late-stage CLL with cytotoxic agent therapy
Cell-mediated immunity	Functional T-cell defects; NK defects; LAK defects; CD4/CD8 inversion	Same as early-stage CLL but secondary effects of therapy pronounced; effect of cortico steroids on lymphocytes and on monocyte-macrophage axis	Same as early-stage CLL but secondary effects of therapy pronounced; inhibition of STATI, differential effect apoptosis between B and T cells, rapid reduction of CD4 cell count by fludarabine; effects on CD4/CD8 lymphocytes and on monocytes by cladribine; inhibition of ADA and effects on lymphocytes, monocytes, macrophages, and NK cells by pentostatin
Low complement	Primary vs secondary; low Cl andC4;Cl esterase inhibitor deficiency; autoimmune features	Same as early-stage CLL	Same as early-stage CLL

* ADA = adenosine deaminase; CLL = chronic lymphocytic leukemia; LAK = lymphokine-activated killer.; NK = natural killer.
† Fludarabine and **pentostatin.**

Tsiodras, S., Samonis, G., Keating, M.J. & Kontoyiannis, D.P. Infection and immunity in chronic lymphocytic leukemia. Mayo Clin Proc 75, 1039-1054 (2000). (Reprodcued with permission from Mayo Clinic Proc.).

Another risk factor of CLL is the development of Richter syndrome. Richter syndrome is the transformation of CLL to an aggressive lymphoma, commonly DLBCL or classic Hodgkin lymphoma [4]. Approximately 2-7% of patients with CLL develop Richter syndrome more frequently seen in patients with NOTCH1 mutations or patients who express certain stereotypical immunoglobulin molecules [4]. The prognosis of patients with Richter syndrome is generally poor, especially for those who are heavily pretreated for CLL and/or who have transformation involving lymphocytes that are clonally related to the underlying CLL [4].

As seen in table 1.4, there is increasing body of evidence suggesting impaired cell-mediated immunity in CLL. These include decreases in T helper activity, increases in T suppressor activity, reversal of CD4/CD8 ratio, increased expression of interleukin-2 (IL-2) receptors, and defects in large granular lymphocytes or natural killer cells [15, 45-48]. Analysis of T cell repertoire of CLL patients have shown that oligoclonality is much more common in CD4 and CD8 T cells in CLL patients than in age matched controls [49]. It is unclear however; if this T cell dysfunction can be reversible in CLL patients. Therefore, it is important to understand the molecular mechanisms leading to this immune dysfunction. To examine the possible mechanisms of T cell defects in CLL patients, Görgün *et al.* performed a global gene expression analysis of highly purified CD4+ and CD8+ T cells from peripheral blood of individuals with CLL compared with age-matched healthy donors [50]. Their analysis of the differentially expressed genes demonstrated a number of abnormalities in specific pathways. For example, in

CD4 cells, Ras-dependent JNK and p38 MAPK pathways were markedly changed [50]. These pathways play a major role in the regulation of CD4 T cell differentiation into T helper (Th) 1 and Th2 cells. The data demonstrated a decrease in the p38 MAPK pathway activator genes, which subsequently can impair CD4 differentiation function [50]. Gene expression analysis of CD8 cells revealed changes in expression of genes responsible for cytoskeleton formation and vesicle trafficking, which leads to decrease in cytotoxicity and effector functions of CD8 cells [50]. Moreover, p38 MAPK pathway was also altered in CD8+ T cells, which regulates the production of tumor necrosis factor alpha (TNF-α), perforin and granzyme [50]. Taken together, these changes in gene expression profile of T cells between CLL and normal donors is likely to contribute to the failure of T cell responses against tumor cells in CLL. A different study demonstrated that CD8 and CD4 T cells from CLL patients have an impaired ability to form an immunological synapse with antigen presenting cells because of defects in actin polymerization [51]. The formation of immune synapse in CD4 T cells allows the directed secretion of IL-2 and other cytokines toward the immune synapse and ultimately T cell proliferation [51]. CD8 cytotoxic cells form immune synapses that could deliver lethal doses of cytolytic granules [51]. Interestingly, a short-term cell contact between CLL cells and healthy allogeneic T cells induced the same immunological synapse defects [51]. In a follow up study Gribben and colleague found that T cell exhaustion could contribute to the T cell dysfunction [52]. T cells from CLL patients had an increased expression of exhaustion markers including CD244, CD160, and

programmed death-1 (PD1) in comparison to healthy donors [52]. In addition, $CD8^+$ cells showed functional defects in proliferation and cytotoxicity caused by impaired granzyme packaging and nonpolarized degranulation [52].

Furthermore, CLL cells themselves have been reported to produce immunosuppressive factors such as transforming growth factor beta (TGF-β) and IL-10 [53, 54]. Very few studies have demonstrated the mechanism by which IL-10 could be playing an immunosuppressive role that leads to CLL disease progression. For example, activated CLL cells were shown to inhibit macrophage TNF production in co-culture assays in an IL-10-dependent manner, but its importance for CLL disease was not tested [55]. In this dissertation we will be investigating multiple hypotheses regarding the role of IL-10 in CLL growth and disease immunosuppression.

(1e) Interleukin-10 producing B cells and CLL

In addition to their roles as antibody-producing or antigen-presenting cells, B lymphocytes are major producers of cytokines such as interleukin-10 (IL-10), IL-6, lymphotoxin alpha (LT-α) and TNF-α [56-58]. They have also been reported to produce other cytokines such as IL-2, IL-4, interferon gamma (IFN-γ) and IL-12 [59-61]. The notion that B cells can markedly influence immunity through the secretion of cytokines gained momentum when it was observed that B cells could differentiate into distinct cytokine-producing subsets termed Be1 and Be2, which could subsequently regulate the differentiation of naïve $CD4^+$ T cells into Th1 cells or Th2 cells, respectively, *in vitro* [62]. In addition, endogenous B cells were found to control immunity through the production of cytokines *in vivo* in two

models of inflammatory diseases, ulcerative colitis (UC) and experimental autoimmune encephalomyelitis (EAE) [63, 64]. In both cases, B cells limited pathogenic immunity, and improved the disease progression through the production of IL-10, a cytokine classically thought to be produced by regulatory T cells and some other innate immune cells [63, 64]. Moreover, it has been shown that B cells can have similar suppressive functions in humans leading to the notion of regulatory B cells (B_{regs}). For example, B cells from patients with relapsing-remitting multiple sclerosis (MS) produce less IL-10 than B cells from healthy patients upon stimulation *in vitro*, which suggests that a defect in this suppressive mechanism can induce onset or progression of MS [65]. In other studies, B cell depletion therapy resulted in exacerbation of UC in some patients who were treated with rituximab (anti-CD20 antibody), wherein the elimination of B cells correlated with a loss of IL-10 expression in the intestinal mucosa [66].

Regulatory B cells that produce IL-10 are now recognized as an important part of the immune system. A subset of B cells producing IL-10 has been recently identified and named B10 cells [67]. There are no unique phenotypic markers for B10 cells as they share surface markers with previously defined B cells including B-1 B cells [67]. They are $CD5^{+}CD19^{hi}CD1d^{hi}$ cells and are defined primarily by their competency to produce IL-10 following appropriate stimulation [67]. B10 cells have been shown to exert a variety of IL-10-dependent regulatory effects that are involved in autoimmune disease. Those effects are mediated by multiple mechanisms affecting both the innate and adaptive immunity. For example, B10 cells have been described to negatively regulate the ability of dendritic cells

(DCs) to present Antigens [68]. Also, B10 cells suppresse IFN-γ and TNF-α responses *in vitro* and IFN-γ responses *in vivo* by $CD4^+$ T cells [68, 69]. The important regulatory effects of B10 cells have been demonstrated in a variety of mouse models of human autoimmune diseases including but not limited to, experimental autoimmune encephalomyelitis, inflammatory bowel disease, collagen-induced arthritis and systemic lupus erythematosus [68, 70-72]. Therefore, it was proposed that we can either improve autoimmune diseases by promoting the IL-10 mediated regulatory functions of B cells or we can improve antimicrobial or even antitumor immunity by turning down these inhibitory processes.

As discussed earlier, B-1 cells are thought to be a possible cellular origin of CLL cells. One of the interesting features of B-1 cells is their ability to produce IL-10 constitutively, which is unlike B10 cells [56, 73]. The production of IL-10 by B-1 cells has been shown to act in an autocrine manner and inhibit proliferation responses of B-1 cells to toll like receptor (TLR) stimulation by blocking degradation of IκBα and translocation of RelA to the nucleus [73]. Interestingly, CLL cells have been shown to share this characteristic with B-1 cells in constitutively producing IL-10 [54]. However, not much is known about the role of IL-10 produced by CLL cells. In this dissertation, we will test a few hypotheses in regard to the role of CLL-induced IL-10.

(1f) Importance of IL-10 in the immune system

IL-10 was first described as cytokine synthesis inhibitory factor (CSIF) because it was produced by Th2 cells that inhibited Th1 cell activation and

cytokine production [74]. Later on IL-10 was shown to exert its anti-inflammatory effects by inhibiting antigen presentation by DCs and limiting their secretion of proinflammatory cytokines, such as IL-1, IFN-γ and TNF-α [75, 76].

IL-10 binds as a homodimer to its receptor, which is a tetramer formed of two α (IL-10R1) and two β (IL-10R2) chains [77]. IL-10R1 is the ligand binding subunit, while IL-10R2 activates downstream signaling involving Janus tyrosine kinases Jak1 and Tyk2 [77]. JAK1 and Tyk2 phosphorylate the cytoplasmic tail of IL-10R1, which leads to the recruitment of the signal transducer and activator of transcription 3 (STAT3) to the IL-10R [78]. Ultimately, phosphorylated STAT3 homodimerizes and translocates to the nucleus to promote the expression of IL-10 responsive genes [78].

IL-10 is now known to be produced by multiple types of cells including macrophages, DCs, B cells, and various subsets of $CD4^+$ and $CD8^+$ T cells [79]. In addition to its role in inhibiting the production of proinflammatory cytokines, IL-10 is involved in inhibiting the production of various chemokines involved in inflammation [80]. Furthermore, IL-10 regulates T cell responses indirectly through its effects on macrophages and monocytes, inhibiting their MHC class II and costimulatory molecule B7-1/B7-2 expression and limiting their production of proinflammatory cytokines and chemokines [79]. IL-10 can also act directly on T cells, inhibiting proliferation and production of IL-2, IFN-γ, IL-4, IL-5 and TNF-α [81, 82]. Activation of T cells in the presence of IL-10 can induce anergy in T cells, which cannot be reversed by IL-2 or stimulation by anti-CD3 and anti-CD28 [83]. Therefore, IL-10 appears to play an essential role in the regulation of

different immune responses. Here we show that IL-10 is also produced by chronic lymphocytic leukemia cells. The question here remains as to the role of IL-10 in in this leukemic disease sitting.

(1g) Molecular mechanisms involved in IL-10 production

As noted earlier, IL-10 is expressed by various cell types, which account for the complexity of its regulation. Many cells of the innate and adaptive immune response produce IL-10 and the molecular mechanisms for its regulation differ according to the cell type and stimulant, although some common mechanisms exist [84]. IL-10 is expressed by macrophages and myeloid DCs, but not by plasmacytoid DCs, in response to microbial antigens. The extracellular signal regulated kinase 1/2 (ERK1/2) and p38 MAPkinase pathways are two of the main signaling pathways activated in these cells resulting in IL-10 expression [84].

In macrophages and DCs, the expression of IL-10 can be induced by TLR or non-TLR signaling [84]. Activation of TLRs and their adaptor molecules, myeloid differentiation primary-response protein 88 (MYD88) and TIR-domain-containing adaptor protein inducing IFNβ (TRIF), leads to the activation of ERK1 and ERK2, p38 and nuclear factor-κB (NF-κB) pathways, which in turn result in induction of IL-10 expression [84]. In myeloid DCs, non-TLR can signal through DC-specific ICAM3-grabbing non-integrin (DC-SIGN) and RAF1, which leads to increase in TLR2-induced IL-10 production [84]. Moreover, activation of dectin 1, which signals through spleen tyrosine kinase (Syk) and ERK results in IL-10 production [84]. In macrophages, nucleotide-binding oligomerization domain 2 (NOD2) signaling downstream of TLR2 has been shown to induce IL-10

production [84]. Macrophages from neonatal and aged mice produce more IL-10 than those from young adults [85].

In T helper cells, molecular mechanisms of IL-10 production have been studied to a lesser extent than in macrophages and DCs. Normally, IL-10 production is accompanied by the expression of the another signature cytokine for each subset of Th cells. For example, IL-10 production was first described in Th2 cells, where its expression is accompanied by the expression of IL-4, IL-5 and IL-13 [74]. Indeed IL-10 production in Th2 cells seem to be regulated by the same signaling pathways and transcription factors involved in the main Th2 type cytokine secretion, which include IL-4, STAT6 and GATA3 [84, 86, 87]. In Th1 cells, the expression of IL-10 can be induced only under certain conditions [84, 88]. Strong T cell receptor (TCR) stimulation and endogenous IL-12 have been shown to be required for the differentiation of IL-10-producing Th1 cells [89]. Moreover, IL-10 expression by Th1 cells is dependent on STAT4 and ERK activity [89]. Another T helper cell subset that has been shown to be induce to produce IL-10 is the Th17 subset and thus attenuate their pro-inflammatory activity [90]. IL-10 expression by Th17 cells appears to occur in a STAT3 and STAT1 dependent manner [91]. Furthermore, TGF-β have been shown to induce the production of IL-10 by forkhead box P3 $(FOXP3)^+$ T regulatory (TReg) cells *in vivo* and this cytokine can also promote the development of IL-10 producing $FOXP3^-$ T cells from naïve T cells [92].

Taken together, Th1, Th2 and Th17 cells require the same signals needed for their differentiation to produce IL-10. Interestingly, the production of IL-10 by

these subsets requires ERK activation, which indicates a common mechanism for IL-10 production by T helper cells. But unlike macrophages and DCs, p38 signaling pathway is not required for the production of IL-10 by T helper cells [89]. In addition, it appears that all T cell subsets can produce IL-10; however, this depends on the environmental context and the strength of stimulus. For other immune cells such as B cells and mast cells, the exact signaling cascades leading to IL-10 production remain elusive. A summary of IL-10 expression by different immune cells in provided in figure 1.3 [84].

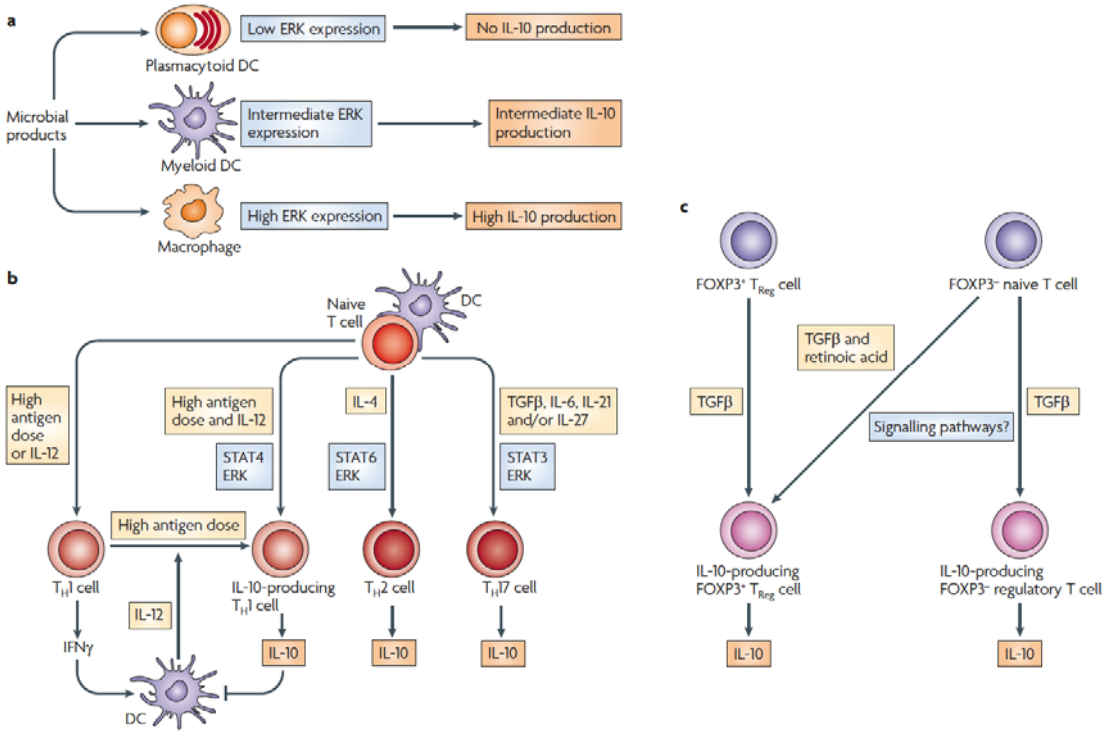


Figure 1.3: Interleukin-10 production by immune cells

Saraiva, M. & O'Garra, A. The regulation of IL-10 production by immune cells. *Nat Rev Immunol* **10**, 170-181 (2010). (Reproduced with permission from Nature).

(1h) Transcription factors that regulate IL-10 expression

There are many transcription factors that have been described to regulate the expression of *IL10* in APCs and T helper cells. Figure 1.4 represents both the signaling molecules (outer circle) and the transcription factors (inner circle) with a validated role in *IL10* expression [84]. As relevant to the work presented in this dissertation, the transcription factors specific protein 1 (Sp1), Sp3, CCAAT/enhancer binding protein-β (C/EBPβ), IFN-regulatory factor 1 (IRF1) and STAT3 all have been shown to bind and transactivate *IL10* in macrophage and T cell lines of mouse and human origin [84]. In addition, in human T cell lymphoma cell lines, the NF-κB p50 subunit has been describe to bind the *IL10* promoter [93]. More importantly, many of these findings are dependent on the cell type and the stimulus. For instance, when THP1 cells (a human pro-monocytic cell line) were stimulated with LPS, *IL10* promoter activity was dependent on an Sp1 site located between positions -636 and -631 relative to the initiation site [94]. On the other hand, stimulating the same cells with cyclic AMP shows that *IL10* promoter activity relies on the C/EBP5 motif located between the TATA box and the translation start point [95]. Overall, vast number of studies provided evidence that IL-10 is not regulated in a simple manner, which is in agreement with its functions in regulating various immune responses.

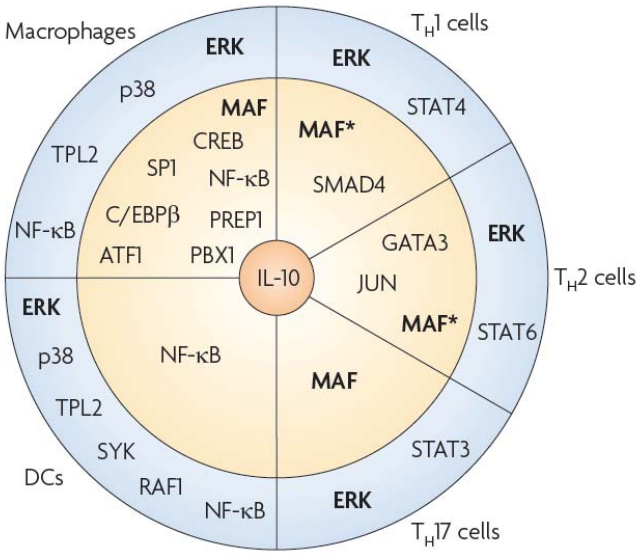


Figure 1.4: Transcription factors involved in IL-10 production

Saraiva, M. & O'Garra, A. The regulation of IL-10 production by immune cells. *Nat Rev Immunol* **10**, 170-181 (2010). (Reproduced with permission from Nature).

(1i) Mechanisms of IL-10 production by normal and malignant B cells

As alluded to earlier, in addition to macrophages, DCs and $CD4^+$ T cells, other cells of the immune system are also known to express IL-10. Both normal and malignant B cells are amongst these cells [54, 55, 73]. It is however not clear whether the molecular mechanisms required for the induction of IL-10 by these cells are regulated by the same or similar factors that regulate IL-10 production by T helper cells, macrophages and DCs. Therefore, the mechanisms involved in IL-10 production by normal B cells and CLL cells remain understudied. A recent report indicated the importance of B-cell-activating factor of the tumor necrosis factor family (BAFF) and its receptor, transmembrane activator and cyclophilin ligand interactor (TACI), for IL-10 production by normal and leukemic B cells [96].

Stimulation of both human and mouse CLL cells with BAFF led to an increase in IL-10 production [96]. In addition, splenic cells from TACI-deficient mice were unable to secrete IL-10 following TLR stimulation [96]. Another study attempted to define the molecular mechanisms involved in the variation in levels of IL-10 produced by human CLL cells. Their data suggested an epigenetic control of IL-10 production, in which differential IL-10 gene methylation was responsible for the variability of IL-10 production by human CLL cells [97]. In addition, they found that IL-10 induction by CpG stimulation requires STAT3 activity [97].

Due to the importance of tonic B cell receptor signaling in the survival of normal B cells, as well as its impact on the survival and growth of malignant B-cell clones in CLL and in other non-Hodgkins lymphomas, here we investigated the role of BCR signaling in IL-10 production by CLL cells [98-101]. We discovered a novel role of BCR signaling in IL-10 production by CLL cells [54]. Our studies described in the thesis showed that BCR dependent constitutive activation of Src or Syk family kinase is required for constitutive IL-10 production by both mouse and human CLL cells. The studies to understand the molecular pathways leading to IL-10 production CLL cells by BCR signaling could provide valuable information on possible targets for IL-10 manipulation and the modulating of the immune response in CLL.

(1j) Chronic lymphocytic leukemia study models

Expression of T-cell leukemia oncogene 1 (*TCL1*) has been described as a molecular marker of aggressive disease and poor outcome in patients with CLL [9]. In 1994, Giandomenico Russo and colleagues were studying the *TCL1* locus

on human chromosome 14q32.1 that was found to be commonly involved in chromosomal translocations and inversions with one of the TCR loci in human T cell leukemias and lymphomas [102]. They eventually discovered a gene coding for a 1.3 kb transcript, which is expressed only in a restricted subset of cells within the lymphoid lineage while expressed at high levels in leukemic cells with specific translocations or inversions within the chromosome regions 14q11 and 14q32 [102]. This gene is known as the *TCL1* gene, which is preferentially expressed early in T and B cell differentiation [102]. TCL1 has been described as a novel Akt kinase coactivator, which facilitates the oligomerization and activation of Akt *in vivo*, promoting Akt-dependent cell survival [103]. To study the role of *TCL1* in the generation of T cell malignant transformation, a transgenic mouse model that expresses the human *TCL1* gene under the transcriptional control of the T cell specific gene promoter $p65^{lck}$ was generated [104]. The *lck-TCL1* transgenic mice developed mature T cell leukemias at old age (15-20 months), while younger mice presented with pre-leukemic T cell expansions expressing *TCL1* [104]. After the demonstration that *TCL1* overexpression causes mature T cell proliferation in transgenic mice, many studies revealed strong expression of the gene in almost all tumor cells of B cell lineage, which indicated a possible role for *TCL1* in B cell proliferation [105]. For example, *TCL1* was found to be expressed in lymphoblastic lymphoma, chronic lymphocytic leukemia, mantle cell lymphoma, follicular lymphoma, Burkitt's lymphoma, diffuse large B cell lymphoma, and primary cutaneous B cell lymphoma [106]. Following these studies, a new mouse model

with *TCL1* expressed at similar levels in both B and T cells was generated [107]. These transgenic mice developed Burkitt-like lymphoma and diffuse large B cell lymphoma beginning 4 months of age and only one mouse developed a T cell malignancy at 15 months, which is consistent with a longer latency for transformation of T cells by *TCL1* [107]. Finally, to elucidate the role of *TCL1* in B cell development and B cell malignancies, Bichi *et al.* generated the Eμ-*TCL1* transgenic mice that overexpress the human oncogene *TCL1* under the control of the B-cell-specific μ enhancer and the IGVH promoter to target gene expression specifically to B cells (Figure 2.1) [108]. Starting at 2 months of age, $CD5^+$ B cells were detected in the peritoneal cavity of Eμ-*TCL1* mice, which became evident in the spleen by 3-5 months and in the bone marrow by 5-8 months [108]. At 13-18 months of age, the Eμ-*TCL1* mice display an overt disease with an expansion of $CD5^+CD19^+$ B cell population in both lymphoid and non-lymphoid organs, associated with splenomegaly, hepatomegaly and lymphadenopathy, recapitulating the main features of human CLL [108]. The Eμ-*TCL1* transgenic mouse became the first of a number of engineered mice to develop a CLL-like disease but one of the very few to closely resemble the real human disease [109].

Several other mouse models mimicking genetic lesions found in CLL (13q14 deletion), transgenic for genes that are overexpressed in the disease (including *APRIL*, *BCL2 X traf2dn*, *ROR1*), or driven by ectopic oncogene expression (*IgH.T* and *IgH.TEμ*) have been generated to model CLL [109]. In all of these mouse models including the Eμ-*TCL1* mouse, the disease develops late

in life and resembles the indolent disease course of CLL. In addition, transformation to CLL appears to be driven by the expansion of peritoneal B-1a cell, as suggested by the expression of CD5 and of unmutated IGHV genes, high levels of IgM, and low levels of IgD and CD23 [109]. However, the most notable difference among these CLL mouse models and the Eμ-*TCL1* mouse is the penetrance of the phenotype. The penetrance is highest in the Eμ-*TCL1* mice, intermediate in the *13q14-MDR* and the *APRIL* transgenic mice and lowest in the *ROR1* transgenic mice (5%) [109].

In this dissertation, we utilized the Eμ-*TCL1* mouse model for our studies due to the close resemblance to human CLL disease and the high penetrance of the disease. CLL cells in mice also respond to various types of therapeutic regimens effective for human CLL (e.g., fludarabine, ibrutinib, *etc*.). More importantly, T cell dysregulation such as decrease in T cell activation and increase in regulatory T cell numbers are characteristics of both Eμ-*TCL1* mice and human CLL patients [110]. CLL development in these transgenic mice is associated with similar impairment of T cell function and alteration of gene expression in CD4 and CD8 T cells to that observed in human patients with CLL [111]. T cell dysfunction in Eμ-*TCL1* mice and in CLL patients has been shown to contribute to the immunosuppression status that is associated with disease progression and susceptibility to infections, which are the leading causes of death in CLL patients [13, 111-113]. Finally, it is worth noting that in our Eμ-*TCL1* mice cohort, mice develop a full CLL-like disease at 10-15 months of age. The similarities with human CLL validate the use of Eμ-*TCL1* mice as a model for

further analyses of ways to prevent and reverse cancer-induced immune dysfunction.

(1k) Study aims

As discussed above, chronic lymphocytic leukemia disease is associated with an immunosuppression status, which is the leading cause of death in CLL patients [13-15]. Many mechanisms of immunosuppression have been investigated in CLL. CLL cells themselves secrete an immunosuppressive factor, IL-10. In this study, we utilized the Eμ-*TCL1* mouse model as well as primary human CLL cells and the human CLL cell line MEC-1 to investigate the role of IL-10 in both survival and immune responses in CLL by addressing the following specific aims:

1. IL-10 regulates the proliferation responses to normal B-1 cells, which are thought to be the cellular origin of CLL. Similar to normal B-1 cells, Eμ-*TCL1* CLL cells also produce IL-10 constitutively. Here we will test the hypothesis that IL-10 may regulate the survival and proliferation of CLL cells.
2. Since IL-10 in a well-known immunosuppressive cytokine, we will test the hypothesis that CLL-derived IL-10 may have role in suppression of host anti-CLL immune response.
3. The mechanism of IL-10 production by B cells is understudied. We will investigate these possible mechanisms of IL-10 production by CLL cells with the aim of identifying therapeutic targets.

CHAPTER 2

Materials and Methods

(2a) Mice and cells

C57BL/6J, B6.129S2-Il10rb^{tm1Agt}/J (IL-10R$^{-/-}$), and NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (NSG) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). NOD.Cg-Prkdc^{scid} Il2rg^{tm1Sug}/JicTac (NOG) mice were obtained from Taconic (Hudson, NY). Eμ-*TCL1* mice in BL/6 background were provided by Dr. John Byrd (Ohio State University, OH) and bred in house. Mice were housed under specific pathogen free conditions in micro-isolator cages under the Institutional Animal Care and Use Committee (IACUC) approved protocol. The University of Kentucky IACUC protocol number for this study is 2011-0904. The described studies are approved under this protocol. Cohorts of Eμ-*TCL1* mice were maintained and monitored regularly for CLL cells in blood by flow cytometry. Mice were bled by submandibular bleeding using a lancet [114]. Blood was collected in K2 EDTA Microtainer tubes (BD #365974, San Diego, CA). Most Eμ-*TCL1* mice developed CLL between 6-9 months of age (at least 30% CD5^{+}CD19^{+} B cells in the blood). Mice were euthanized when CLL cells were 80-90% or when their body condition score (BCS) was ≤2. BC scoring technique is as followed: with BCS=5 the mouse is smooth and bulky, bone structure disappears under flesh and subcutaneous fat; BCS=4 the mouse spine is a continuous column and vertebrae palpable only with firm pressure; BCS=3 the mouse vertebrae and dorsal pelvis is not prominent but palpable with slight

pressure; BCS=2 the mouse segmentation of vertebral column is evident and the dorsal pelvic bones are readily palpable; BCS=1 the mouse skeletal structure extremely prominent with little or no flesh cover and the vertebrae is distinctly segmented [115]. In addition to using CLL cells from primary Eμ-*TCL1* mice, we adoptively transfer CLL cells from spleens of euthanized Eμ-*TCL1* mice with 80-90% CLL into syngeneic mice, which lead to a reliable and consistent development of the disease in the recipient mice within weeks of injection and splenomegaly (Figure 2.1). Adoptive transfer of Eμ-*TCL1* cells consisted of transferring 1-10x10^6 Eμ-*TCL1* splenic cells into C57BL/6J mice via retro-orbital injection.

For *in vitro* experiments, Eμ-*TCL1* CLL cells were cultured in RPMI 1640 medium (Corning #10-040-CV, New York, NY) supplemented with 10% Fetal Bovine Serum (FBS) (Atlanta Biological Systems, Flowery Branch, GA). The human CLL cell line, MEC1, was obtained from Dr. Natarajan Muthusamy at Ohio State University. MEC1 cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) (ThermoFisher Scientific #12440061, Waltham, MA) supplemented with 10% FBS.

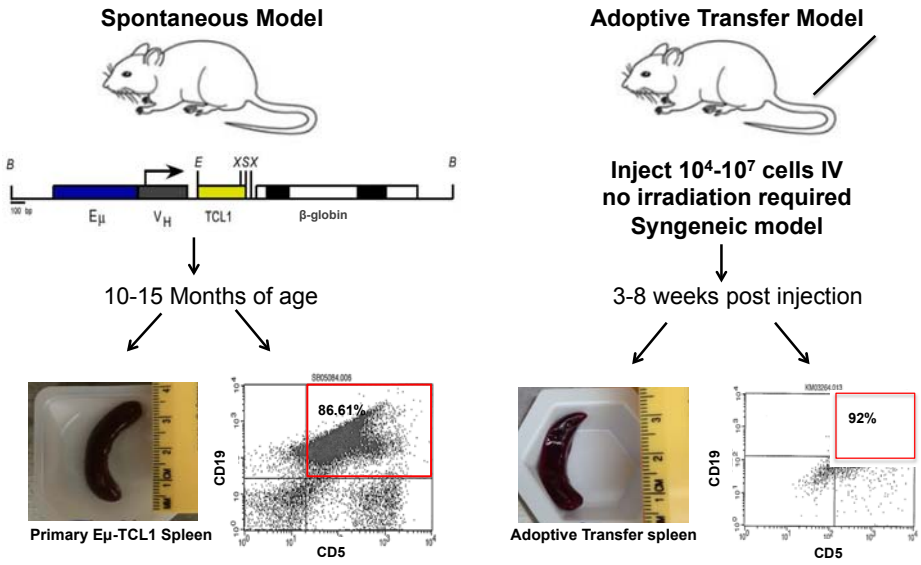


Figure 2.1: Production of Eμ-*TCL1* transgenic mice and the adoptive transfer model.

Gene organization for the transgene was adapted from Bichi, R. *et al.* Human chronic lymphocytic leukemia modeled in mouse by targeted TCL1 expression. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 6955-6960 (2002). Copyright © 2002, The National Academy of Sciences

(2b) Patients

Patients with CLL were recruited from the University of Kentucky Markey Cancer Center. All 18 patients gave informed consent according to protocols approved by the University of Kentucky Institutional Review Boards. Blood CLL cells were purified using Ficoll-Paque PLUS density gradients (GE HealthCare #17-1440-02, Pittsburgh, PA). CLL preparations were always >90% CD5+CD19+ B cells. For healthy controls, Leukopak units were obtained from the Kentucky blood bank. B cells were enriched using Ficoll-Paque PLUS density gradients and human CD19+ Microbeads (Miltenyi Biotech #130-050-301, San Diego, CA). For *in vitro* studies, human CLL cells were cultured in RPMI 1640 medium supplemented with 10% FBS.

(2c) Reagent

PMA (P1585), Ionomycin (407952), LPS (L2018), Thiazolyl Blue Tetrazolium Bromide (MTT) (M5655), and monoclonal anti-β-actin antibody (A5441) were from (Millipore Sigma-Aldrich, St. Louis, MO). Purified anti-mouse IL-10 (554463) and IL-10R (550012) antibodies were obtained from (BD PharMingen, San Diego, CA). AffiniPure F(ab')2 Fragment Goat anti-Mouse IgM (115-006-020) and AffiniPure F(ab')2 Fragment Goat anti-human IgM (109-006-129) antibodies were purchased from (Jackson ImmunoResearch Laboratories, West Grove, PA). Mouse anti-CD19 MicroBeads (130-052-201), anti-CD8a MicroBeads (130-049-401) and CD4+ T cell isolation kits were purchased from Miltenyi Biotech. Dasatinib (0003-0528-11) was manufactured by (Bristol-Myers Squibb Company, Seattle, WA). Syk inhibitor IV (Bay 61-3606) (57-471-42MG) was obtained from (EMD Millipore Calbiochem, Billerica, MA). Mithramycin A (BML-GR305-0001) was purchased from (Enzo Life Sciences, Farmingdale, NY). Ibrutinib (A3001) and ERK1/2 inhibitor (A3805) were obtained from (APExBIO, Houston, TX). Phosphate buffered saline (PBS) (#SH30256.FS) was obtained from GE HealthCare. Carboxyfluorescein succinimidyl ester (CFSE) (C1157) was purchased from ThermoFisher Scientific. Mouse fluor-conjugated anti-CD5 (100606 or 100608), anti-CD19 (115520 or 115508 or 115512), anti-CD45 (103114 or 103110), anti-CD11b (101212), anti-CD4 (100412 or 100510), anti-CD8 (100706 or 100708), anti-IL-10R (112706), anti-IFN-γ (505806), and anti-IL-10 (505010) as well as human anti-CD5 (364022), anti-CD19 (302208) and anti-CD45 (368512) antibodies, fixation buffer (420801) and intracellular staining

permeabilization wash buffer (421002) were all acquired from (BioLegend, San Diego, CA). Mouse Anti-CD90.2 (Thy1.2) (553013) was purchased from BD PharMingen. Antibodies to P-Syk (2711), total Syk (2712), P-p38 (9211), total p38 (9212), P-ERK1/2 (9101), P-STAT3 (9145), total STAT3 (4904) and GAPDH (2118) were obtained from (Cell Signaling Technology, Danvers, Massachusetts). Antibodies to IL-10 (SC-365858), Sp1 (SC-17824), Lyn (SC-15), and total ERK1 (SC-94) were acquired from (Santa Cruz Biotechnology, Santa Cruz, CA). Peroxidase coupled goat anti-rabbit (SC-2004) and anti-mouse (SC-2005) Ig secondary antibodies were also acquired from Santa Cruz Biotechnology. Mouse IL-10 (5261) and SimpleChIP kit (9003) were purchased from Cell Signaling Technology.

(2d) Immunofluoresence analysis and cell sorting

Single-cell lymphocyte suspensions from mouse tissues were prepared as described before. Spleens were pressed through a 40μm strainer using the plunger end of a syringe in Hank's buffered salt solution (HBSS) (Millipore Sigma-Aldrich #H6136-10XL). Tibiae and femora were harvested from mice. The bones were flushed with a 26G syringe in HBSS to obtain bone marrow single cell suspension. Peritoneal cells were obtained by peritoneal lavage with ~30mL of HBSS. Mouse blood mononuclear cells were isolated by centrifugation after subjecting 100-200μl of blood to RBCs lysis. Cells were resuspended in RPMI 1640 medium supplemented with 10% FBS. For multi-color immunofluorescence analysis, single-cell suspensions of mononuclear cells (10^6 cells) were incubated with normal rat IgG (10μg/10^6 cells) at 4°C for 15 min to block Fcγ receptors. The

cells were then labeled with fluorochrome-conjugated anti-mouse antibodies for 30 minutes on ice. Cells were washed with staining buffer (2% FBS in 1xPBS). Cells with the forward and side light scatter properties of single viable lymphocytes were analyzed using Becton Dickinson (San Jose, CA) LSRII flow cytometer and CellQuest Pro software. Anti-CD19, anti-CD11b and anti-CD5 were used to identify and sort B-1a (CD19+ CD5+ CD11b+), B-1b (CD19+ CD5- CD11b+), B-2 (CD19+ CD5- CD11b-) cells from the peritoneum of C57BL/6J mice using iCyt Synergy sorter system from Sony Biotechnology (San Jose, CA). CD45 staining was used to gate on lymphocytes and myeloid cells. Anti-CD19 and anti-CD90.2 antibodies were used to identify and sort T cells (CD19- CD90.2+) for the adoptive transfer experiments also using the iCyt Synergy sorter. Intracellular staining was performed according to Biolegend protocol. Briefly, cells were stimulated with PMA (20ng/ml) and Ionomycin (1µg/ml) for 4 hours in RPMI media supplemented with 10%FBS at 37°C. After surface staining, cells were fixed with 1X PBS solution containing 4% paraformaldehyde for 20 minutes at room temperature, permeabilized using BioLegend Intracellular Staining Permeabilization Wash Buffer, and finally stained with the antibody of interest for 30 minutes on ice.

(2e) Enzyme-linked immunosorbent assay (ELISA)

For cytokine analysis, normal B-1, murine CLL and human CLL cells were cultured in triplicate ($2x10^6$ cells/mL) for 24 hours in 96-well plate with various stimulants or treatments. Cells were removed by centrifugation and supernatants were assayed immediately or stored at -80°C. Plasma from mice was obtained

by centrifuging blood collected in EDTA tubes at the time of euthanization by cardiac puncture and the samples were stored at -80°C. IL-10 levels in supernatants or plasma were quantified using IL-10 OptEIA ELISA kit (BD #555252). IFN-γ levels were measured using IFN-γ OptEIA ELISA kit (BD# 555138). Human plasma or secreted IL-10 levels were quantified using IL-10 ELISA MAX set (BioLegend #430601).

(2f) In vitro cell survival and proliferation assays

CLL cell survival was determined by MTT assay [116]. Splenic CLL cells were stimulated with 5μg/ml LPS in the presence or absence of anti-IL-10R (10μg/ml) or anti-IL-10 (10μg/ml) antibodies. After 48 hours, media was changed and cultured with MTT for 4 hours followed by solubilization in acidic isopropanol and spectrophotometric measurements at 560nm and 690nm. To calculate final optical density (OD) counts, measurements at 690nm are subtracted from 560nm measurements to account for background readings.

For T cell proliferation and differentiation studies, CD8+ cells were purified using anti-CD8+ Microbeads using the autoMACS cell separator (Miltenyi Biotec). Cells then were and cultured (1-2 x 10^5 cells) with irradiated mouse CLL cells (25Gy) (2 x 10^5 cells) for 72 hours. Irradiation was performed in a Mark I-68 Cesium γ-irradiator (J.L Shepherd and Associates) on a rotating platform. The cultures were pulsed with 1.0 μCi of ^{3}[H] thymidine for 4 hours. The incorporated radioactivity was measured after harvesting cells onto a 96-well-plate by using a Matrix 96 β-counter (Packard, Downers Grove, IL). For IFN-γ analysis, supernatants were collected after 24 hours. All cell cultures were set in triplicates

in 96-well-plate at cell density of $2x10^6$cell/mL.

(2g) Immunoblotting

CLL cells (mouse or human) were cultured at $5x10^6$ cells/well in a 6 well plate for treatments indicated in figures. At each time point, cells were collected and washed twice using HBSS. Cells were then lysed in Cell Signaling lysis buffer (#9803) containing 1mM PMSF (Sigma #P7626), 2mM NaF (Sigma #S-1504), 2mM Na_3VO_4 (Sigma #S-6508) and 1x protease inhibitor cocktail (Roche #5892953001) (Indianapolis, IN) for 15 minutes on ice and collected by centrifuging at highest speed setting. Protein concentration in cell lysates was estimated by the Bicinchoninic Acid (BCA) assay kit (Thermo Scientific #23227). Protein lysates were diluted in 4x sodium dodecyl sulfate (SDS) sample buffer (100mM Tris-HCl, pH 6.8, 30% glycerol, 4% SDS, 5% 2-ME and 0.01% W/V bromophenol blue) to a 1x final concentration and boiled for 10 min. The BIO-RAD Mini PROTEAN Tetra System was used for both gel electrophoresis and transfer. 30µg total protein/sample of total lysate was subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. 7µl of Precision Plus Protein dual color ladder (BIO-RAD #1610394, Hercules, CA) with a size range spanning 10-250 kDa was used as a size standard for every gel. 10% or 12% polyacrylamide gels were run with running buffer (25mM Tris, 192mM glycine, 0.1% SDS, pH 8.3) at 100Volts and ~150mA for 10 min to stack the proteins, and later at 150Volts and ~150mA for 1 h to separate the proteins. Separated proteins were transferred to polyvinylidene difluoride membranes (EMD Millipore #IPVH00010) with transfer buffer (25mM Tris, 192mM glycine, 20% methanol, pH

8.3). Transfer was performed at 100V and ~150mA for 1.5 h at 4°C. Membranes were blocked at room temperature for 1hr with 5% milk or 3% bovine serum albumin (when probing for phosphorylated proteins) in 1x TBST that was diluted from 10x TBST (0.5M Tris, 1.5M NaCl and 1% Tween-20). The membranes were then probed with appropriate primary antibodies at 4°C overnight, followed by horseradish peroxidase-conjugated secondary antibodies at room temperature for 1hr. The blots were developed with HyGLO chemiluminescence reagent (Denville Scientific #E2400, Holliston, MA) and exposed to HyBlot CL autoradiography film (Denville Scientific #E3012). Band densitometry analysis was performed using the NIH ImageJ program. Protein expression was normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-actin or total target protein expression as appropriate.

(2h) CLL and T cell adoptive transfer and CFSE Labeling

Adoptive transfers were performed by transferring 1-10x10^6 Eμ-*TCL1* splenic cells into C57BL/6J or IL-10R KO or NSG mice (without any preconditioning) intravenously via retro-orbital injection. CLL disease was monitored by periodic submandibular bleeding and CD5+CD19+ cells were quantified by flow cytometry [117]. For CFSE Labeling, CLL cells were resuspended at 10^7 cells/ml in 1xPBS + 10mM CFSE, incubated at 37°C for 20 min, and then washed with 1xPBS to prepare for retro-orbital injection [118].

For total T cell adoptive transfer experiments, C57BL/6J and IL-10R KO mice were injected with 4 x 10^6 CLL cells for priming. 14-17 days after injection, splenic T cells were sorted using anti-CD90.2 antibody. 4 x 10^6 T-depleted CLL

cells along with sorted 0.5 x 10^6 T cells (NSG) or 0.25 x 10^6 T cells (NOG) were adoptively transferred and disease was monitored weekly by bleeding. For CD8+ T cell adoptive transfer, C57BL/6J and IL-10R KO mice were injected with 4 x 10^6 CLL cells for priming. 14 days after injection, splenic CD8+ T cells were isolated using CD8+ microbeads and the autoMACS cell separator. 4 x 10^6 T-depleted CLL cells along with sorted 0.125 x 10^6 T cells (NSG) were adoptively transferred and disease was monitored weekly by bleeding.

(2i) Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated from Eμ-*TCL1* CLL cells using TRI reagent (Sigma-Aldrich #T9424). RNA was quantified and 2μg of total RNA was subsequently used to make cDNA using qScript cDNA SuperMix (Quanta Biosceince #95048-100, Gaithersburg, MD) according to the manufacturer's protocol. iTaq Universal SYBR Green Supermix (BIO-RAD #172-5121) was used to carryout the qRT-PCR reaction. qRT-PCR was performed and analyzed (comparative C_T ($\Delta\Delta C_T$) method) on StepOnePlus Real-Time PCR System from (Applied Biosystems, Foster City, CA). Primer sequences used are described in Table 2.1. The 18s-specific primers were used for loading control. All primers were obtained from (IDT technologies, Coralville, IA).

Table 2.1: List of qRT-PCR Primers

Primer	Sequence	Manufacturer
IL-10 Forward	5'-ACTGGCATGAGGATCAGCAG-3'	IDT technologies
IL-10 Reverse	5'-AGAAATCGATGACAGCGCCT-3'	IDT technologies
SP1 Forward	5'-TGCCACCATGAGCGACCAAGATCA-3'	IDT technologies
SP1 Reverse	5'-TGCTGCTGCTTCGAGTCTGAGAAA-3'	IDT technologies
18S Forward	5'- CGCCGCTAGAGGTGAAATTCT -3'	IDT technologies
18S Reverse	5'-CGAACCTCCGACTTTCGTTCT-3'	IDT technologies

(2j) Short hairpin RNA (shRNA) sequence and cell infection

Lyn shRNAs in the pLKO.1 lentiviral vector were validated and selected by the RNAi Consortium and glycerol stocks were purchased from ThermoFisher Scientific (#RHS4533). MEC1 cells were seeded at a density of 1.5 x 10^6 cells/ml in a 6-well assay plate, infected with 25-50µl of concentrated shRNA lentiviral supernatants with the addition of polybrene (10µg/ml) and centrifuging for 90 minutes at 2800rpm and 10°C. Virus and cells were incubated for 24hrs at 37°C and then fresh media was replenished. Puromycin antibiotic selection began at day 3 and remained in culture for entire period of experimentation after proper titration. All lentiviral infections were assayed by Western blot analysis with anti-Lyn antibody. Lyn shRNA Clone# TRCN0000010101 is represented in this paper.

(2k) Chromatin Immunoprecipitation (ChIP) for qChIP analysis

ChIP analysis was performed using Cell Signaling SimpleChIP Enzymatic Chromatin IP kit following manufacturer's protocol. The proteins and DNA were cross-linked with 1% formaldehyde, lysed, and the DNA was sheared into 150-900 bp fragments. Proteins linked to the DNA were immunoprecipitated with anti-Sp1 antibody (using rabbit IgG antibody as control). Subsequently, immune complexes were eluted from the beads, protein-DNA crosslinks were reversed, and DNA was isolated after phenol/ chloroform/isoamyl alcohol extraction followed by ethanol precipitation. For qChIP, RT-PCR was performed on the eluted DNA using SYBR Green Reaction Mix as described in *(section 2i)*. The primers used to amplify specific regions of the IL-10 promoter described to contain Sp1 binding site [119] were as follows; forward, 5'-

GCAGAAGTTCATTCCGACCA-3’; reverse, 5’-GGCTCCTCCTCCCTCTTCTA-3’. For calculating fold changes, we used the fold enrichment method. This normalization method is also called 'signal over background' or 'relative to the no-antibody control'. With this method, the ChIP signals are divided by the no-antibody signals, representing the ChIP signal as the fold-increase in signal relative to the background signal. The assumption of this method is that the level of background signal is reproducible between different primer sets, samples, and replicate experiments.

(2l) Tissue Histology and Disease Scoring

Colons were dissected from euthanized mice and fixed in 10%-buffered formalin (Fisher Scientific #SF93-4, Fair Lawn, NJ), embedded in paraffin, and stained with hematoxylin and eosin (H&E) by the University of Kentucky histology services. Histological scoring was based on the method described previously by Berg *et al*. [120]. Briefly, a score from 0 to 4 was based on criteria summarized in table 2.2 [120].

Table 2.2: Criteria for histological scoring of the colon

Grade	Criteria
0	No change from normal tissue.
1	Few multi-focal mononuclear cell infiltrates in the lamina propria. Minimal epithelial hyperplasia. Slight to no depletion of mucus from goblet cells.
2	Several multifocal, mild inflammatory cell infiltrates in the lamina propria composed primarily of mononuclear cells with a few neutrophils. Mild epithelial hyperplasia and mucin depletion. Occasional small epithelial erosions. Inflammation rarely involving the submucosa.
3	Lesions involved a large area of the mucosa. Moderate inflammation often involving the submucosa but rarely transmural. Inflammatory cells are a mixture of mononuclear cells as well as neutrophils. Crypt abscesses are present. Moderate epithelial hyperplasia and mucin depletion. Occasionally observed ulcers.
4	Lesions involved most of the intestinal section. Severe Inflammation, including mononuclear cells and neutrophils, and was sometimes transmural. Epithelial hyperplasia marked with crowding of epithelial cells in elongated glands. Few mucin containing cells. Crypt abscesses and ulcers are present.

(2m) Statistical analysis

GraphPad Prism 7 was used for statistical analyses (GraphPad Software, Inc., La Jolla, CA). Statistical significance of differences between groups was evaluated by Student's t test or Tukey's multiple comparisons test as appropriate and p values < 0.05 were considered significant.

CHAPTER 3

Chronic lymphocytic leukemia cells produce IL-10 constitutively, which does not affect their survival *in vitro*

Peritoneal B-1 cells were shown early on to have the ability to produce Interleukin-10 constitutively [56]. In a recent study, a human $CD11b^+$ B-1 cell subset was also found to constitutively secrete IL-10 [121]. This constitutive nature of IL-10 production by B-1 cells differs from the newly described B10 subset, which can produce IL-10 but requires further activation in order to do so, such as anti-CD40, CPG and LPS stimulation [122, 123]. As described earlier, B-1 cells are further subdivided into B-1a and B-1b based on the differences in CD5 expression [29]. Among the different subsets of peritoneal B-1 cells, B-1a cells produced the highest amount of IL-10 constitutively, followed by B-1b cells [73]. Splenic B-1a cells produced much less IL-10 than peritoneal B-1a cells but more than splenic B-2 cells [73]. We previously demonstrated that IL-10 has autoregulatory effects in peritoneal B-1 cells through an inhibitory feedback mechanism, which affects their proliferation response to stimulation with LPS [73]. This autoregulation was found in response to TLR4 as well as ligation of TLR2, TLR3, TLR7, and TLR9 receptors [73]. Splenic B-1 cells did not exhibit this autoregulation, as their TLR responses were not enhanced by anti–IL-10R antibodies [73]. The autoregulatory effect extends to the differentiation response of B-1 cells. The autoregulation was found to be a result of inhibition of the classical NF-κB signaling by IL-10 [73]. In a recent study, the well-known

hyporesponsiveness of B-1 cells to BCR signaling was also found to be a result of the feedback-inhibitory effects of B-1 cell–derived IL-10 [54]. As described earlier, B-1 B cells are thought to be a possible cell of origin for murine CLL cells. Normal B-1 B cells and the Eμ-*TCL1* CLL cells share many characteristics. Preliminary studies showed that Eμ-*TCL1* CLL cells constitutively produce IL-10 [54]. Since IL-10 has autoregulatory effects in B-1 cells, we hypothesized that IL-10 could be playing a similar role in Eμ-*TCL1* CLL cells as they are also known to be hyporeactive to BCR and TLR ligation. Therefore, in this chapter we further characterized the production of IL-10 by Eμ-*TCL1* CLL cells and tested if the constitutively produced IL-10 plays a role in the survival and proliferation of Eμ-*TCL1* CLL cells *in vitro*.

Results

(3a) Constitutive IL-10 production by Eμ-TCL1 CLL cells

Eμ-*TCL1* CLL cells (or simply CLL cells) are defined by the co-expression of the surface molecules CD5 and CD19. For the studies performed here we use splenic cells from the primary Eμ-*TCL1* mouse as well as splenic cells from the CLL adoptive transfer mice described in the methods. Figure 3.1A shows a representative flow cytometry dot plot of spleen cells from both a normal C57BL/6 mouse (top) and an adoptively transferred mouse with CLL (bottom) stained with anti-CD5 and anti-CD19 antibodies. The majority of the splenic cells from the adoptively transfer mouse are CLL cells and are defined by ≥80% $CD5^+CD19^+$ cells, while normal C57BL/6 mouse spleen would only have <10% of $CD5^+CD19^+$ cells (normal B-1 cells). TCL1 expression can also be used to distinguish between normal B-1 cells and CLL cells as shown in Figure 3.1B.

Since CLL cells obtained from each Eμ-*TCL1* mouse are unique (based on V_H gene expression), we first tested if all Eμ-*TCL1* CLL cells produced IL-10 constitutively. Cells from spleens of Eμ-*TCL1* mice share a similar functional phenotype with peritoneal $CD5^+CD19^+CD11b^+$ B-1a cells in that they both secrete IL-10 constitutively, although this constitutive production is small in CLL from some Eμ-*TCL1* mice with CLL (Figure 3.2A). Here we confirmed that this constitutive production is a property of CLL cells themselves by purifying CLL cells using $CD19^+$ microBeads from spleens of Eμ-*TCL1* mice. Purified $CD19^+$ CLL cells produced a significant amount of IL-10 after 24 hours in culture, which was comparable to the unpurified splenic cells of Eμ-*TCL1 de novo* and adoptive transfer models (Figure 3.2A). Since the majority of normal B-1 cells are derived

from the peritoneal cavity, we also tested CLL cells derived from the peritoneal cavity for their ability to secrete IL-10. We found that peritoneal CLL cells also secrete IL-10 constitutively (Figure 3.2A). Finally, we demonstrated that LPS enhances IL-10 production by B-CLL cells, another property shared with normal B-1 cells (Figure 3.2B). In addition, even the CLL cells that produce low levels of IL-10 constitutively produce high levels of IL-10 upon LPS stimulation.

(3b) Neutralization of IL-I0 or blocking with anti-IL-10R antibody does not affect the survival of the Eμ-TCL1 CLL cells

While it is known that B-CLL cells produce IL-10, very few studies address the direct effects of CLL-induced IL-10 on CLL cells growth and survival. We have previously reported that IL-10 produced by normal B-1 cells regulated their proliferation responses to TLR stimulation as well as to BCR ligation [54, 73]. However, neutralization of CLL-derived IL-10 using anti-IL-10 antibodies or anti-IL-10R antibodies did not affect the survival of the CLL cells as shown by MTT assay (Figure 3.3A) or their proliferation responses shown by Thymidine uptake experiments (Figure 3.3B). LPS stimulation enhanced CLL survival but only had a modest effect on their proliferation. Neutralization of IL-10 did not affect the LPS induced increases in survival or proliferation. Thus, the CLL cells appear to be distinct from normal B-1 cells in not responding to IL-10-mediated suppressive affects *in vitro*. This lack of response to blocking IL-10 signal may be due to absence of IL-10R on CLL cells and therefore inability to signal. Hence, we measured the levels of IL-10R on the surface of CLL cells and the functional ability of the receptor to induce downstream signaling. Flow cytometry analysis of

IL-10R on the surface of CLL cells derived from Eμ-*TCL1* mice confirmed the presence of IL-10R on CLL cell surface (Figure 3.3C). Then to confirm the functionality of the IL-10R, we tested if exogenous IL-10 would induce the well-known downstream signaling by inducing phosphorylation of STAT3 transcription factor. Indeed, treatment of CLL cells with exogenous IL-10 induced phosphorylation of STAT3 in comparison to untreated cells (Figure 3.3D). Thus IL-10 receptor appears to be functional in CLL cells.

Figure 3.1A

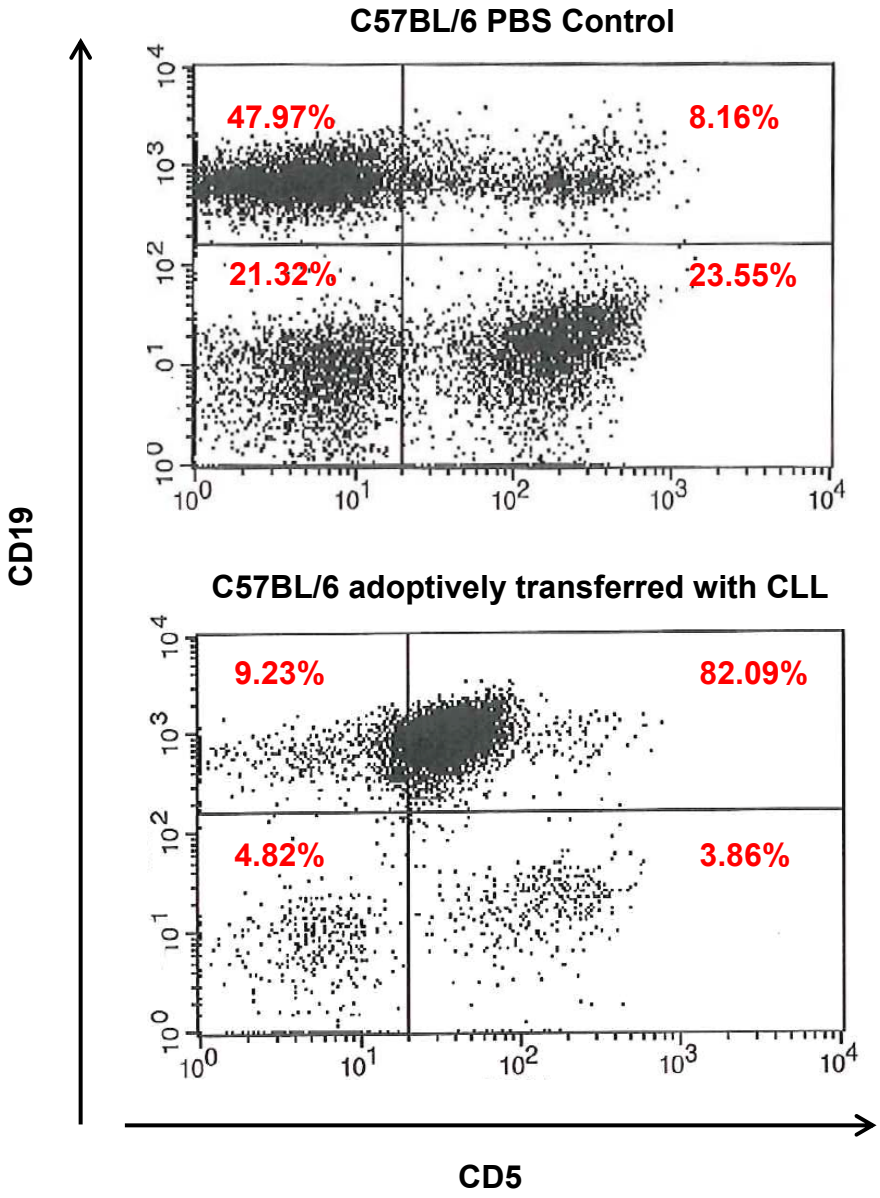

C57BL/6 PBS Control
47.97%
8.16%
21.32%
23.55%
C57BL/6 adoptively transferred with CLL
9.23%
82.09%
4.82%
3.86%
CD19
CD5

Figure 3.1B

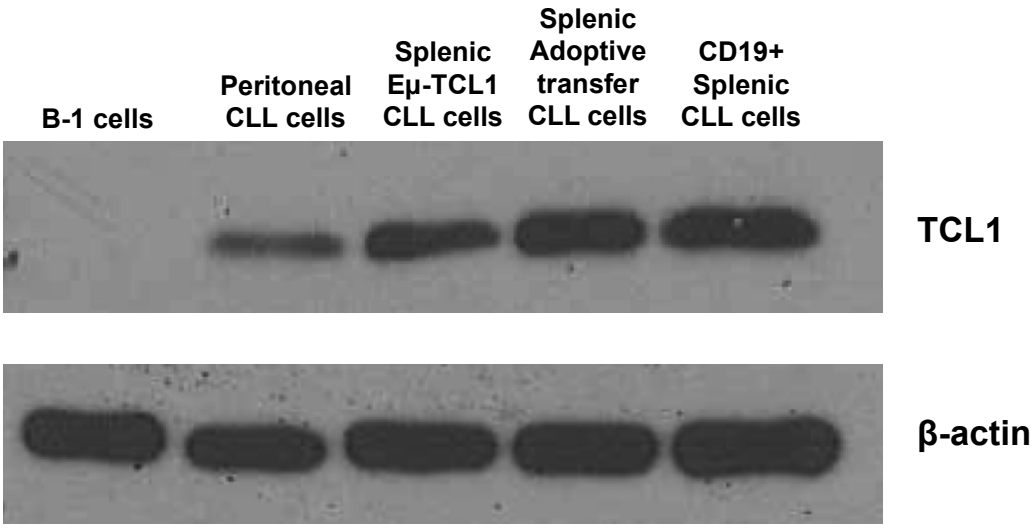


Figure 3.1: Flow cytometry and western blot representation of CLL cells

A) C57BL/6 mice injected IV with either PBS (top) or $4x10^6$ CLL cells (Bottom). After 4 weeks, both splenocytes from recipient mice were stained with CD5 mAb and CD19 mAbs. Representative dot plots for one-mouse show frequencies of $CD5^+CD19^+$ cells among total $CD45^+$ cells. In a typical experiment, 80-100% of recipient mice develop CLL in 4-10 weeks after transfer of CLL cells. **B)** CLL cells were harvested from the peritoneal cavity or the spleen of Eμ-*TCL1* or adoptive transfer mice. B-1 cells were isolated from the peritoneal cavity of C57BL/6J WT mice. Purified $CD19^+$ CLL cells were obtained by using $CD19^+$ microbeads and the autoMACS cell separator cell separation. Protein lysates from these cells were analyzed for the levels of TCL1 by Western blot. β-actin is used for loading control.

Figure 3.2A

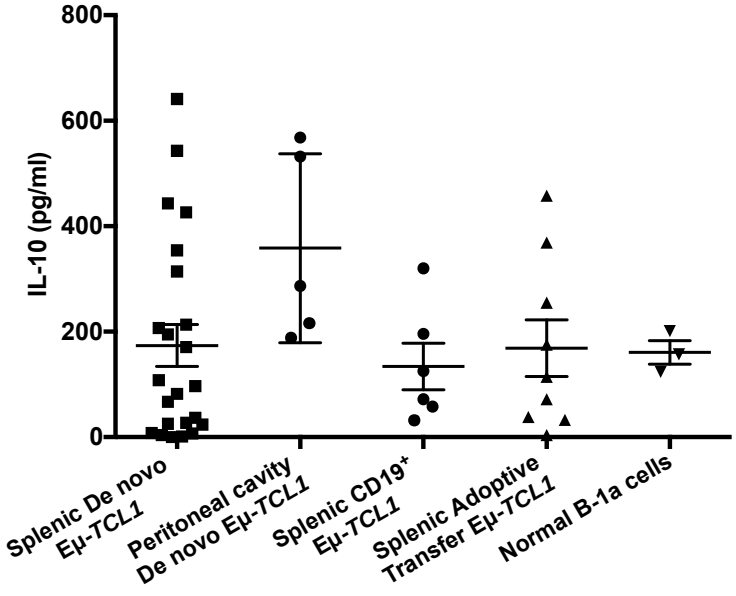


Figure 3.2B

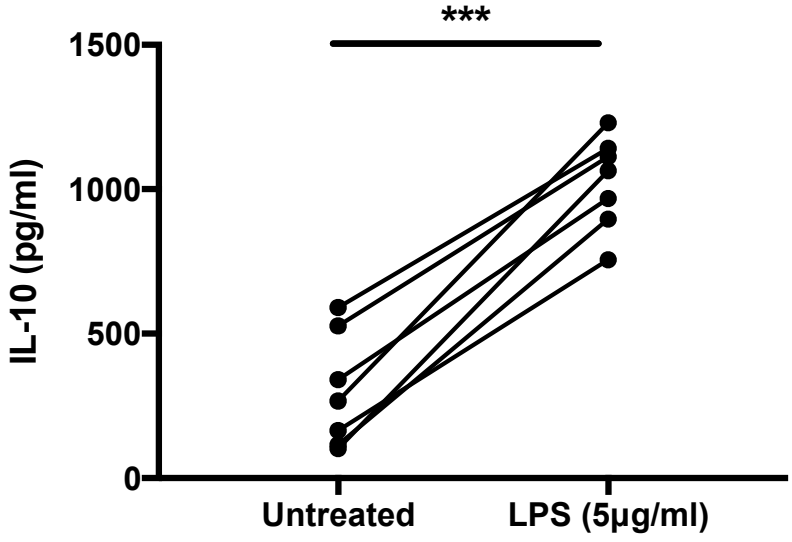


Figure 3.2: Constitutive IL-10 production by CLL cells

A) CLL cells were harvested from the spleen (n=23) or peritoneal cavity (n=5) of Eμ-*TCL1* or adoptive transfer mice (n=9). B-1a cells were isolated from the peritoneal cavity of C57BL/6J WT mice (n=3). Purified $CD19^+$ Eμ-*TCL1* CLL cells were obtained by using $CD19^+$ microbeads and the autoMACS cell separator cell separation. Cells were cultured for 24 hours (no stimulation) and IL-10 was measured in the supernatant by ELISA. **B)** Splenic CLL cells (n=7) were treated with or without LPS (5μg/ml) for 24 hours. Supernatant was collected and IL-10 was measured by ELISA. Values represent mean ± SE of number of mice indicated. ***$p<0.001$

Figure 3.3A

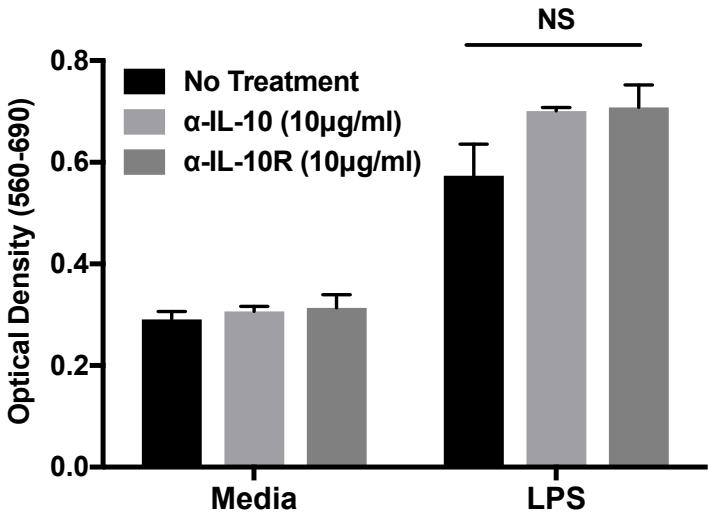


Figure 3.3B

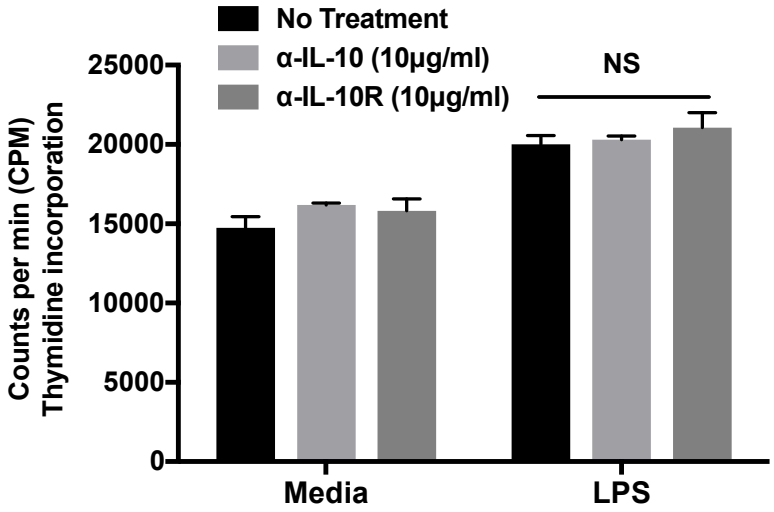


Figure 3.3C

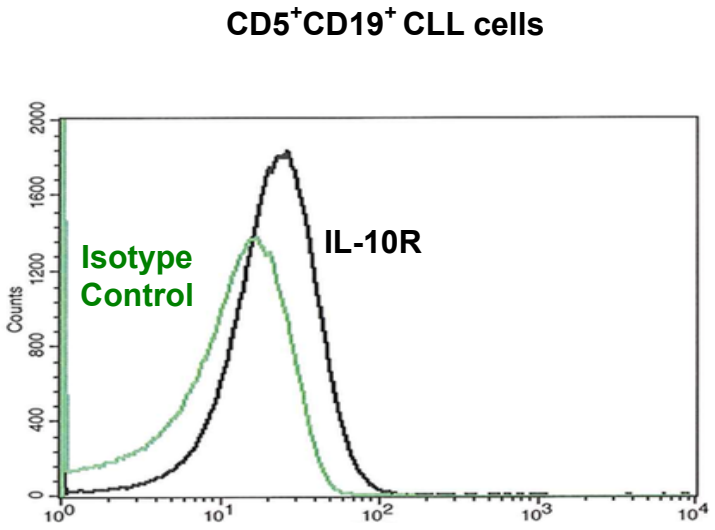


Figure 3.3D

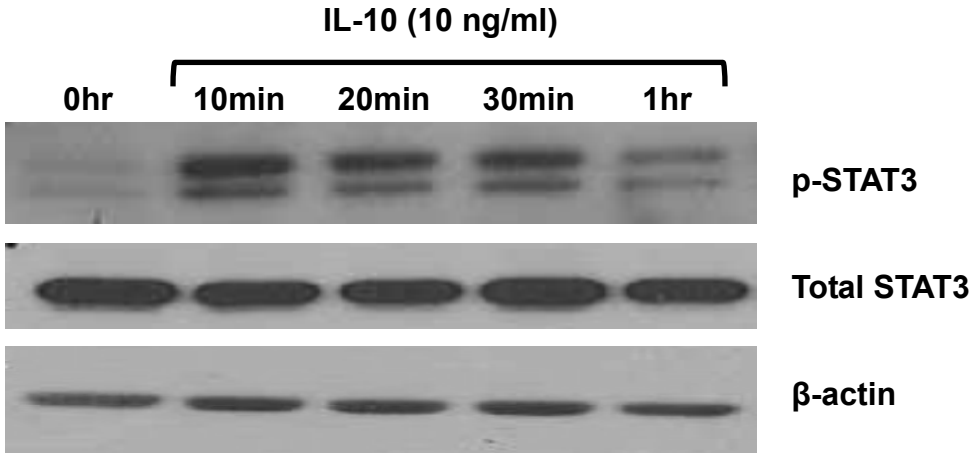


Figure 3.3: Inhibiting IL-10 signaling does not affect the survival of the Eμ-*TCL1* CLL cells *in vitro*

A) Splenic Eμ-*TCL1* CLL cells were cultured with αIL-10 or αIL-10R antibodies with or without LPS (5μg/ml) for 48 hours. Survival of CLL cells was measured by MTT. Values represent mean ± SD of triplicate cultures. **B)** Splenic Eμ-*TCL1* CLL cells were cultured with αIL-10 or αIL-10R antibodies with or without LPS (5μg/ml) for 48 hours. Tritiated thymidine was added at the last 4 hours of culture. Proliferation was measured by tritiated thymidine incorporation using a beta plate reader. Values represent mean ± SD of triplicate cultures. **C)** Eμ-*TCL1* CLL cells stained with anti-IL-10R or isotype control antibody and analyzed by flow cytometry. **D)** Protein lysates of Eμ-*TCL1* CLL cells stimulated with exogenous IL-10 for indicated time points were analyzed for the levels of p-STAT3 and total STAT3 by Western blot. β-actin is used for loading control. NS; not significant.

Summary

During the course of our studies, we made the observation that splenic cells isolated from our CLL model, the Eμ-*TCL1* mouse, constitutively secreted IL-10. We found that peritoneal CLL cells also secrete IL-10 constitutively and that LPS enhances this IL-10 production. On average CLL cells from both spleen and peritoneal cavity produced similar amounts of IL-10. This is unlike normal B-1 cells, wherein peritoneal but not splenic B-1 cells produced IL-10 constitutively or upon TLR4 stimulation. As discussed above, IL-10 secretion by normal B-1 cells regulates their proliferation responses to TLR stimulation as well as BCR ligation. Here we found that neutralization of IL-10 using anti-IL-10 antibodies or anti-IL-10R antibody did not affect the survival or proliferation of the CLL cells despite the fact that IL-10 receptor appears to be functional in CLL cells. An interesting result seen here is the amount of variability of constitutive IL-10 production by the different Eμ-*TCL1* mice. This could be due to differences in V_H subfamily expression between the different Eμ-*TCL1* CLL cells. Hence, we sequenced a number of Eμ-*TCL1* CLL cells for the expression of V_H subfamilies. We found no linkage between the basal level of IL-10 and V_H subfamily expression (unpublished data). Worth noting that studies performed in this work always used CLL cells that constitutively produced IL-10 with at least 150pg/ml levels. In future chapters we will be discussing if IL-10 has a function in the growth of CLL cells *in vivo*.

CHAPTER 4

The role of IL-10 during immune responses to CLL

CLL is associated with a profound immune defect, which results in increased susceptibility to infections as well as a failure to mount effective antitumor immune responses. Infections in CLL patients have been recognized as a common cause of morbidity and mortality [13-15]. Many mechanisms of immunosuppression have been described in CLL. As seen in chapter 3, CLL cells constitutively produce IL-10, an immunosuppressive factor. However, CLL-induced IL-10 had no effect on CLL cell survival *in vitro*. This led us to hypothesize that the CLL-derived IL-10 may have a suppressive effect on immune responses against CLL. IL-10 is known to regulate T cell responses indirectly through its effects on macrophages and monocytes, inhibiting their MHC class II and costimulatory molecule B7-1/B7-2 expression and limiting their production of proinflammatory cytokines and chemokines [79]. IL-10 can also act directly on T cells, inhibiting proliferation and production of IL-2, IFN-γ, IL-4, IL-5 and TNF-α [81, 82]. Studies presented in this chapter investigate the role of adaptive immune responses in CLL and the effects of IL-10 on the microenvironment and T cell responses in CLL using the Eμ-*TCL1* mouse model.

Results

(4a) Immune responses control CLL growth

CLL has been shown to be associated with defects in T-cell function, resulting in failure of antitumor immunity and increased susceptibility to infections [50]. Here we hypothesized that IL-10 could be playing a role in this T-cell dysfunction. First, we wanted to confirm the role of adaptive tumor immunity against CLL in the Eμ-*TCL1* mouse model. Utilizing our CLL adoptive transfer model, we injected CLL cells into C57BL/6J wild type (WT) mice and NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (NSG) mice that lack mature B and T cells, as well as natural killer (NK) cells with secondary defects in macrophages and DCs [124, 125]. We hypothesized that lack of adaptive immunity would lead to a change in the engraftment pattern of CLL cells. Indeed, comparing the percentage of CD5$^+$CD19$^+$ CLL cells in peripheral blood (PB), CLL cells in NSG mice were detectable as early as day 7 post injection (1-3% in CLL injected mice versus <0.15% in PBS injected NSG recipients) and had to be euthanized at day 16 due to poor body condition with an average of 70% CD5$^+$CD19$^+$ cells in PB (Figure 4.1A). On the other hand, CLL cells were detectable only at day 21 post injection in WT mice and at day 28 an average of 50% CD5$^+$CD19$^+$ cells were present in PB (Figure 4.1A). The percentages of CLL cells in blood and spleen of both C57BL/6J and NSG mice at the time of euthanization are shown in Figure 4.1B. Kaplan-Meier analysis showed that upon CLL injection C57BL/6J mice survived longer than NSG mice ($p<0.05$) (Figure 4.1C), revealing an important role of adaptive immunity against CLL cells.

(4b) CLL cell growth is reduced in IL-10R null mice

Now that we confirmed the presence of anti-tumor immunity against CLL cells, we investigated if CLL-derived IL-10 could inhibit immune responses against CLL. In our hypothetical model as shown in Figure 4.2, IL-10 could be playing a role in inhibiting immune responses in CLL by either affecting innate immune cell functions in the CLL microenvironment such as their proinflammatory cytokine secretion or by directly affecting adaptive immune responses such as cytotoxic T cell role in anti-tumor immunity. To test this hypothesis, first using our adoptive transfer model, we injected CLL cells into WT and IL-10R KO mice both on the C57BL/6J background. Lack of IL-10 may enhance pro-inflammatory environment and increase CLL growth in IL-10R KO mice compared to wild type. For the possibility that IL-10 directly suppresses T cells, IL-10R KO mice will have more robust anti-CLL T cell responses leading to reduced CLL growth. Comparing the percentages of $CD5^{+}CD19^{+}$ CLL cells in PB of these mice, we found that CLL cells grew at a slower rate in IL-10R KO mice than in WT mice with the most difference at day 16 and day 20 (Figure 4.3A), though the differences in blood CLL levels between the two recipients at days 13, 16, 20 and 25 were all statistically significant. After euthanization, CLL tumor burden in different tissues collected including spleen, bone marrow (BM), and peritoneal cavity (PC) was higher in WT mice than in IL-10R KO mice ($p<0.05$) (Figure 4.3B-D). Thus the presence of IL-10 signaling in WT mice appears to inhibit anti-tumor immunity leading to a higher growth rate and engraftment of CLL in these mice compared to mice unable to receive IL-10 mediated

immunosuppressive signals. Since it has been reported that at 12 weeks of age, approximately 60% of IL-10R KO mice develop chronic colitis and increased numbers of splenocytes resulting in splenomegaly [126], we performed our experiments soon after weaning, 3-4 weeks old mice. At the end of the experiment, colon sections from both WT and IL-10R KO mice showed no significant difference in inflammation by histopathology (Figure 4.3E). Moreover, there was no significant difference in plasma IL-10 levels between WT and IL-10R KO mice at the end of the experiment (Figure 4.3F).

IL-10 is known to inhibit the function of macrophages and monocytes by limiting their production of proinflammatory cytokines and chemokines, which are thought to affect localization and survival of CLL cells in microenvironmental niches. We investigated this possibility in our model by testing if IL-10 is affecting the migration of CLL cells to the different mouse tissues *in vivo*. Using an adoptive transfer of CFSE labeled CLL cells, we found no significant difference in the total number of $CFSE^+$ cells in the spleen, peritoneal cavity, bone marrow and lymph node between WT and IL-10R KO mice 3 days post injection, suggesting that differences in CLL growth in IL-10 sufficient and deficient mice are not due to any effects on early localization of the transferred cells (Figure 4.4).

(4c) Decrease in T-cell function in wild type compared to IL-10R null mice

We tested if the decrease in CLL growth in the IL-10R KO mice is due to changes in T cell levels and/or function. Indeed, frequencies of $CD4^+$ and $CD8^+$ T cells in the spleen were higher in the IL-10R KO mice in comparison to WT mice

injected with CLL (Figure 4.5). Therefore, to further understand the inhibitory effects of IL-10, we investigated the effects of CLL-derived IL-10 on T cell function during the course of the disease by measuring their ability to proliferate or secrete γ-IFN in response to stimulation with autologous CLL cells. We adoptively transferred CLL cells into WT and IL-10R KO mice and euthanized a set of mice at days 13 and 20 post injection. $CD8^+$ cells were purified from the spleen of the mice using anti-CD8 antibody coupled microbeads. We found that proliferation of $CD8^+$ cells isolated from IL-10R KO mice upon restimulation with irradiated CLL cells was significantly higher than proliferation of $CD8^+$ T cells from WT mice at both time points (Figure 4.6A). $CD8^+$ T cells isolated from IL-10R KO mice exhibited a higher capacity to secrete IFN-γ than $CD8^+$ T cells from WT mice upon restimulation with CLL cells *in vitro* (Figure 4.6B). In addition, intracellular staining of IFN-γ after a short-term (4 hours) stimulation *ex vivo* with PMA and ionomycin revealed higher percentages of both $CD4^+IFN\text{-}\gamma^+$ and $CD8^+IFN\text{-}\gamma^+$ cells in IL-10R KO in comparison to WT mice injected with CLL cells (Figure 4.6C). These data suggest a role for IL-10 in inhibiting T cell responses against CLL *in vivo*.

(4d) T cells from IL-10R KO mice controlled CLL growth significantly longer than T cells from WT mice

To further demonstrate the functional differences between WT and IL-10R null T cells generated after CLL injection, we used the model of adoptive transfer of CLL cells into NSG mice. Here, we primed both WT and IL-10R KO mice with CLL cells. Then we isolated total T cells ($Thy1.2^+CD19^-$) from the spleens of the

mice after two weeks and adoptively transferred these T cells along with CLL cells used for priming into NSG mice at a ratio of one T cell to 8 CLL cells (experimental model shown in Figure 4.7). Overall, injection of T cells along with CLL cells delayed onset of disease in PB by a significant amount of time, no matter the source of the T cells (Figure 4.8A). At the time of euthanization, the number of CLL cells in the spleens indicated a difference between mice that received T cells from WT vs. IL-10R KO mice and was only significantly different between mice that received IL-10R KO T cells and mice that received no T cells (Figure 4.8B). 100% of the mice that received T cells from IL-10R KO mice survived at the end of the experiment while only 40% of the mice that received T cells from WT mice survived (Figure 4.8C). Thus IL-10R null T cells that cannot respond to IL-10 were more effective in controlling CLL disease than IL-10 responsive wild type T cells. Similar results were obtained when NOG mice, an independently derived T cell, B cell and NK cell deficient mice, were injected with CLL cells and wild type or IL-10R KO T cells primed with CLL cells as above. The only difference was that the T cell to CLL ratio was decreased to 1:16. Despite this lower T cell to CLL ratio, none of the mice receiving CLL primed IL-10R null T cells developed CLL even after 184 days while only three out of five mice receiving primed wild type T cells developed CLL by day 50. All the NOG recipients receiving CLL cells without any T cells developed CLL by day 27.

(4e) The adoptive transfer of CD8+ T cells was sufficient in controlling CLL development and CD8+ T cells from IL-10R KO mice controlled CLL growth significantly longer than T cells from WT mice

To help us identify the possible subset of T cells responsible for the anti-CLL immune response, we injected isolated CD8+ T cells from WT and IL-10R KO mice primed with CLL as above along with CLL cells at a ratio of 1 T cell to 32 CLL cells into NSG mice. Interestingly, CD8+ T cells were sufficient in delaying CLL growth and CD8+ T cells from IL-10R KO mice were better at controlling disease development than CD8+ T cells from WT mice (Figure 4.9A). At the time of euthanization, the number of CLL cells in the spleens indicated a significant difference between mice that received CD8+ T cells from WT vs. IL-10R KO mice; however, no significant difference was observed between group receiving T cells from IL-10R KO mice and PBS control mice (Figure 4.9B). 100% of the mice that received No T cells or CD8+ T cells from WT mice developed disease by day 30 post injection while only 16.7% of the mice that received CD8+ T cells from IL-10R KO mice developed disease by that time (Figure 4.9C).

Figure 4.1A

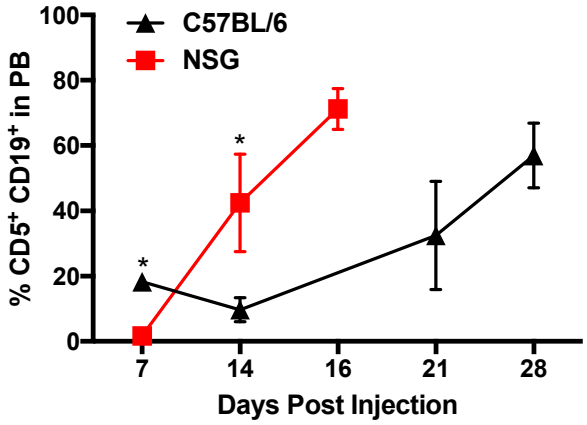


Figure 4.1B

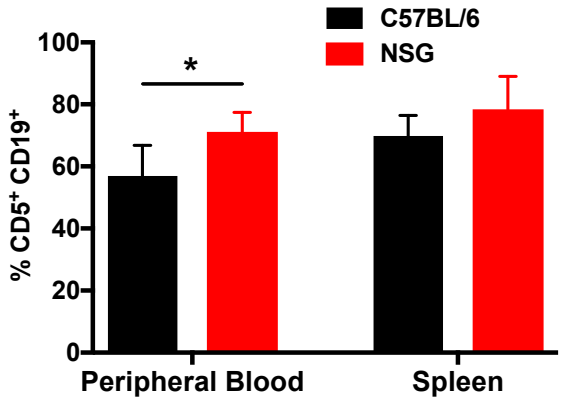


Figure 4.1C

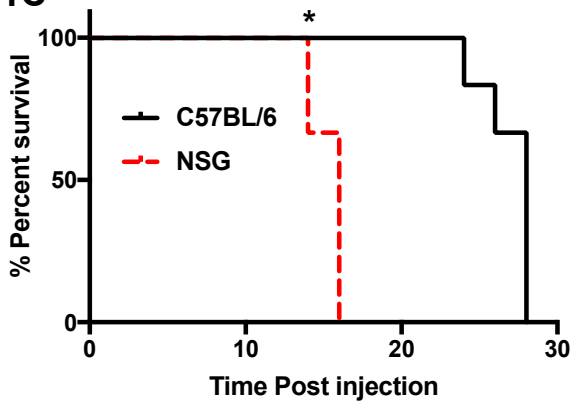


Figure 4.1: Lack of B, T and NK cells leads to an acceleration of CLL growth kinetics

A) Eμ-*TCL1* CLL cells (4 x 10^6) were adoptively transferred into WT and NSG mice by retro-orbital injection. Leukemic status is determined by weekly submandibular bleeding. Graph shows the % $CD5^+CD19^+$ cells in the peripheral blood at indicated time points. Values represent arithmetic mean of six mice per group ± SD. **B)** % $CD5^+CD19^+$ cells in the spleen of C57BL/6 and NSG mice is analyzed at the time of euthanization by flow cytometry. Values represent arithmetic mean of values from six recipient mice per group ± SD. **C)** Kaplan-Meier blot represents the survival of C57BL/6 and NSG mice during the course of the experiment (n=6). * $p < 0.05$ determined by Student's t-test for panels A and B and by Log-rank (Mantel-Cox) test for panel C. Similar results were obtained in another experiment.

Figure 4.2

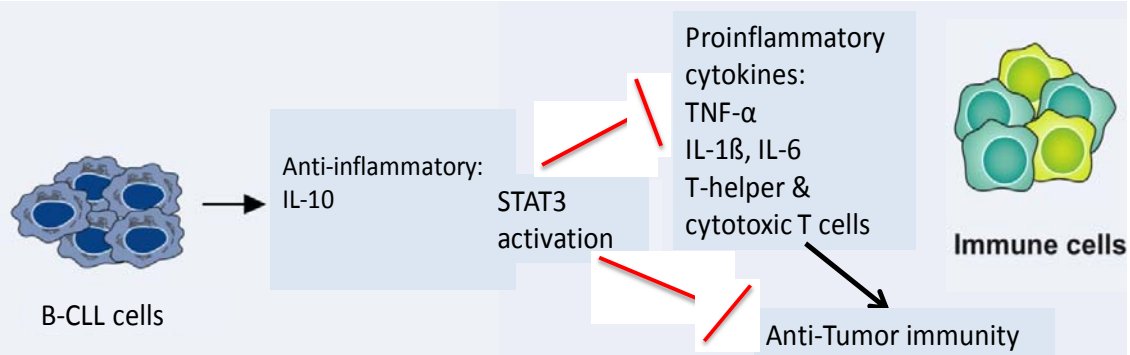


Figure 4.2: Hypothetical model for the effects of IL-10 on the immune system in CLL

Figure 4.3A

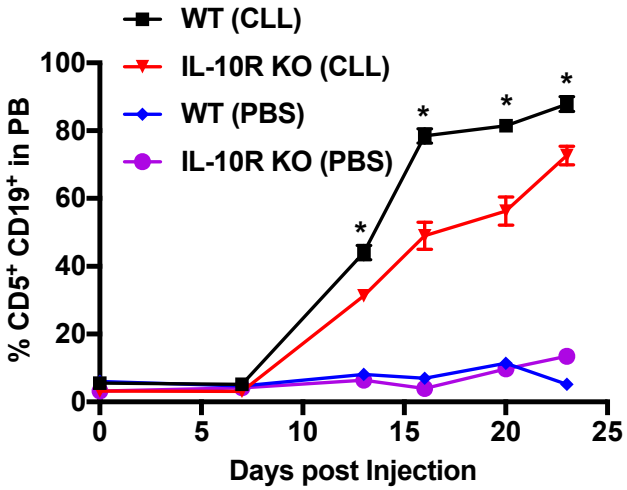


Figure 4.3B

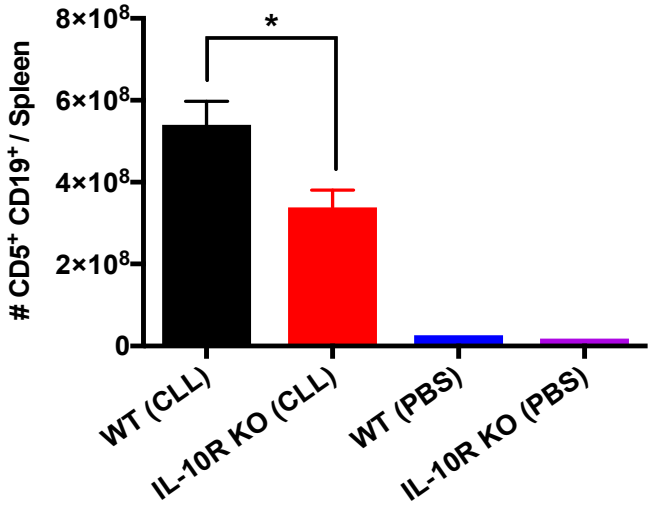


Figure 4.3C

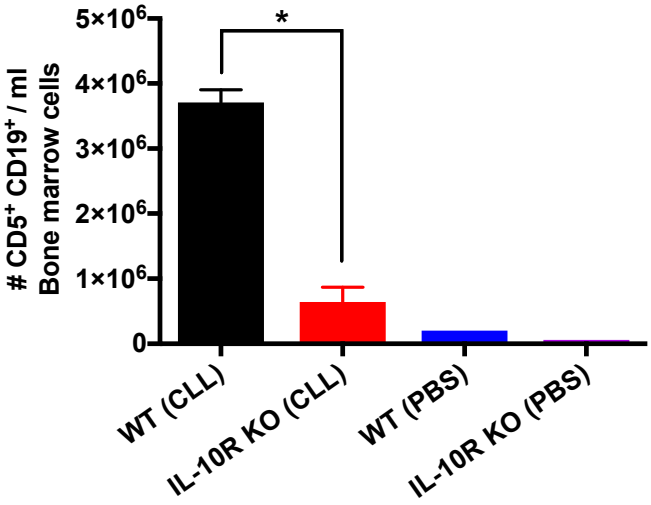


Figure 4.3D

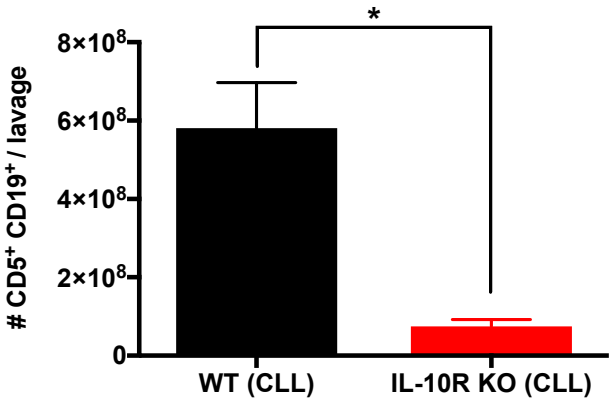


Figure 4.3E

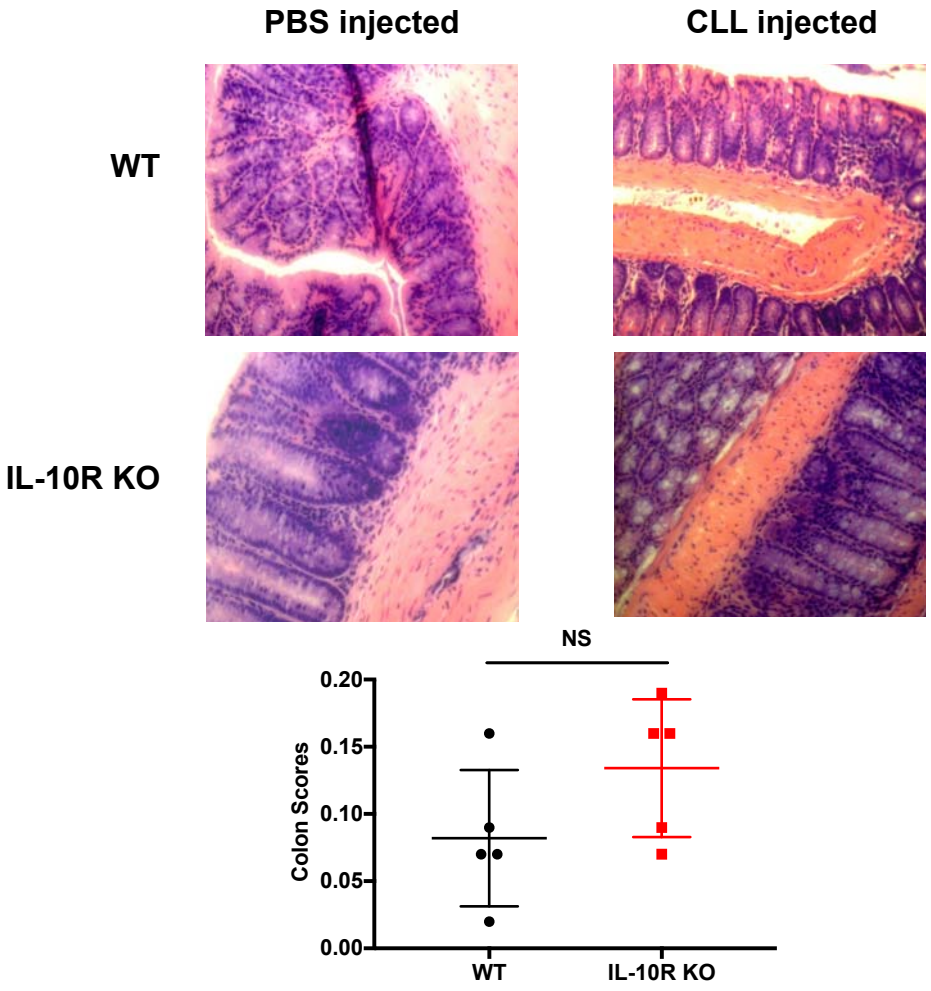


Figure 4.3F

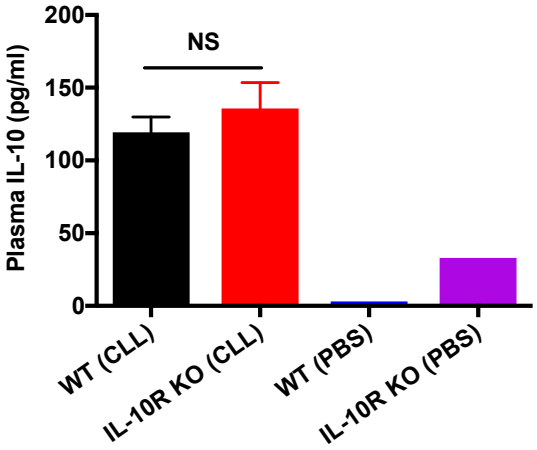


Figure 4.3: CLL cell growth rate is reduced in IL-10R null mice

A) Eμ-*TCL1* CLL cells (4 x 10^6) were adoptively transferred into WT and IL-10R KO mice by retro-orbital injection (n=5 mice/group). Leukemia status was monitored by weekly bleeding and is shown as $CD5^+$ $CD19^+$ cells as a percentage of $CD45^+$ cells in the peripheral blood. Injection of PBS was used as a control. **B-D)** Tumor burden as total number of $CD5^+$ $CD19^+$ cells was calculated based on total cell count and % of $CD5^+$ $CD19^+$ cells per spleen **(B)**, bone marrow (**C)** and peritoneal cavity lavage **(D)**. Values represent arithmetic mean of five mice per group ± SD. *p< 0.05. **E)** Representative colon H & E staining from WT and IL-10R KO mice injected with CLL cells or PBS. Colon sections were scored as described in the methods. NS; not significant. **F)** IL-10 plasma levels from WT and IL-10R KO mice were determined by ELISA at the end of the experiment.

Figure 4.4

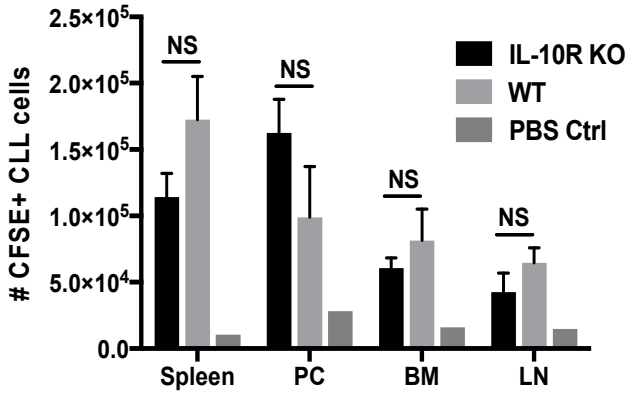


Figure 4.4: No differences in localization of CLL cells between WT and IL-10R KO mice

Eμ-*TCL1* CLL cells were labeled with CFSE (10μM/ml) and adoptively transferred into WT and IL-10R KO mice by retro-orbital injection (n=4/group). Mice were euthanized 3 days post injection and indicated tissues were harvested and stained for CD5 and CD19. Total numbers of CFSE+ CLL cells are represented. Injection of PBS was used as a control. Values represent arithmetic mean of four replicates ± SD. NS; not significant. Results from one of three experiments with similar outcome are shown.

Figure 4.5

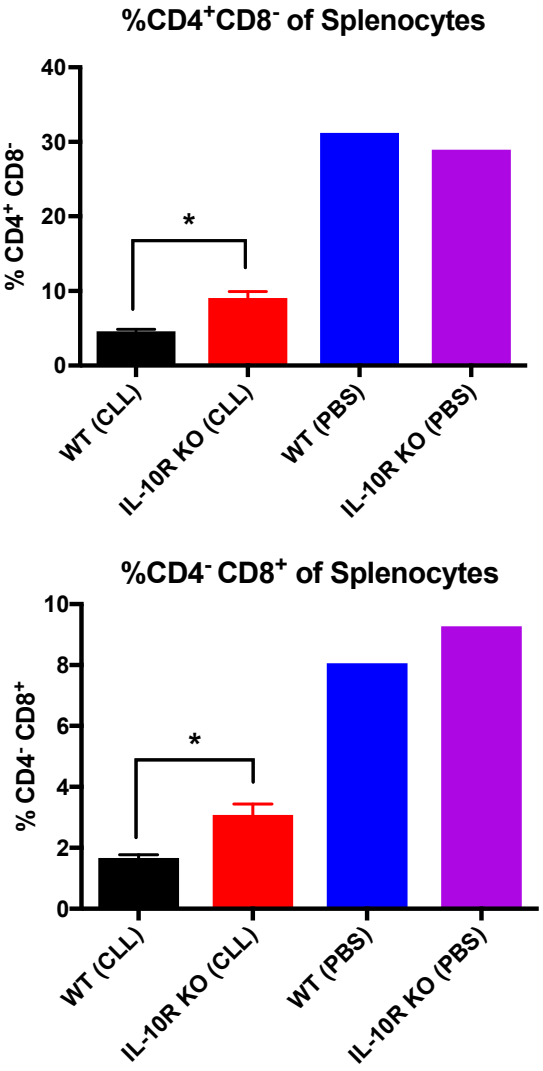


Figure 4.5: Frequency of T cells are reduced in WT mice in compare to IL-10R KO mice injected with CLL

Eμ-*TCL1* CLL cells (4 x 10^6) were adoptively transferred into WT and IL-10R KO mice by retro-orbital injection. At Day 23 post injection, mice were euthanized and splenic cells were stained for CD4 and CD8. Frequencies of CD4+CD8- cells **(Top)** and CD4-CD8+ cells **(Bottom)** are indicated. Values represent arithmetic mean of data from five mice per group ± SD. *p< 0.05.

Figure 4.6A

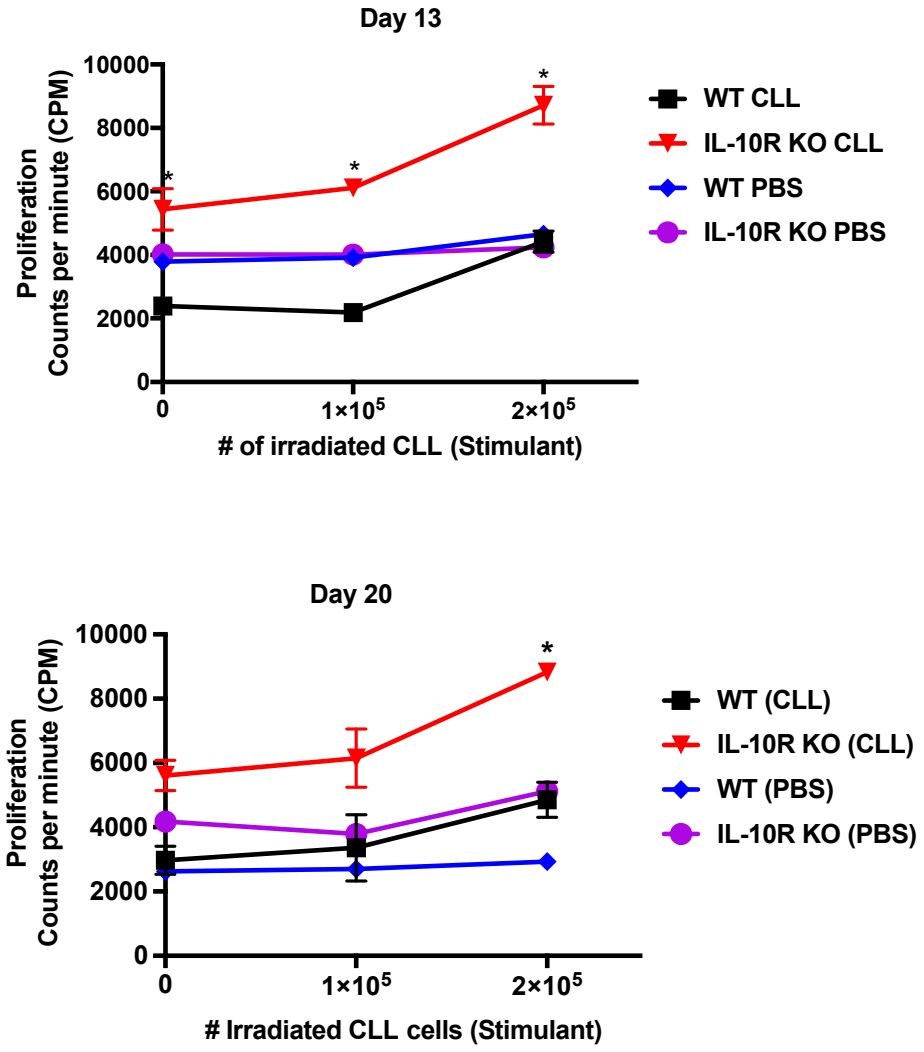

Day 13
Proliferation
Counts per minute (CPM)
10000
8000
6000
4000
2000
0
0
1×10^5
2×10^5
# of irradiated CLL (Stimulant)
*
*
*
WT CLL
IL-10R KO CLL
WT PBS
IL-10R KO PBS
Day 20
Proliferation
Counts per minute (CPM)
10000
8000
6000
4000
2000
0
0
1×10^5
2×10^5
Irradiated CLL cells (Stimulant)
*
WT (CLL)
IL-10R KO (CLL)
WT (PBS)
IL-10R KO (PBS)

Figure 4.6B

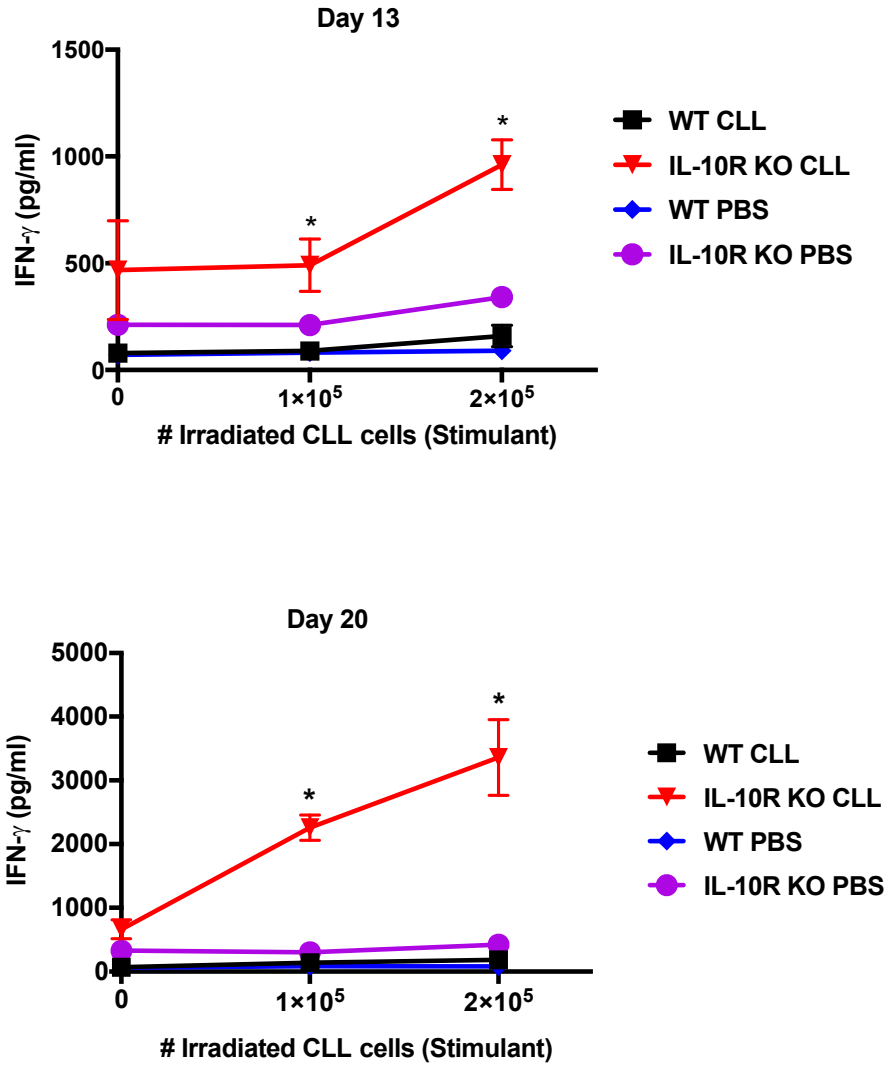
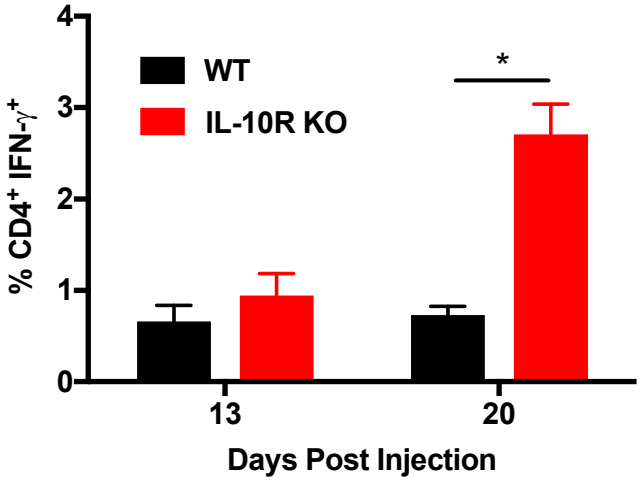

Day 13
1500
1000
500
0
IFN-γ (pg/ml)
*
*
0
1×10^5
2×10^5
Irradiated CLL cells (Stimulant)
WT CLL
IL-10R KO CLL
WT PBS
IL-10R KO PBS
Day 20
5000
4000
3000
2000
1000
0
IFN-γ (pg/ml)
*
*
0
1×10^5
2×10^5
Irradiated CLL cells (Stimulant)
WT CLL
IL-10R KO CLL
WT PBS
IL-10R KO PBS

Figure 4.6C


4
3
2
1
0
% CD4+ IFN-γ+
WT
IL-10R KO
*
13
20
Days Post Injection

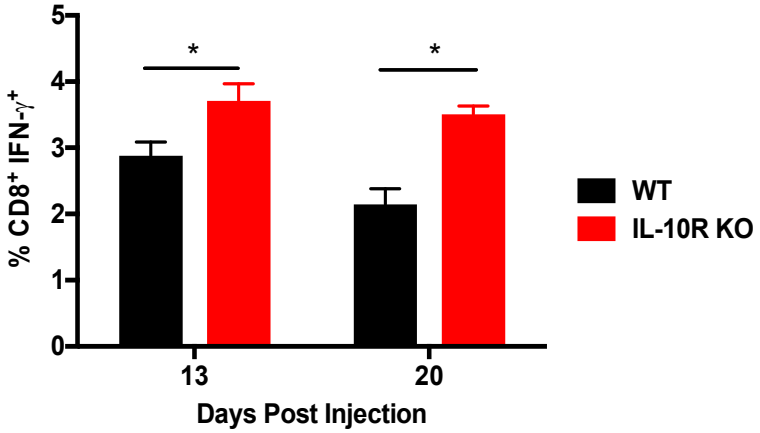
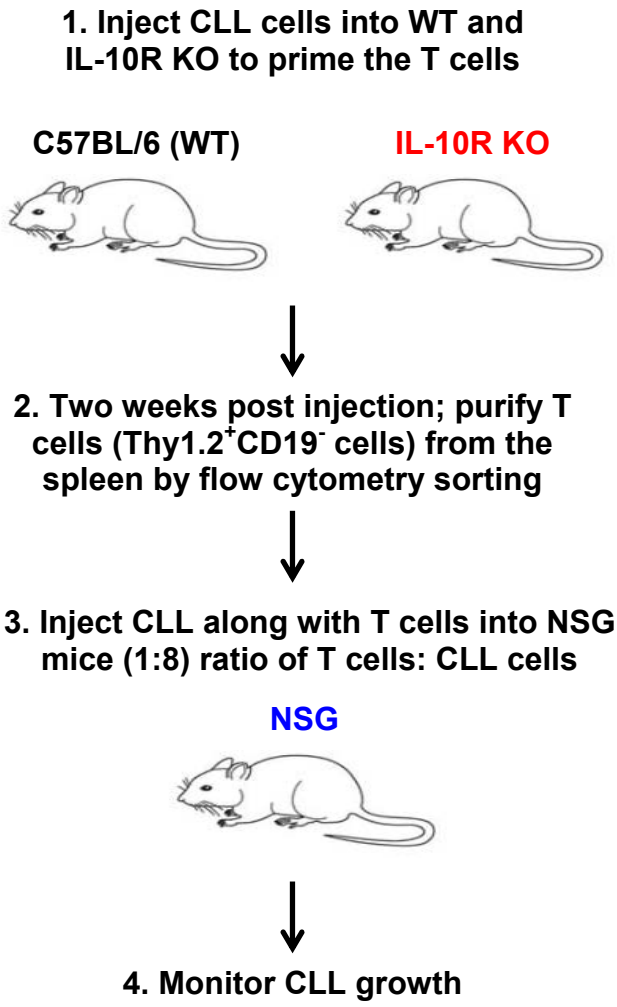

5
4
3
2
1
0
% CD8+ IFN-γ+
*
*
13
20
Days Post Injection
WT
IL-10R KO

Figure 4.6: IL-10 caused a decrease in T cell function in mice injected with CLL cells

$CD8^+$ cells were purified from spleen of WT and IL-10R KO mice using $CD8^+$ microbeads 13 and 20 days post CLL injection. $CD8^+$ cells were cultured alone or with irradiated 1 x 10^5 CLL cells at a ratio of 1:2 or 1:1 for 72 hours. **(A)** Tritiated thymidine was added to culture in the last 4 hours and proliferation was measured by ^{3}[H] incorporation using a beta plate counter. Values represent mean ± SD of triplicate cultures. **B)** CD8+ cells were cultured with irradiated CLL cells at a ratio of 1:2 or 1:1 for 24 hours. Supernatant was collected and IFN-γ was measured by ELISA. Values represent mean ± SD of triplicate cultures. **C)** Surface staining for CD4 (top panel) and CD8 (bottom panel) and intracellular staining of IFN-γ was performed on splenic cells from WT and IL-10R KO mice obtained 13 and 20 days post CLL injection. Cells were stimulated for 4 hours with PMA and ionomycin before staining. Values represent arithmetic mean of four recipient mice ± SE. *p< 0.05. Representative results from one of two experiments are shown.

Figure 4.7



Prediction: Engraftment of CLL will be lower/slower in the mice that received T cells from IL-10R KO mice.

Figure 4.7: Experimental model for T cell adoptive transfer experiment

Figure 4.8A

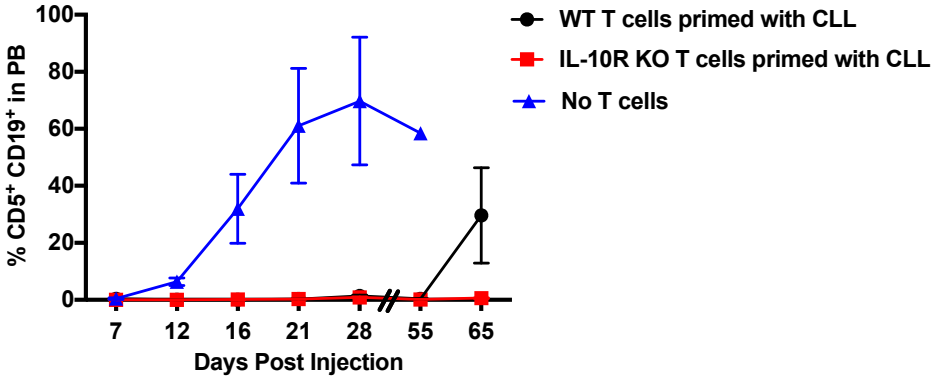


Figure 4.8B

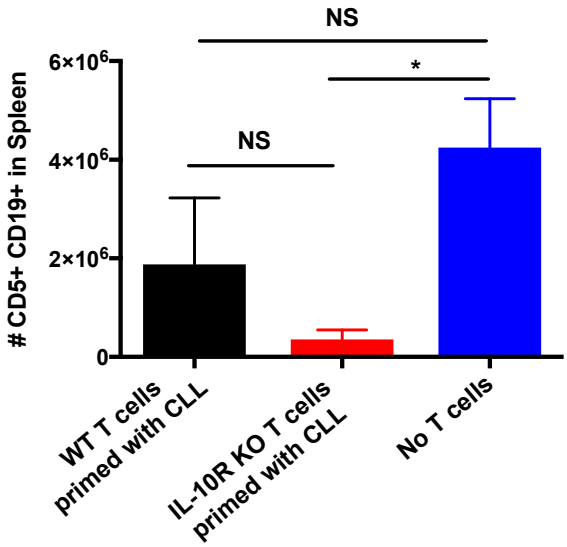


Figure 4.8C

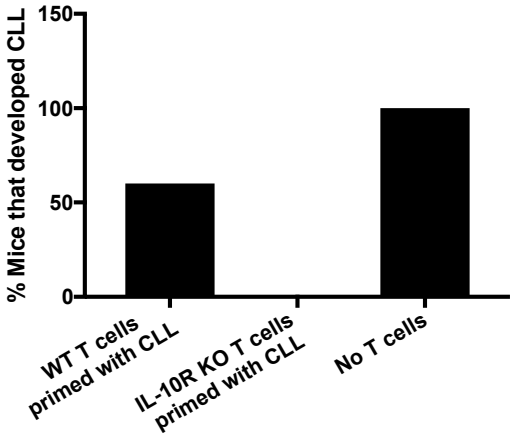


Figure 4.8: Adoptive transfer of CLL primed T cells delayed CLL growth

A) WT and IL-10R KO mice received an IV injection of CLL cells. 17 days post injection; Thy1.2^{+} cells from the CLL recipient mice were sorted by flow cytometry. CLL cells and Thy1.2 cells were then injected into NSG mice at a ratio of one T cell to 8 CLL cells and leukemic status was monitored by staining for CD5^{+}CD19^{+} cells in the blood at the time points indicated. Values represent mean values ± SE (n= 4-5 recipients). **B)** #CD5^{+} CD19^{+} cells are determined in the spleen of NSG mice at the time of euthanization. **C)** Bar graph representing the percentage of mice that developed CLL from each group at the end of the experiment.

Figure 4.9A

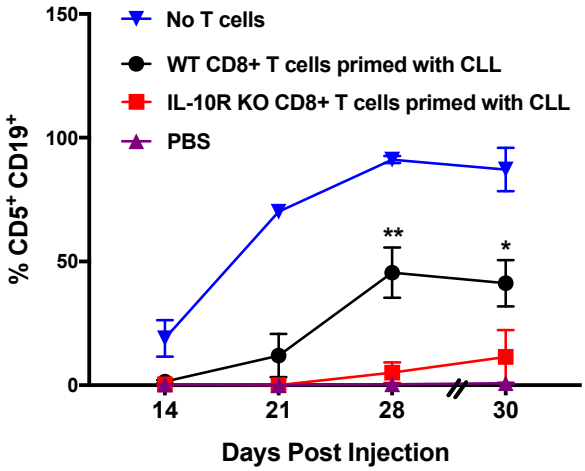


Figure 4.9B

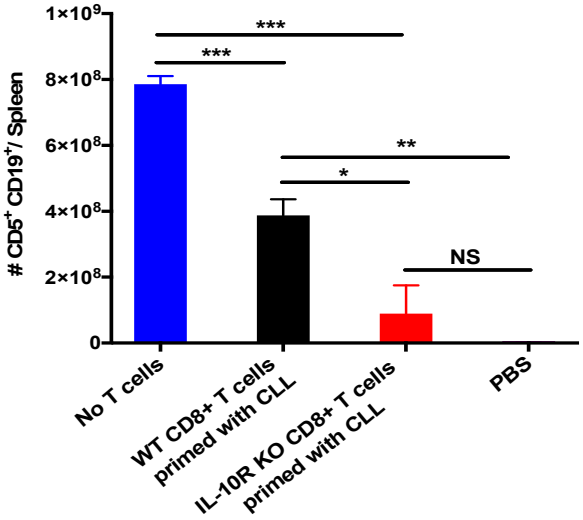


Figure 4.9C

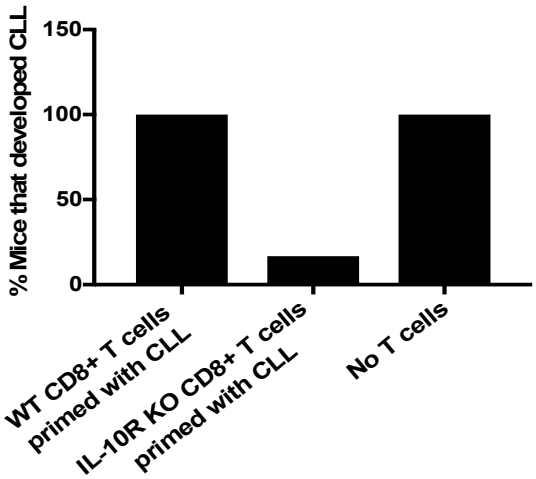


Figure 4.9: The adoptive transfer of CLL primed CD8+ T cells was sufficient in delaying CLL growth

A) WT and IL-10R KO mice received an IV injection of CLL cells. 14 days post injection; CD8+ cells from the CLL recipient mice were isolated with CD8+ microbeads and the autoMACS cell separator. CLL cells and CD8+ T cells were then injected into NSG mice at a ratio of one T cell to 32 CLL cells and leukemic status was monitored by staining for $CD5^{+}CD19^{+}$ cells in the blood at the time points indicated. Values represent mean values ± SE (n=6 recipients). The significance indicated is by comparing the WT CD8+ T cells primed with CLL and the IL-10R KO CD8+ T cells primed with CLL groups. **B)** #$CD5^{+}$ $CD19^{+}$ cells are determined in the spleen of NSG mice at the time of euthanization. **C)** Bar graph representing the percentage of mice that developed CLL from each group at the end of the experiment. *p< 0.05, p**< 0.01, p***< 0.001, NS; not significant.

Summary

The immune system plays an important role in controlling tumor progression. Effective antitumor immune response depends on close interaction of several elements of the immune system. These include antigen-presenting cells, different subsets of T cells, B cells and NK cells. Here as predicted we found that adaptive immune responses played a significant role in chronic lymphocytic leukemia. CLL cell growth *in vivo* was significantly faster in mice that lacked B, T and NK cells in comparison to WT mice with intact immune system. However, eventually CLL cells, given enough time, are able to engraft tissues of the wild type mice equally, which indicates that tumor cells develop a number of mechanisms to escape recognition and elimination by immune system. CLL cells are capable of producing the immunosuppressive protein, IL-10, constitutively. Initially, we observed that engraftment of CLL cells in comparison to WT mice was significantly lower in IL-10R KO mice, in which IL-10 is unable to carry out its immunosuppressive functions. Since IL-10 has been known for its effects on a number of immune cells including APCs and T cells, we first tested if the difference in engraftment was due to IL-10 effects on APCs. Macrophages and monocytes are known to secrete many cytokines and chemokines that affect the localization and survival of CLL cells in the microenvironment. Although is our studies we found no significant difference in CLL cell-localization in IL-10 sufficient and deficient mice, we cannot eliminate the role of these innate cells in CLL. Future studies will investigate the inhibitory effects IL-10 might have on macrophages and monocytes in the CLL model. Next, we examined the effects

of IL-10 on T cell responses in CLL model. We found that the proliferation of T cells isolated from IL-10R KO mice upon restimulation with irradiated CLL cells was significantly higher than proliferation of T cells from WT mice. In addition, $CD8^+$ T cells isolated from IL-10R KO mice exhibited a higher capacity to secrete IFN-γ than $CD8^+$ T cells from WT mice injected with CLL cells. Finally, upon transfer of primed T cells from both WT and IL-10R KO mice into CLL-injected NSG mice, T cells were able to delay the progression of CLL, where T cells from IL-10R KO mice were more effective. Interestingly, CD8+ T cells were sufficient in delaying CLL disease development and CD8+ T cells isolated from IL-10R KO primed with CLL were significantly better at controlling CLL growth than CD8+ T cells isolated from WT mice. Future studies will need to investigate if CD4+ T cells have similar effects in controlling CLL growth.

CHAPTER 5

Role of BCR signaling in constitutive and induced IL-10 production by CLL cells and a novel role of Sp1 in regulating IL-10 production by CLL B cells

After having established a role for IL-10 in CLL growth, we investigated the possibility of targeting IL-10 to overcome its immunosuppressive effects. Therefore, we aimed to identify the possible mechanisms by which IL-10 is produced constitutively by CLL cells, which may help identify targets to prevent IL-10 production and immunosuppression associated with it. The molecular mechanisms involved in IL-10 production by many cells of the immune system have been extensively studied, however, it is not clear whether the molecular mechanisms required for the induction of IL-10 by B cells are regulated by the same factors that regulate IL-10 production by T helper cells, macrophages and DCs. Due to the importance of tonic B cell receptor signaling in the survival of normal B cells, as well as its impact on the survival and growth of malignant B-cell clones in CLL and in other non-Hodgkins lymphomas, here we investigated the role of BCR signaling in IL-10 production by CLL cells [98-101]. We discovered a novel role of BCR signaling in IL-10 production by CLL cells [54]. BCR dependent constitutive activation of Src or Syk family kinase is required for constitutive IL-10 production by both mouse and human CLL cells. This work to understand the molecular pathways leading to IL-10 production CLL cells by BCR signaling would provide valuable information on possible targets for IL-10 manipulation and modulation of the immune response in CLL.

Results

(5a) The novel role of BCR signaling in IL-10 production by Eμ-*TCL1 CLL cells*

During our preliminary studies, we were able to establish a novel role of BCR signaling in IL-10 production by normal B-1 and malignant Eμ-*TCL1* CLL cells (Figure 5.1). For the majority of our Eμ-*TCL1* CLL cells, crosslinking the BCR with anti-IgM led to an increase in IL-10 production (Figure 5.1A). Inhibition of Src, Btk or Syk family kinases that are essential for signal transduction via BCR reduced both constitutive and anti-IgM induced IL-10 production by Eμ-*TCL1* CLL cells in a dose dependent manner (Figure 5.1B).

Additionally, we utilized a human CLL cell line called MEC1 cells in our studies. MEC1 cells grew spontaneously from the peripheral blood of a patient with CLL in prolymphocytoid transformation [127]. MEC1 cell line expresses the same light and heavy chains as the parental CLL cells with similar intensity. MEC1 cells are $CD19^+$, $CD20^+$, $CD21^+$ and $CD22^+$. However, MEC1 cells are $CD5^-$ but also produce IL-10 constitutively and IL-10 was found to be regulated by BCR signaling in MEC1. Constitutive production of IL-10 by MEC1 cells is diminished by Src, Syk or Btk inhibition (Figure 5.1C). Here we also opted to use MEC1 cells in our gene silencing experiments due to the difficulties we faced in transfecting primary mouse or human CLL cells. We used a short hairpin RNA (shRNA) to knock down Lyn, a Src family kinase and one of the earliest enzymes activated with BCR cross-linking. MEC1 cells with 50% lyn knock-down produced less IL-10 than control shRNA treated cells (Figure 5.1D). Stimulation of Eμ-*TCL1* CLL cells by BCR cross-linking led to an increase of IL-10 mRNA levels

and inhibition of Syk reduced those levels (Figure 5.1E), suggesting that IL-10 production is controlled at the transcript level. To further verify IL-10 production by Eμ-*TCL1* cells, we performed IL-10 intracellular staining. 60-80% of Eμ-*TCL1* CLL cells were IL-10 producers, which was reduced by Syk inhibition (Figure 5.2).

(5b) IL-10 production by Eμ-TCL1 CLL cells is dependent on ERK1/2 MAPK and the transcription factor Sp1 but not on p38MAPK or STAT3

Importance of P38/MAPK and STAT3 was tested since they are known to be involved in IL-10 production by myeloid cells. Surprisingly, phosphorylation of the P38/MAPK or the transcription factor STAT3 was not affected by Syk inhibition (Figure 5.3A). On the other hand, phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) was reduced after the inhibition of Syk (Figure 5.3A). In order to find a possible downstream transcription factor, which is involved in BCR induced IL-10 production, we tested the transcript levels of multiple transcription factors known to be required for IL-10 transcription in various immune cells. These included SMAD4, GATA3, CREB, ATF1 and Sp1 [84]. None of these transcription factors except Sp1 were regulated by BCR signaling, as their transcript levels did not change upon stimulation with anti-IgM (Figure 5.3B). Sp1 was the only tested transcription factor found to be significantly enhanced by BCR signaling and reduced by Syk inhibition (Figure 5.3C). Treatment of Eμ-*TCL1* CLL cells with Mithramycin A, a well-known inhibitor of Sp1 [128], reduced IL-10 protein levels in a dose dependent manner (Figure 5.3D-E). Sp1 is proposed to bind and transactivate *IL10* gene in

macrophages and T cells [129]. To further establish its role in IL-10 transcription in CLL cells, we utilized chromatin immunoprecipitation (ChIP) to test if Sp1 binds to the IL-10 promoter in CLL cells. RT-PCR of the ChIP product revealed 8-fold enrichment in binding of Sp1 to IL-10 promoter over the input sample (Figure 5.3F). Taken together, these results indicate that IL-10 production by Eμ-*TCL1* CLL cells is dependent on the transcription factor Sp1.

Previous studies have indicated the important role of ERK1/2 in Sp1 activation [130]. Since we already showed that ERK1/2 activation is regulated by BCR signaling (Figure 5.3A), we hypothesized that ERK1/2 activation is the link between BCR and Sp1. Accordingly, treatment of Eμ-*TCL1* CLL cells with SCH772984, a specific ERK1/2 inhibitor decreased IL-10 production in a dose-dependent manner (Figure 5.4A). Interestingly, Sp1 protein levels were also reduced upon ERK1/2 inhibition (Figure 5.4B). Also, consistent with data using Syk inhibitor, ERK1/2 inhibitor did not affect the activation of STAT3 (Figure 5.4B). To demonstrate a better correlation between reduced ERK1/2 phosphorylation and Sp1 levels, we measured Sp1 transcript levels after ERK1/2 inhibition and found that Sp1 mRNA was significantly reduced after ERK1/2 inhibition (Figure 5.4C). This suggests that IL-10 production by CLL cells is regulated by the activation of ERK1/2 and subsequent activation of Sp1 leading to IL-10 transcription.

(5c) BCR signaling regulates IL-10 production by human CLL cells

We tested the role of BCR signaling in IL-10 production by human CLL cells. Table 5.1 summarizes the properties of the CLL patients' donor pool.

Peripheral blood mononuclear cells (PBMCs) from CLL patients produced a significant amount of IL-10 only after LPS stimulation or BCR cross-linking in comparison to normal human PBMCs, with very little constitutive production (Figure 5.5A). BCR cross-linking with anti-IgM leads to increased IL-10 production by human CLL cells in a dose dependent manner (Figure 5.5B). There was a significant amount of IL-10 in the plasma of CLL patients while it was nearly undetectable in healthy age matched individuals, though there was some variability (Figure 5.5C). In addition, neutralization of LPS or anti-IgM induced-IL-10 did not affect the survival of human CLL cells (Figure 5.5D), consistent with data seen using mouse CLL cells (Figure 3.3). Inhibition of Src, Syk family kinases or Btk led to the complete abrogation of anti-IgM induced IL-10 production by human CLL cells (Figure 5.5E). Similar to mouse CLL cells, inhibition of BCR signaling in human CLL reduced ERK1/2 activation, Sp1 activity and IL-10 levels (Figure 5.5F).

Figure 5.1A

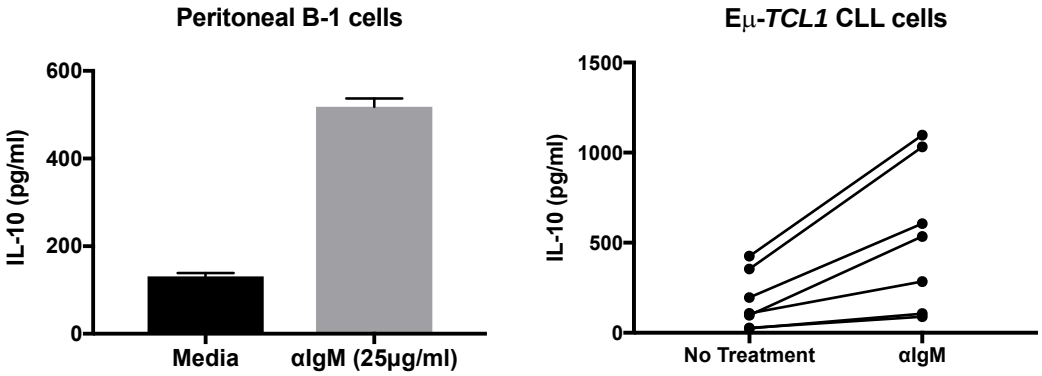


Figure 5.1B

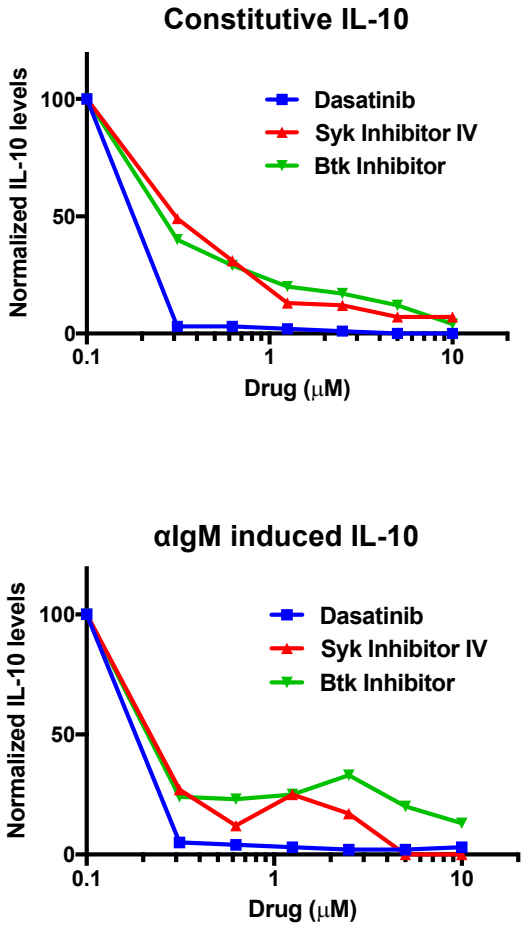


Figure 5.1C

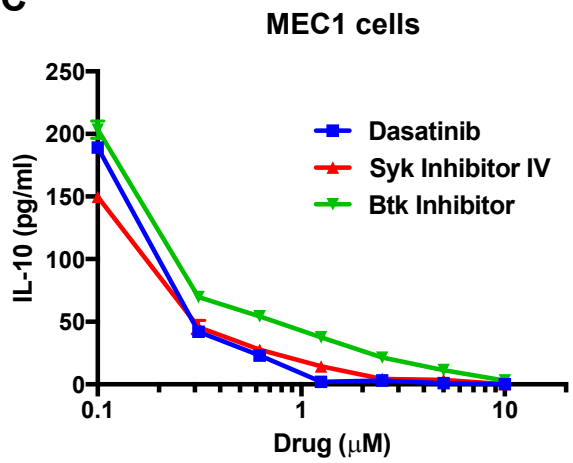


Figure 5.1D

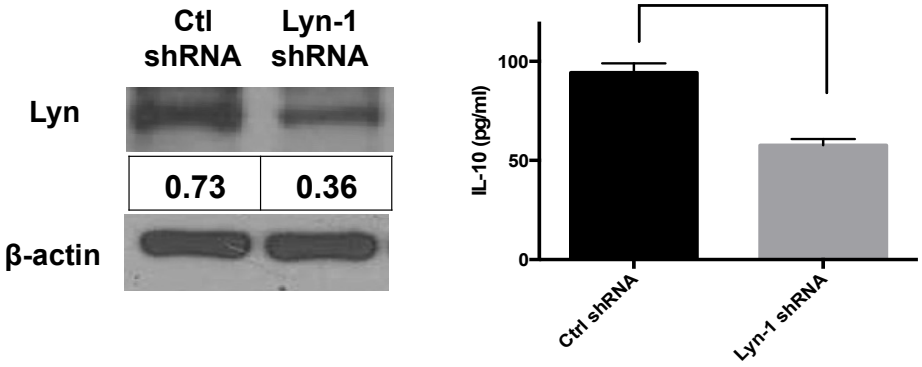


Figure 5.1E

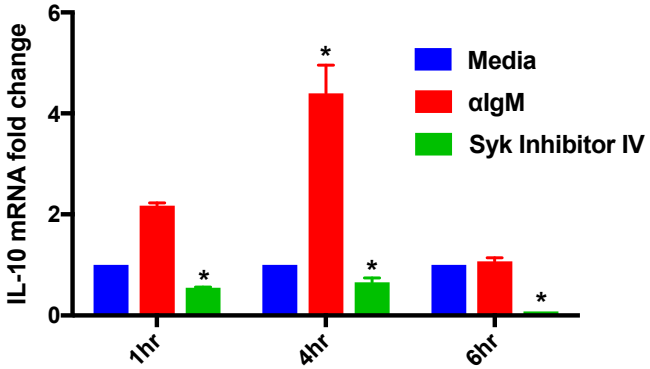


Figure 5.1: The role of BCR signaling in IL-10 production by Eμ-*TCL1* CLL cells

A) Normal peritoneal B-1 cells (Left) and Eμ-*TCL1* CLL cells (Right) were cultured with or without αIgM (25μg/ml) for 24 hours. Supernatants were collected and IL-10 levels were measured by ELISA. Each line represents a single clone of Eμ-*TCL1* CLL cells (clone=cells from one individual Eμ-*TCL1* mouse) **B)** Eμ-*TCL1* CLL cells were cultured without (top) or with (bottom) αIgM (25μg/ml) and then treated with indicated doses of dasatinib (A SRC family kinase inhibitor), Syk inhibitor IV (BAY 61-3606) or Btk inhibitor (Ibrutinib) for 24 hours. Supernatants were collected and IL-10 levels are measured by ELISA. Values are normalized to the no drug control and set to 100%. Values represent mean ± SD of triplicate cultures. Results are representative of 4-8 experiments. **C)** MEC1 cells were cultured with inhibitors indicated as in Panel B and supernatants were collected after 24 hours. IL-10 levels were measured by ELISA. Values represent mean ± SD of triplicate cultures. Results are representative of two experiments. **D)** Western blot showing a reduction in Lyn in MEC1 cells expressing Lyn specific shRNA. Lyn protein values were normalized to β-actin (Left). IL-10 levels were measured in the supernatant of MEC1 cells expressing either control shRNA or Lyn shRNA (Right). **E)** IL-10 mRNA levels are determined by qRT-PCR in Eμ-*TCL1* CLL cells treated with or without αIgM (25μg/ml) in the presence or absence of Syk inhibitor IV (5μM) for time points indicated. IL-10 mRNA expression was normalized to mouse 18S RNA. Values

represent mean ± SD of triplicate determinations. *p< 0.05. Results are representative of 2-4 experiments.

Figure 5.2

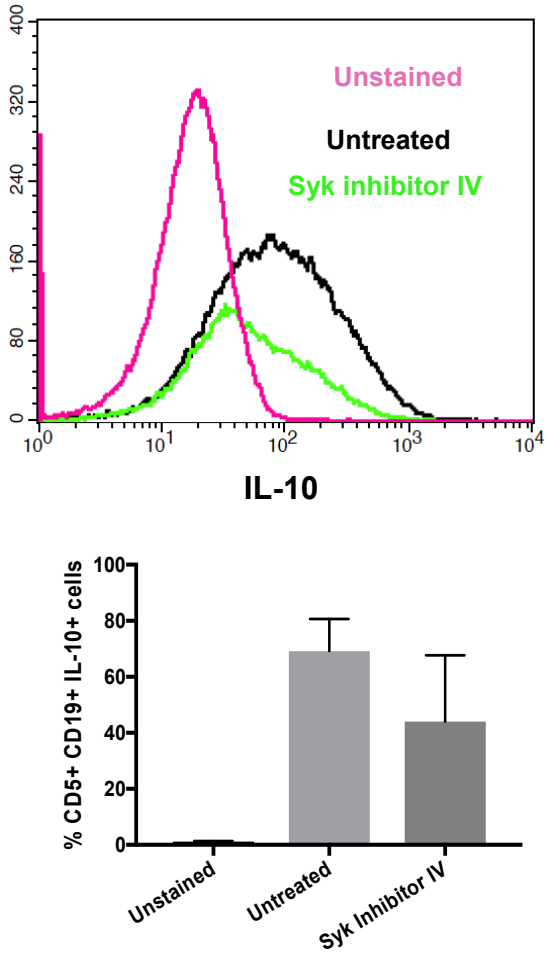


Figure 5.2: Syk inhibition leads to decrease in the number of IL-10 producing CLL cells

Eμ-*TCL1* CLL cells were cultured with or without Syk inhibitor IV (2μM) for 24 hours. Then stimulated with PMA (20ng/ml) and Ionomycin (1μg/ml) for 4 hours and intracellular IL-10 staining was performed. A representative IL-10 histogram overlay of unstained, untreated, and Syk inhibitor treated samples after gating on viable CD5+ CD19+ cells is shown on the top. The bar graph represents an average of IL-10 intracellular staining of 3 Eμ-*TCL1* mice CLL cells with or without Syk inhibition (bottom). Values represent mean ± SEM.

Figure 5.3A

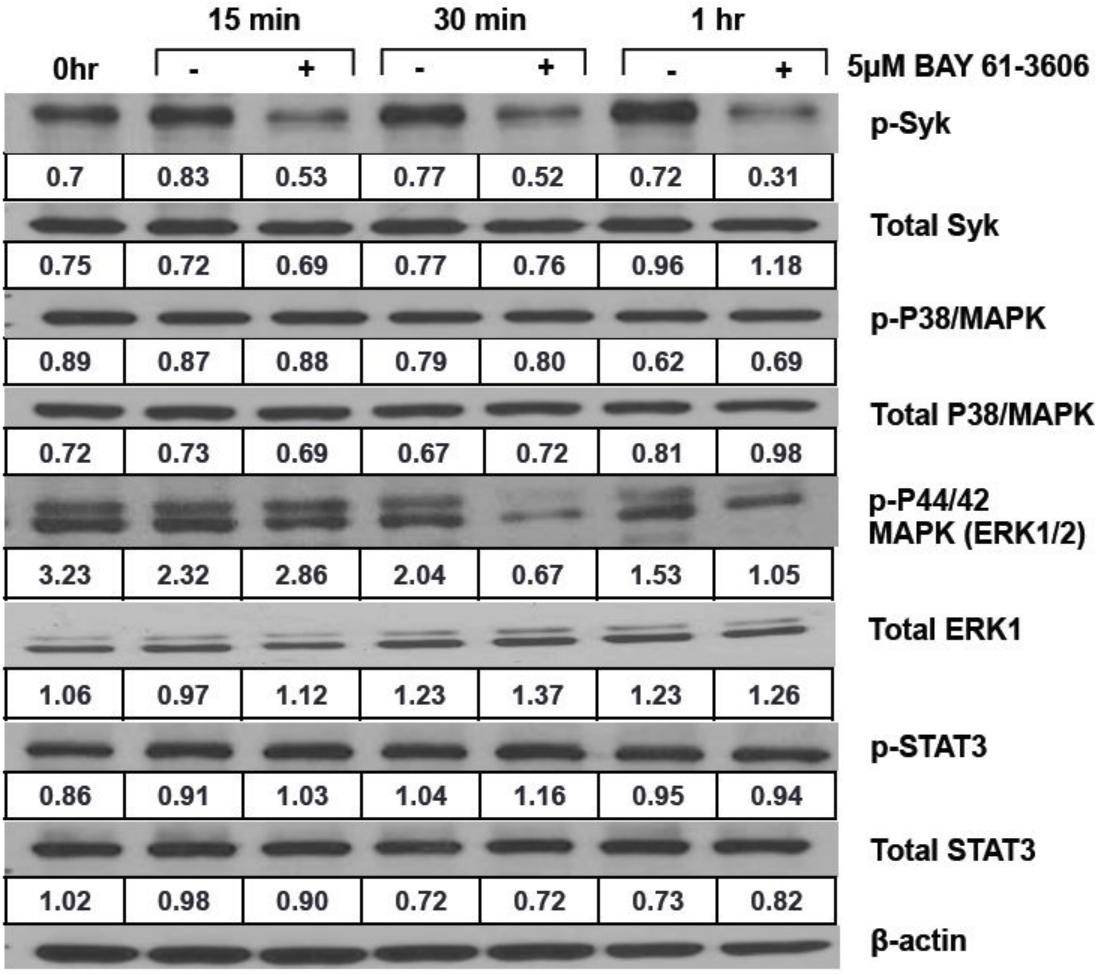

15 min
30 min
1 hr
0hr
-
+
-
+
-
+
5μM BAY 61-3606
p-Syk
0.7
0.83
0.53
0.77
0.52
0.72
0.31
Total Syk
0.75
0.72
0.69
0.77
0.76
0.96
1.18
p-P38/MAPK
0.89
0.87
0.88
0.79
0.80
0.62
0.69
Total P38/MAPK
0.72
0.73
0.69
0.67
0.72
0.81
0.98
p-P44/42
MAPK (ERK1/2)
3.23
2.32
2.86
2.04
0.67
1.53
1.05
Total ERK1
1.06
0.97
1.12
1.23
1.37
1.23
1.26
p-STAT3
0.86
0.91
1.03
1.04
1.16
0.95
0.94
Total STAT3
1.02
0.98
0.90
0.72
0.72
0.73
0.82
β-actin

Figure 5.3B

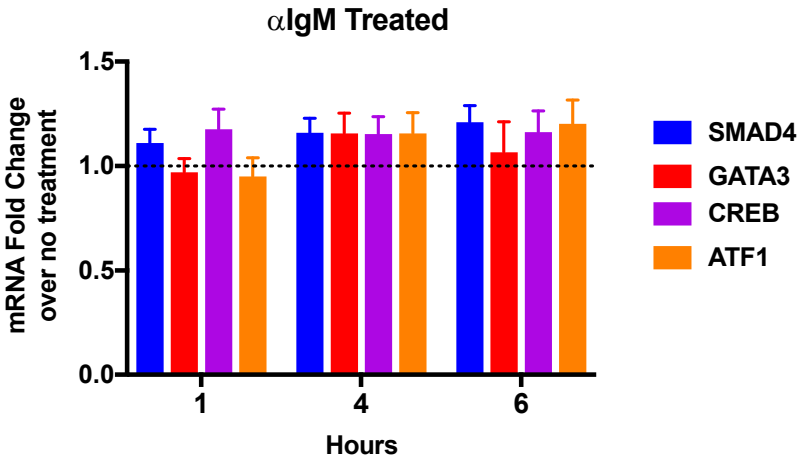


Figure 5.3C

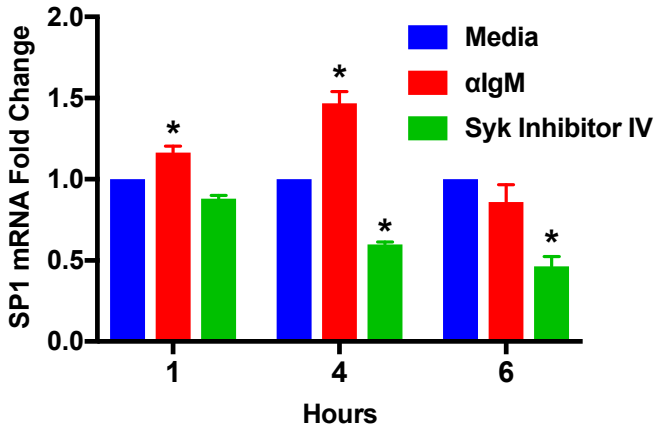


Figure 5.3D

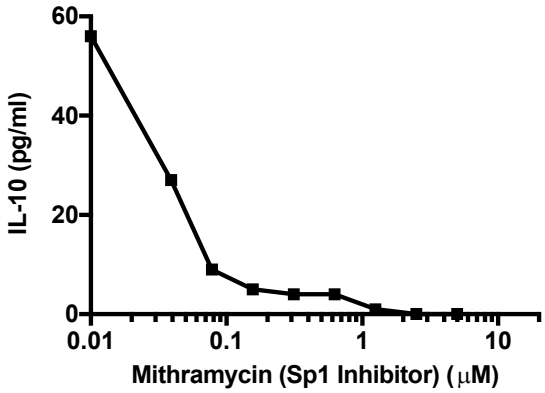


Figure 5.3E

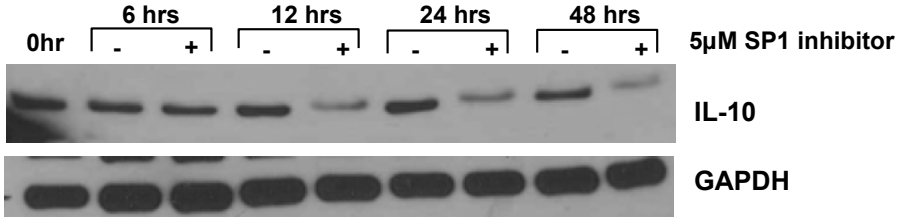


Figure 5.3F

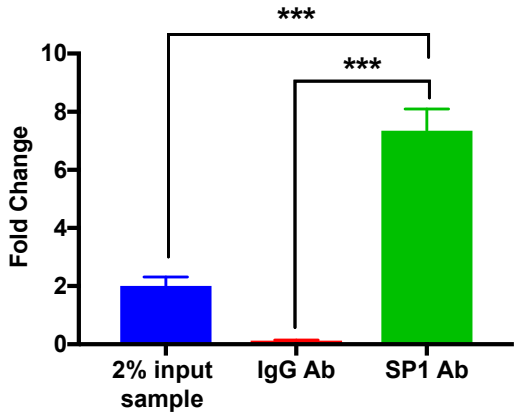


Figure 5.3: IL-10 production by Eμ-*TCL1* CLL cells is dependent on ERK1/2 MAPK and the transcription factor Sp1 but not on p38MAPK or STAT3

A) Eμ-*TCL1* CLL cells were treated with 5μM of Syk inhibitor IV for indicated time periods. Levels of p-Syk, total Syk, p-P38 MAPK, total P38 MAPK, p-ERK1/2, total ERK1/2, p-STAT3, and total STAT3 were quantified by Western blot. Phospho-protein levels were normalized to total protein. Total protein levels were normalized to β-actin. Numbers indicate quantification of band intensity by ImageJ software. Results are representative of three experiments. **B)** mRNA levels of transcripts indicted were quantified by qRT-PCR after treatment of Eμ-*TCL1* CLL cells with αIgM (25μg/ml) for 1, 4, or 6 hours. Fold change was normalized to the no-treatment group (dashed line). Values represent mean ± SD of triplicate determinations. **C)** Sp1 mRNA levels were quantified by qRT-PCR after treatment of Eμ-*TCL1* CLL cells with αIgM (25μg/ml) or Syk inhibitor IV (5μM) for 1, 4, or 6 hours. Fold change was normalized to the no-treatment group. Values represent mean ± SD of triplicate determinations. **D)** Eμ-*TCL1* CLL cells were treated with various doses of mithramycin A, an Sp1 inhibitor for 24 hours. Culture supernatants were collected and IL-10 was measured by ELISA. **E)** Western blot analysis of IL-10 protein levels in CLL cells after treatment with mithramycin A (5μM) for indicated time points. GAPDH was used for loading control. **F)** ChIP was carried out as described in the methods. Antibodies against Sp1 and IgG (control) were used for Chromatin IP. qRT-PCR was performed on the ChIP DNA product using primers specific for the consensus Sp1 binding site sequence in the IL-10 promoter. Data is calculated using the Fold Enrichment

Method. This normalization method is relative to the no-antibody control (2% input sample). *p< 0.05, ***p<0.001

Figure 5.4A

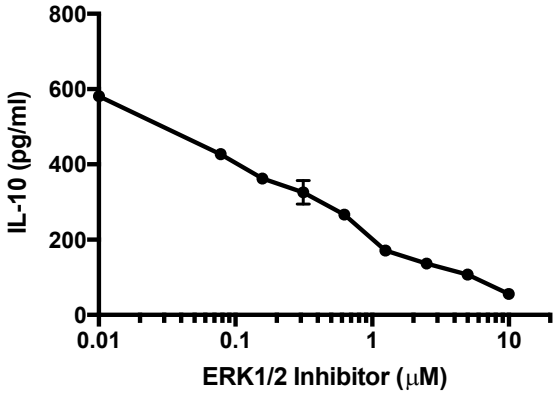


Figure 5.4B

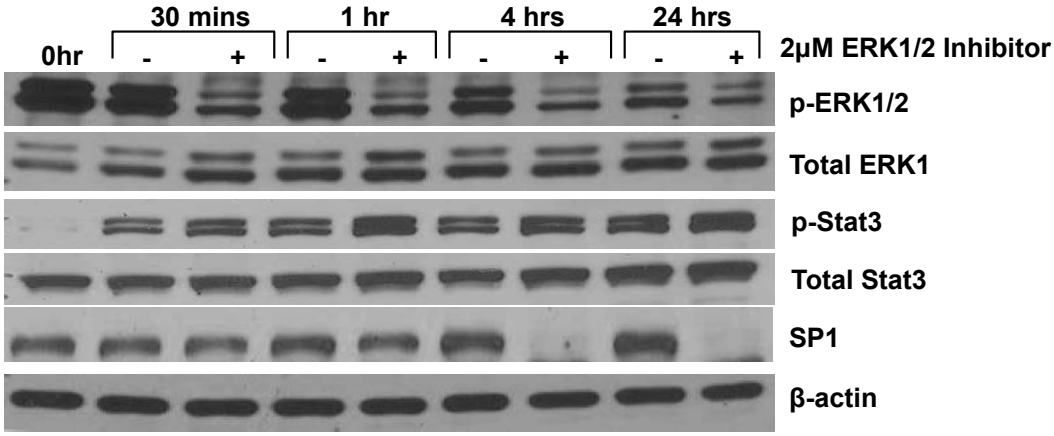


Figure 5.4C

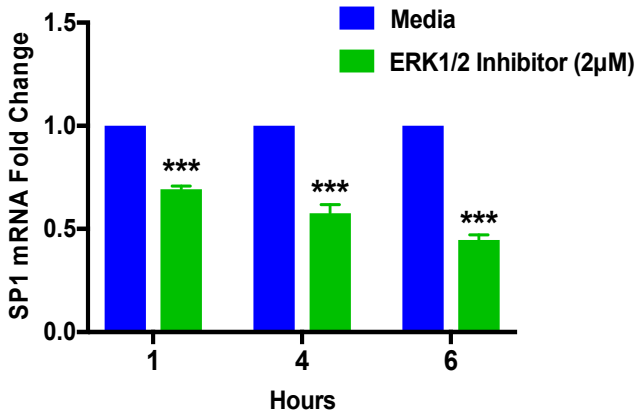


Figure 5.4: ERK1/2 inhibition leads to Sp1 degradation and inhibition of IL-10 production in CLL cells

A) Eμ-*TCL1* CLL cells were cultured with the ERK1/2 inhibitor (SCH772984) and supernatants were collected after 24 hours. IL-10 levels were measured by ELISA. Values represent mean ± SD of triplicate cultures. **B)** Eμ-*TCL1* CLL cells were treated with ERK1/2 inhibitor (2μM) for indicated time points. Levels of p-ERK1/2, total ERK1, p-STAT3, total STAT3 and Sp1 were quantified by Western blot analysis. β-actin was used for loading control. Results are representative of three experiments. **C)** Sp1 mRNA levels were quantified by qRT-PCR after treatment of Eμ-*TCL1* CLL cells with ERK1/2 inhibitor (2μM) for 1, 4, or 6 hours. Fold change was normalized to the no-treatment group. Values represent mean ± SD of triplicate determinations. ***$p<0.001$

Table 5.1: Properties of the CLL patients' donor pool

Patient#	Age	Sex	WBC (K/μL)	%CD5+ CD19+	CD38 Status	Treatment	IGHV mutation
1	82	F	20.7	90.54	Negative	No	M-CLL
2	56	F	ND	82.35	ND	No	U-CLL
3	36	M	38.3	88.08	Negative	No	M-CLL
4	46	M	41	44.01	Negative	No	M-CLL
5	52	M	15.2	81.37	ND	Yes	M-CLL
6	69	M	29	83.88	Positive	No	M-CLL
7	57	M	40	50.33	ND	Yes	U-CLL
8	62	F	83.7	10.55	ND	No	U-CLL
9	69	M	30.2	95.71	ND	No	M-CLL
10	70	M	17.4	80.45	ND	No	M-CLL
11	63	M	34.8	96.97	ND	No	M-CLL
12	53	M	12.1	84.73	Positive	Yes	U-CLL
13	55	M	6	13.64	ND	Yes	M-CLL
14	76	F	36.6	37.23	ND	Yes	M-CLL
15	80	M	142	97.73	ND	Yes	U-CLL

ND; Not determined

Figure 5.5A

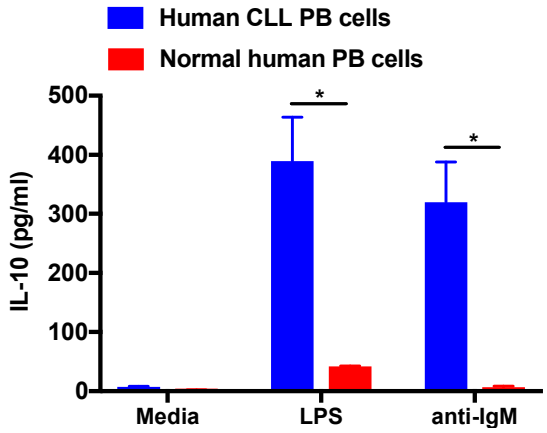


Figure 5.5B

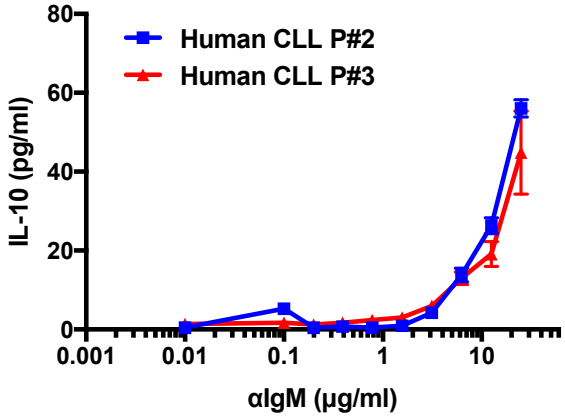


Figure 5.5C

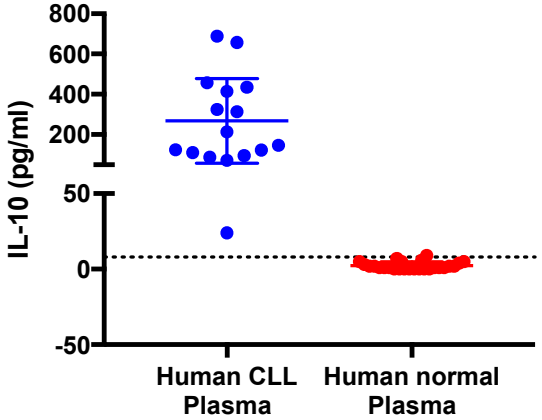


Figure 5.5D

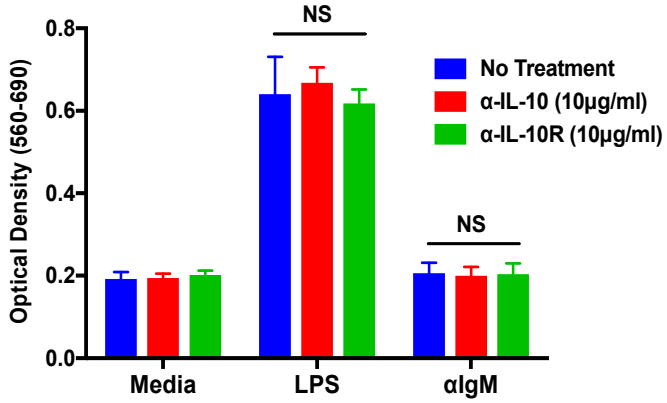


Figure 5.5E

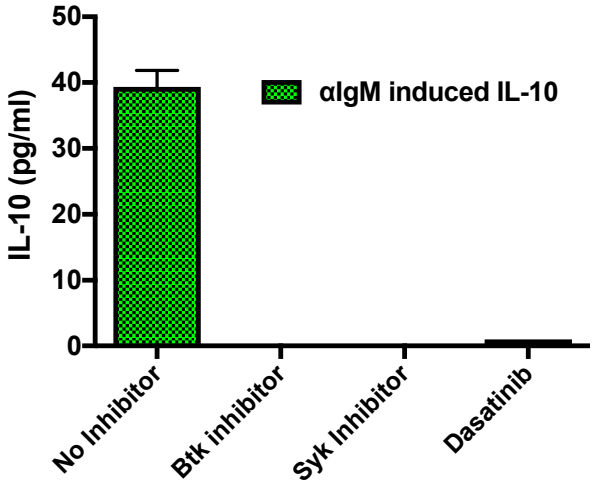


Figure 5.5F

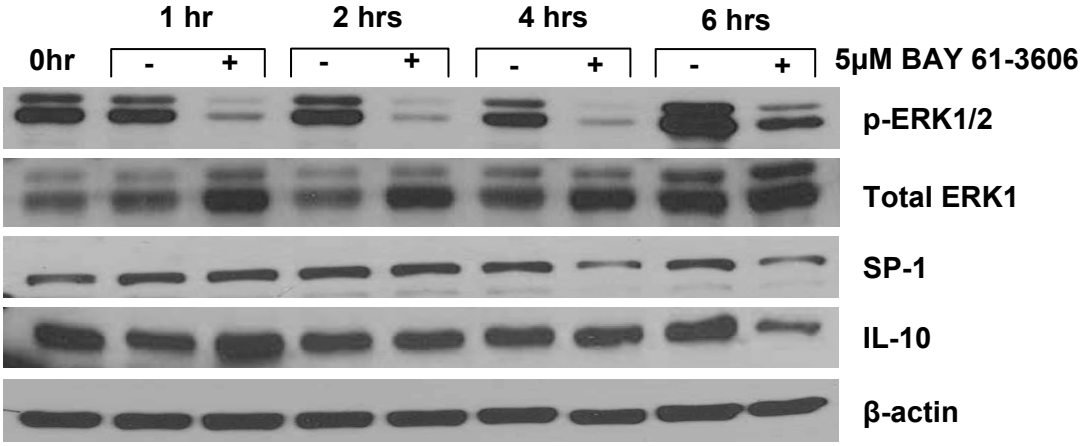


Figure 5.5: Human CLL cells share similar mechanisms to mouse CLL cells for IL-10 production

A) Human CLL cells obtained from peripheral blood of CLL patients were cultured and stimulated with LPS (5μg/ml) or αIgM (25μg/ml) for 24 hours. IL-10 was measured in the supernatant of the cells by ELISA. Values represent mean ± SD of triplicate cultures. **B)** Human CLL cells were cultured and stimulated with increasing doses of αIgM for 24 hours. IL-10 was measured in the supernatant of the cells by ELISA. The graph represents data from 2 human CLL samples. Values represent mean ± SD of triplicate cultures. **C)** Human CLL patients (n=16) as well as normal donors (n=28) plasma levels of IL-10 were measured by ELISA (n=16). **D)** Human CLL cells were cultured with αIL-10 or αIL-10R antibodies with or without LPS (5μg/ml) or anti-IgM (25μg/ml) for 48 hours. Survival of CLL cells were measured by MTT. Values represent mean ± SD of triplicate cultures. NS; not significant. **E)** Human CLL cells stimulated with αIgM (25μg/ml) and treated with or without Btk inhibitor, Syk inhibitor or dasatinib for 24 hours. Supernatants were collected and IL-10 levels were measured by ELISA. Values represent mean ± SD of triplicate cultures. **F)** Human CLL cells were treated with Syk inhibitor (5μM) for indicated time points. Levels of p-ERK1/2, total ERK1, Sp1 and IL-10 were quantified by Western blot analysis. β-actin was used for loading control. Results were reproducible with 3 CLL patient samples.

Summary

In this chapter we demonstrated a novel role of BCR signaling in the constitutive production of IL-10. Since BCR signaling is important in CLL subtypes such as U-CLL, BCR-induced IL-10 could be a factor in their more aggressive nature. We found that inhibition of the major kinases in the BCR signaling pathway including Src, Syk and Btk leads to the significant reduction of IL-10 production by Eμ-*TCL1* CLL cells. We also discovered that inhibition of BCR signaling led to a decrease in ERK1/2 MAP kinase activation, which is consistent with a role for ERK1/2 in IL-10 production seen in T cells [84]. However, BCR signaling mediated IL-10 production was STAT3 independent. In addition, we were able to reveal a novel role for the transcription factor Sp1 in BCR signaling dependent IL-10 production by Eμ-*TCL1* CLL cells. Sp1 was found to bind to the IL-10 promoter in Eμ-*TCL1* cells and inhibition of Sp1 led to a decrease in IL-10 production in a dose dependent manner. We were also able to link the activation of ERK1/2 to Sp1 activity, which is consistent with regulation of Sp1 by ERK1/2 in other cell types [130, 131]. We found that ERK1/2 inhibition leads to Sp1 degradation and inhibition of IL-10 production in both murine and human CLL cells. Many studies have indicated that phosphorylation, acetylation, sumoylation, ubiquitination and glycosylation are among the posttranslational modifications that influence the transcription activity and stability of Sp1 [132]. For example, recent studies indicated that c-Jun NH(2)-terminal kinase 1 (JNK1) phosphorylates Sp1 at Thr278 and Thr739, protecting Sp1 from ubiquitin-dependent degradation and increase its stability during mitosis in tumor cells

lines [133]. Here since we demonstrate that ERK1/2 inhibition leads to degradation of both RNA and protein levels of Sp1, we hypothesize that ERK1/2 might be controlling Sp1 RNA stability in CLL cells. To test this hypothesis, we will utilize luciferase assay using a plasmid expressing the promoter for Sp1. After transfecting the plasmid in our CLL cells, we will use the ERK1/2 inhibitor to determine its effects on Sp1 transcription. The regulation of IL-10 production by mouse CLL cells was found to be similar in human CLL cells. Despite the fact that there are significantly higher levels of plasma IL-10 in human CLL patients compared to healthy donors, PBMCs from human CLL patients do not constitutively produce IL-10. However, treatment with anti-IgM leads to an increase in IL-10 production by human CLL cells, which is completely abrogated by the inhibition of kinases in the BCR signaling pathway. As seen in the murine system B cells from healthy human patients, which are likely to be B-2 cell population produce very little IL-10 even after stimulation by BCR cross-linking. Moreover, similar to mouse CLL cells, inhibition of BCR signaling in human CLL reduced ERK1/2 activation, Sp1 activity and IL-10 levels.

CHAPTER 6

Discussion

Immune cells and humoral factors comprise two intertwined systems, innate and acquired. Immune cells scan the existence of any molecule that is considered to be nonself, which include nonself antigens presented by cancer cells. A specific immune response is generated, which results in the proliferation of antigen-specific lymphocytes. Immunity is considered acquired when antigen specific antibodies and T cells are upregulated. Both innate and acquired immune systems interact to initiate antigenic responses against tumors. A key driver of anti-tumor immunity is T cell. Many important discoveries had led to an increase in our understanding of the role of T cells in cancer. T cells are either cytotoxic (CD8+) or helper (CD4+). CD8+ T cells react with peptide antigens that have been digested from endogenous proteins and presented by MHC molecules on the surface of cells. CD8+ T cells then destroy the cell by perforating its membrane and by delivering apoptosis triggering molecules. The CD8+ T cell will move to another cell expressing the same MHC-peptide complex and destroys it as well. Ideally, CD8+ T cells create a very specific and robust response against tumor cells. Cytotoxic T cells are considered to be essential effectors of the cell-mediated immune response. The ability to identify and destroy tumors as a central part of the immune system function is defined as the immune surveillance theory, which was put forward in the 1960s [134]. Later on, the theory received major doubts as a study demonstrated no increase in tumor incidence in athymic nude mice [135]. However, extensive work in the last couple of decades has

shown that athymic nude mice were not necessarily an appropriate model for studying immune surveillance due to the fact that nude mice do not completely lack functional T cells. On the other hand, the use of genetically modified mice generating defined and stable immune defects has fully supported the theory of surveillance. For example, mice with genetic alterations leading to deficiencies in B and T cells are more prone to spontaneous and induced carcinogenesis than wild type mice [136]. In addition, patients with acquired immune deficiencies such as acquired immune deficiency syndrome (AIDS) and post transplant immune suppression display a dramatic increase in the incidence of several tumor types, including lymphoid tumors, lung cancer and tumors related to viral infections [137]. Immune surveillance mechanisms are ideal for limiting cancer development; unfortunately, they are not completely efficient. Tumors that eventually arise are typically poorly or not immunogenic [138]. Lately, it has been become evident that a tumor can develop many defense mechanisms an immune attack. The ability to evade the immune response is a major hallmark of cancer [139].

Now, it is well established that the development of cancer is associated with alterations in the number and function of immune cells in the periphery and especially at the sites of tumor progression. In this dissertation work, I studied B cell chronic lymphocytic leukemia, which is a disease caused by a clonal expansion of small, mature B lymphocytes. Although it is often detected as a consequence of a lymphocytosis in otherwise asymptomatic patients, patients with more advanced disease can exhibit a variety of symptoms including weight

loss, sweats, lymphadenopathy, splenomegaly, and bone marrow failure [2]. A major feature of CLL is that patients are susceptible to recurrent infections, which are a major cause of morbidity and mortality in this disease [13, 14]. The goal of this study was to further investigate the underlying reasons for the immunosuppression seen in CLL patients as well as to identify mechanisms that could be used to enhance immune responses against CLL.

(6a) Immunosuppression in chronic lymphocytic leukemia

The immune deficiency seen in CLL is quite varied, resulting in increased susceptibility to bacterial, viral and fungal infections and failure to mount an effective antitumor immune response [13, 14]. Nevertheless, one of the earliest observations of the immune system in CLL is an increase in the number of circulating T cells, which is primarily accounted for by an increased number of CD8+ T cells, resulting in a decreased CD4:CD8 ratio [46, 140, 141]. These T cells show diverse phenotypic and functional abnormalities. Phenotypically, they show an increase in CD57, CD69 and HLA-DR expression as well as a decrease in CD28 and CD62L expression, which suggest activation and a shift towards a differentiated effector-memory subtype [142, 143]. Other studies demonstrated oligoclonal expansions of both CD4+ and CD8+ T cells, specifically within the CD57+ subset [49, 144-146]. Interestingly, it has also been shown that T cells from CLL patients have specificity for cytomegalovirus (CMV), in which these CMV-specific T cells dominate the T cell repertoire in seropositive patients, particularly after chemotherapy [147, 148]. In spite of this, patient survival is reduced by almost 4 years in the CMV+ cohort. However, CMV+ CLL patients do

not exhibit symptoms of CMV-induced disease; the negative impact has been suggested to be due to CMV-specific T cell expansion constricting the overall T-cell repertoire [149].

Functionally, CD4+ and CD8+ T cells from CLL patients have been shown to secrete an increased amount of IL-4 [150]. IL-4 has been demonstrated to protect CLL B cells from apoptosis by upregulating the anti-apoptotic molecule Bcl-2 [151-153]. In addition, IL-4 producing CD8+ T cells from CLL patients show increased expression of CD30 [154]. The ligation of CD30L on the surface of CLL cells stimulates their production of TNF-α causing CLL cells to proliferate [154]. Also, binding to CD30L on the surface of CLL cells impairs isotype class switching and increases their sensitivity to FasL-mediated cell death [155]. Furthermore, expansion of CD4+CD25+ regulatory T cells (T_{regs}) may contribute to the immune defects in CLL. Number of T_{regs} is increased in CLL, mostly in patients with advanced disease [112, 156, 157]. Higher frequencies of T_{regs} have been shown to correlate with decreased T cell responses against viral and tumor antigens [112]. T_{regs} are also shown to decrease cellular immunity by soluble IL-2 receptor (CD25) secretion, depriving T effector cells of IL-2 and therefore inhibiting anti-tumor T cell responses [158].

Further functional defects in T cells from patients with CLL have been reported. A global gene expression profiling demonstrated that T cells from patients with CLL show a number of differentially expressed genes when compared with age-matched healthy donor T cells [50]. These altered genes were involved in cell differentiation and cytoskeletal formation in CD4+ T cells,

and cytoskeletal organization, vesicle trafficking and cytotoxicity pathways in CD8+ T cells [50]. Interestingly, these alterations in cytoskeletal formation pathways could be induced in healthy allogeneic T cells by co-culturing them with CLL cells, in a contact dependent manner [50]. In addition, these changes in the expression of cytoskeletal genes translated into a functional defect in actin polymerization, which caused the T cells from CLL patients to exhibit a defective immunologic synapse formation with antigen presenting cells [51]. These gene changes were comparable between T cells from the Eμ-*TCL1* mouse and those from human CLL patients [111]. This work also revealed that CLL cells are the main driver for the changes in T cells, as introduction of malignant CLL cells into young and healthy Eμ-*TCL1* animals induced the gene expression and functional defects similar to those seen in mice with frank leukemia [111]. Subsequent studies also demonstrated that the Eμ-*TCL1* mouse accurately mimics the shift towards an antigen-experienced phenotype observed in human CLL disease [110].

(6b) Strategies to reconstitute the immune response in CLL

Reconstituting the immune response in CLL, especially T cell response, can provide many benefits to patients. According to the immune surveillance hypothesis, in order to present a clinically detectable disease, CLL cells must have evolved strategies for suppressing the immune system [138]. If we are able to restore the immune response to CLL, patients can have durable clinical responses. Also, T cells are known to provide help to B cells as part of their normal healthy function. They stimulate B cells to proliferate, induce B cell

antibody class switching and promote plasma cell differentiation [159]. In CLL, there is evidence that T cells have been skewed to provide help for the malignant B cells. Successful immune reconstitution should reduce the availability of T cell help to CLL, which could possibly lead to starvation of the CLL cells and apoptosis. Finally, as mentioned before infections are considered to be the leading cause of death in CLL. Therefore, immune reconstitution would help patients by enabling them to fight infections more effectively and possibly counteract the immune suppression induced by both the disease and current therapies.

In this dissertation work, I investigated the mechanisms underlying the immunosuppression induced by CLL as well as possible targets for the reconstitution of the immune response in CLL. During the course of my studies, I made the observation that splenic cells isolated from our CLL model, the Eμ-*TCL1* mouse, constitutively secreted IL-10. IL-10 is a well-known immunosuppressive cytokine, which has been shown to induce its effects on a number of immune cells including suppression of T cells. Initially, I found that neutralization of IL-10 using anti-IL-10 antibodies or anti-IL-10R antibody did not affect the survival or proliferation of the CLL cells *in vitro* despite the fact that IL-10 receptor appears to be functional in CLL cells. On the other hand, I found that engraftment of CLL cells in comparison to WT mice was significantly lower in IL-10R KO mice. IL-10 is known to inhibit the function of macrophages and monocytes by limiting their production of proinflammatory cytokines and chemokines, which could affect localization and survival of CLL cells in

microenvironmental niches. When I tested this possibility in our model, I found no significant difference of CLL cell migration between WT and IL-10R KO mice. However, this does not completely eliminate the effects of IL-10 on cells found in the CLL microenvironment. In preliminary data from our lab, we found that the spleen serves as a niche for CLL cell growth. Splenomegaly determined by ultrasound imaging, was observed in the CLL adoptive transfer model before CLL cells were detected in the peripheral blood. Current studies are investigating the unique stromal cells present in the spleen and their role in CLL growth. With finding such cells, we can further study the effects IL-10 might have on them.

Subsequently, I investigated the possible effects of IL-10 on T cell responses using our *in vivo* adoptive transfer model of CLL. Interestingly before this study, there was no direct evidence for involvement of CLL-derived IL-10 in T cell dysfunction. Here for the first time, I show a clear link between CLL-derived IL-10 and dysfunction of T cell responses to CLL cells. I demonstrated that T cells isolated from IL-10R KO mice primed with CLL proliferate and differentiate better than T cells isolated from an environment where IL-10 signaling was intact. In addition, injection of primed T cells along with CLL cells into NSG mice significantly reduced incidence of the disease. T cells from IL-10R KO mice were better than wild type T cells at controlling CLL growth.

Previous reports have shown contradictory results regarding the requirement of IL-10 for the survival and proliferation of B-CLL cells. Fluckiger *et al* reported that exogenous IL-10 inhibited the proliferation of human CLL cells and decreased the survival of CLL cells in culture by inducing apoptosis [160].

They demonstrated that the addition of exogenous IL-10 to human CLL cells in culture decreased the viable cell recovery of the samples tested [160]. In addition, after one week in culture, cells cultured with IL-10 were completely lost while those cultured without IL-10 survived [160]. Finally, flow cytometric analysis, DNA gel electrophoresis, and Giemsa staining all revealed that IL-10 induced death of CLL cells by apoptosis [160]. This observation is similar to normal B-1 cell regulation by IL-10, as we have demonstrated before that normal B-1 cell derived IL-10 inhibits their proliferation responses to TLR stimulation or BCR ligation [54, 73]. On the contrary, Kitabayashi *et al* reported that IL-10 enhanced the survival of CLL cells in a dose dependent manner by preventing the apoptotic cell death of CLL cells [161]. This was also seen in a study, where IL-10 was required for the growth of a malignant B-1 cell clone, LNC, isolated from NZB mice and adapted for *in vitro* culture [162]. Although LNC was of B-1 cell origin, it does not represent CLL, as LNC but not Eμ-*TCL1* cells or human CLL cells undergo extensive proliferation *in vitro.* In my study both mouse and human CLL cells appear to be unique in not responding directly to IL10-mediated suppressive effects *in vitro* but that IL-10 affects CLL growth indirectly by suppressing anti-CLL T cells.

(6c) Multiple immunosuppression mechanisms in CLL

The question then remains of why T cells in the *de novo* CLL disease do not completely control disease development. T cells isolated from CLL patients have been found to have higher expression of checkpoint molecules such as cytotoxic T-lymphocyte-associated protein- 4 (CTLA-4) and programmed cell

death protein-1 (PD-1) [163, 164]. This possibility of multiple pathways of immunosuppression raises the need for combination therapy to target multiple modulators of immune suppression. For example, the use of anti-IL-10 and checkpoint inhibitors should be considered, however, this could only be useful in tumors that produce IL-10 such as CLL and other B-lymphomas known for their IL-10 production. This also alludes to the fact that careful investigation of changes due to immunotherapy drugs such as changes in the expression pattern of cytokines, chemokines, tumor antigens, and other immune-related factors should be considered as they could provide answers to some of the compensatory immunosuppressive mechanisms that could be targeted by combination therapy. Indeed, future studies need to investigate other cytokines produce by CLL cells and their role in either CLL growth or immune responses to CLL. Surprisingly, there are only few studies that explore cytokines and chemokines produced by CLL cells themselves. Notably, the production of IL-6 has been investigated in the context of CLL. One study examined the correlation between serum IL-6 and IL-10 levels and outcome in CLL [165]. The levels of IL-6 and IL-10 were higher in CLL patients compared to healthy individuals [165]. They also found that cytokine levels correlated with clinical features and survival [165]. Similar to the inconsistent results found in literature about the role of IL-10 in CLL growth, IL-6 shares the same problem. In an opposing study, authors demonstrated that the addition of recombinant human IL-6 significantly decreased the TNF-induced CLL growth [166]. Therefore according to this study, IL-6 is not a growth stimulatory factor but an effective inhibitor of the TNF-

induced proliferation of the leukemic B-CLL cells [166]. Another cytokine produced by CLL cells, which is worth noting, is TGF-β. CLL B cells have been found to overexpress TGF-β, in which it functions as an autocrine growth inhibitor possibly accounting for the reduced proliferative responses of these leukemic cells to different stimuli, such as anti-IgM and anti-CD40 stimulation [53].

(6d) T cell adoptive transfer for the therapy of CLL

As seen from data in chapter 4, the transfer of primed T cells in combination with CLL cells into immunodeficient mice significantly delayed CLL disease progression, whereas T cells from IL-10R KO mice were able to completely abrogate disease. In fact, NOG mice injected with a ratio of 1 T cell (from primed IL-10R KO mice) to 16 CLL cells showed no sign of CLL even after 150 days post injection, showing promise for long-term effectiveness. Interestingly, CD8+ T cells were sufficient in delaying CLL disease development and CD8+ T cells isolated from IL-10R KO primed with CLL were significantly better at controlling CLL growth than CD8+ T cells isolated from WT mice. Re-challenging experiments will be done in the future to investigate if memory T cells are still present in these mice. This is such an important observation, as the idea of T cell adoptive transfer in treating a number of cancers has been a hot topic of immunotherapy in the recent years. Specifically, chimeric antigen receptor (CAR) T cell therapy has been an interesting area of investigation. It is the adoptive transfer of T cells with specificity to tumor antigens. There are two main strategies for generating tumor-specific T cells. The first involves gene transfer of TCRs with known specificity into autologous or allogeneic T cells, which are then

expanded *in vitro* and infused into patients [167]. This approach has seen some success in melanoma and in the use of T cells specific to Epstein-Barr virus to treat posttransplant lymphoproliferative disorders [168-170]. The challenge with this approach is the fact that recognition of tumor antigens is MHC-restricted, so the use of these T cells must be individualized to each patient according to their MHC type. Also, with this approach there is a risk that a subunit of the transgenic TCR could associate with an endogenous TCR, which leads to changing the specificity of the T cell possibly leading to autoimmunity. The second strategy uses an antibody-derived antigen binding part, usually a single chain variable fragment, fused with an internal signaling domain such as CD3ζ to form a chimeric antigen receptor or CAR [171, 172]. This strategy eliminates MHC restriction, enabling the same CAR to be used in different patients. In addition, the use of an antibody receptor allows the potential targets to include a variety of surface proteins, sugars and lipids [172]. Nevertheless, the choice of these targets for CARs must be selected carefully in which the antigen is not also expressed on normal nonmalignant tissues [172]. In the context of CLL, a number of targets have the potential to be used for CAR development; those include CD19, CD20, CD23 and receptor tyrosine kinase-like orphan receptor 1 (ROR1). CLL B cells express high levels of CD19 and relatively reduced expression of CD20. However, these molecules are also expressed by normal B cells, so CAR T cells targeting them will also eliminate normal B cells, causing impaired humoral immunity worsening the immunodeficiency already seen in CLL patients [173]. Anti-ROR1 CAR CD8+ T cells have been successfully generated

from patients with CLL [174]. ROR1 has the advantage of being selectively expressed by malignant B cells, although it is also expressed by undifferentiated embryonic stem cells and in adipose tissue at low levels [174]. Likewise, anti-CD23 CAR T cells generated from CLL patients have shown cytotoxicity against autologous and allogeneic CLL cells as well as *in vivo* antitumor effect in a xenograft murine model [175]. Currently, there are a number of phase 1 and 2 clinical trials utilizing anti-CD19 CAR T cells for the treatment of B cell malignancies [172]. In preclinical studies, anti-CD19 CAR T cells were able to effectively lyse a broad panel of human CD19+ tumor cell lines and primary malignant B cells, in addition to demonstrating anti-lymphoma effects in a murine model [176, 177]. In a specific clinical trial, a group treated eight patients with relapsed CLL in two cohorts [178]. The first cohort had three patients who were treated without cyclophosphamide conditioning and showed no response [178]. The second cohort was treated with cyclophosphamide conditioning with a reduced dose of T cells. Three of these patients showed disease stabilization or lymph node responses [178]. In addition, this cohort showed some persistence of the anti-CD19 CAR T cells, which were detectable by immunohistochemistry in bone marrow up to 2 months after infusion [178]. This trial has pointed out the importance of the conditioning regimen in promoting T cell engraftment and activation. For instance, it may be important to eliminate T_{regs}, which are known to be expanded in CLL as well as eliminating other cell populations such as immature dendritic cells and any cell populations that could compete for the same survival and stimulatory cytokines as those T cells [156, 173]. Adoptive

transfer of anti-CD19 CAR T cells is a promising new approach for treating CLL. However, future studies will need to further identify CLL antigens that are recognized by T cells, which could possibly be utilized in developing new CAR T cell therapy for CLL. A recent study has shown promise in this area in which they were able to analyze the landscape of naturally presented HLA class I and II ligands of primary CLL, in which a novel category of tumor-associated T-cell antigens were identified [179]. Specific expression of these HLA ligands exclusively in CLL patients correlated with the frequencies of immune recognition by patient T cells [179]. In addition, patients displaying immune responses to multiple antigens exhibited better survival than those with responses to one or fewer antigens [179].

(6e) Molecular Mechanisms involved in IL-10 production

The fact that IL-10 was found to inhibit T cell responses to CLL makes it an attractive target for enhancement of this anti-CLL immune response as seen in our mouse model, where the lack of IL-10 signaling caused an increase in T cell activity and a reduction in engraftment of CLL. Unfortunately, therapy using anti-IL-10 or anti-IL-10R antibodies has not been successful in human patients [180]. This is due to the fact that IL-10 plays such an important role in regulating a wide range of immune cells. Manipulation of IL-10 must be balanced carefully to enhance anti-tumor responses but with the need to minimize host tissue damage. Importantly, the pleiotropic effect of IL-10 on different cell-types must also be considered, as the function of various immune cells may be up or down regulated simultaneously. Therefore, I aimed to understand the specific

molecular events that regulate the production of IL-10, which can help in designing new strategies of immune intervention. A previous study has found that BAFF stimulation via TACI receptor enhanced IL-10 production by leukemic B cells in CLL patients and Eμ-*TCL1* mice [96]. Another study demonstrated an epigenetic control of IL-10 production, in which differential *IL-10* gene methylation was responsible for the variability of IL-10 production by human CLL cells [97]. My studies demonstrated a novel role of BCR signaling in the constitutive production of IL-10. Since BCR signaling is important in CLL subtypes such as U-CLL, BCR-induced IL-10 could be a factor in their more aggressive nature. I found that inhibition of BCR signaling led to a decrease in ERK1/2 MAP kinase activation, which is consistent with a role for ERK1/2 in IL-10 production seen in T cells [84]. BCR signaling mediated IL-10 production was STAT3 independent. Moreover, a novel role for the transcription factor Sp1 in BCR signaling dependent IL-10 production by CLL cells is described here. Sp1 bound to the IL-10 promoter in CLL cells and inhibition of Sp1 led to a decrease in IL-10 production in a dose dependent manner. I was also able to link the activation of ERK1/2 to Sp1 activity, which is consistent with regulation of Sp1 by ERK1/2 in other cell types [131, 181]. My results introduce a new rationale to therapeutics targeting the ERK-Sp1 pathway.

In this study I utilized Mithramycin A as the Sp1 inhibitor [128]. Mithramycin A is an antitumor compound produced by *Streptomyces argillaceus* that has been used for the treatment of several types of tumors [128]. Mithramycin A is a DNA binding agent with relative specificity for Sp1 [182]. Its

mode of action involves its interaction in a noncovalent way with GC-rich DNA regions located in the minor groove of DNA [182]. By doing so, it prevents Sp1 from binding to a variety of promoters [182]. It was discovered in 1961 and approved for use as anticancer drug in 1970 [182]. However, despite showing strong response rates, it has not been in use in recent years due to its adverse effects. Mithramycin A has a limited therapeutic window because active doses cause toxic effects [182]. Fortunately, the development of a number of derivatives of Mithramycin A is underway [182, 183]. So, future studies need to investigate some of these derivatives and their effects on IL-10 production, in which they could be possibly utilized in animal experiments as well as human clinical trials with limited side effects.

In addition to development of new therapeutic targets for IL-10 production, studies need to be performed to study the effects of currently used therapies in CLL. For example, BCR signaling inhibitors, such as ibrutinib have been FDA approved and widely used in the treatment of CLL. Ibrutinib was found to enhance antitumor immune responses induced by intratumoral injection of CpG in a mouse lymphoma model as well as to enhance generation of CAR T-cells for adoptive immunotherapy [184, 185]. My finding that ibrutinib inhibits IL-10 production by CLL cells provides a potential mechanism by which ibrutinib treatment is enhancing anti-tumor immunity. This mechanism is likely to extend to other BCR signaling inhibitors such as fostamatinib and idelalisib.

Summary and future directions

The study presented here demonstrates that CLL derived IL-10 promotes immunosuppression utilizing BCR signaling, a key mechanism for the survival of B cells, for its regulation (Figure 6.1). I also found that CLL derived IL-10 inhibits T cell responses to CLL allowing for the rapid growth of CLL (Figure 6.1). Hence the inhibition of IL-10 pathway could be used to restore anti-tumor immunity. For future studies, several ideas need to be explored:

1. T cells had a robust effect in controlling the development of CLL cells in our adoptive transfer mouse model, however, T cells in the *de novo* CLL disease do not completely control disease development. There is a higher expression of checkpoint molecules such as PD-1 and CTLA-4 on T cells isolated from CLL patients. Therefore, the use of anti-IL-10 and checkpoint inhibitors could be useful in tumors that produce IL-10 such as CLL.
2. Further investigation of other immunosuppressive mechanisms in CLL should be done, as it could provide answers to some of the compensatory immunosuppressive mechanisms that could be targeted by combination therapy.
3. During my T cell adoptive transfer experiments; T cells were able to completely prevent the development of CLL in a number of mice. Re-challenging experiments will help investigate if memory T cells are present in these mice.

4. Although I was able to observe anti-CLL tumor response by T cells, future studies need to explore the identity of these T cells. Expansion of specific type of T cells can allow us to find out the CLL antigens that are recognized by these T cells and therefore generate new CAR T cells for the treatment of CLL.
5. BCR signaling played a significant role in the production of IL-10 by CLL cells via the ERK1/2-Sp1 pathway. Another potential for combination therapy is the use of Sp1 inhibitors with current approved therapy to test the synergistic or additive effects it might have on disease development.

Figure 6.1

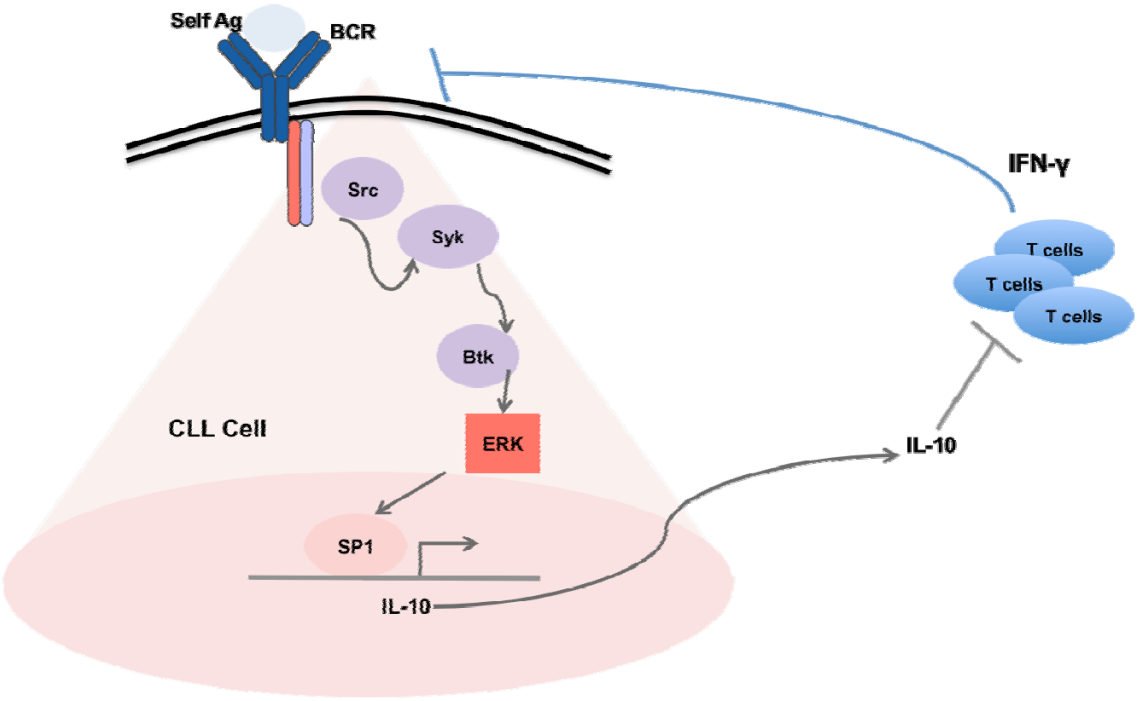


Figure 6.1: Mechanistic model of IL-10 production by CLL cells and IL-10 effects on T cell responses

BCR signaling controls IL-10 production by activation of the Syk-ERK1/2-Sp1 signaling pathway. IL-10 inhibits IFN-γ production by T cells leading to the inhibition of anti-tumor immunity and the rapid growth of CLL cells.

APPENDIX A

List of Abbreviations

NHLs - Non-Hodgkins lymphomas

CLL - Cell Chronic Lymphocytic Leukemia

DLBCL – diffuse large B-cell lymphoma

BCR - B cell receptor

IGVH - immunoglobulin variable heavy chain

U-CLL - Unmutated IGHV CLL

M-CLL - Mutated IGHV CLL

BTK - Bruton tyrosine kinase

PI3K - Phosphoinositide 3-kinase

BCL-2 - B-cell lymphoma 2

GC - Germinal center

MZ - Marginal Zone

AMyIIA - Non-muscle myosin IIA

IL-2 - Interleukin-2

Th - T helper

TNF- α - Tumor necrosis factor alpha

PD-1 - Programmed death-1

TGF- β - Transforming growth factor beta

IL-10 - Interleukin-10

LT-α - Lymphotoxin alpha

IFN-γ - Interferon gamma

UC - Ulcerative colitis

EAE - Experimental autoimmune encephalomyelitis

B_{regs} - regulatory B cells

MS - Multiple sclerosis

DCs - Dendritic cells

TLR - Toll like receptor

CSIF - Cytokine synthesis inhibitory factor

IL-10R - IL-10 receptor

STAT3 - Signal transducer and activator of transcription 3

ERK - Extracellular signal regulated kinase

MYD88 - Myeloid differentiation primary-response protein 88

TRIF - TIR-domain-containing adaptor protein inducing IFNβ

NF-κB - Nuclear factor-κB

DC-SIGN - DC-specific ICAM3-grabbing non-integrin

Syk - Spleen tyrosine kinase

NOD2 - Nucleotide-binding oligomerization domain 2

TCR - T cell receptor

FOXP3 - Forkhead box P3

TReg - T regulatory

Sp1 - Specific protein 1

C/EBPβ - CCAAT/enhancer binding protein-β

IRF1 - IFN-regulatory factor 1

BAFF - B-cell-activating factor of the tumor necrosis factor family

TACI - Transmembrane activator and cyclophilin ligand interactor

TCL1 - T-cell leukemia oncogene 1

IL-10R KO - B6.129S2-Il10rb^{tm1Agt}/J

NOG - NOD.Cg-Prkdc^{scid} Il2rg^{tm1Sug}/JicTac

IACUC - Institutional Animal Care and Use Committee

BCS - Body condition score

FBS - Fetal Bovine Serum

IMDM - Iscove's Modified Dulbecco's Medium

MTT - Thiazolyl Blue Tetrazolium Bromide

PBS - Phosphate buffered saline

CFSE - Carboxyfluorescein succinimidyl ester

HBSS - Hank’s buffered salt solution

ELISA - Enzyme-linked immunosorbent assay

OD - Optical Density

BCA - Bicinchoninic Acid

SDS - Sodium dodecyl sulfate

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

qRT-PCR - Quantitative Real-Time PCR

shRNA - Short hairpin RNA

CHIP - Chromatin Immunoprecipitation

H&E - hematoxylin and eosin

WT - Wild type

NK - Natural killer

PB - Peripheral blood

BM - Bone marrow

PC - Peritoneal cavity

PBMCs - Peripheral blood mononuclear cells

JNK1 - c-Jun NH(2)-terminal kinase 1

AIDS - acquired immune deficiency syndrome

CMV - cytomegalovirus

CTLA-4 cytotoxic T-lymphocyte-associated protein- 4

CAR - chimeric antigen receptor

ROR-1 - receptor tyrosine kinase-like orphan receptor 1

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VITA

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PLACE OF BIRTH

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EDUCATION

08/2011 - 05/2013	Master of Science in Medical Sciences from University of Kentucky, Lexington, KY
01/2007 - 05/2011	Bachelor of Science in Medical Technology from Marshall University, Huntington, WV

PROFESSIONAL EXPERIENCES

08/2011 - Present	Graduate Research Assistant, University of Kentucky, Lexington, KY
01/2011 - 05/2011	Medical Laboratory Scientist Intern, St. Mary's Medical Center, Huntington, WV
08/2010 - 12/2010	Medical Laboratory Technician Intern, Cabell Huntington Hospital, Huntington, WV

SCHOLASTIC AND PROFESSIONAL HONORS

2006 – Present	Full Scholarship support from Saudi Arabian Cultural Mission
12/2016	University of Kentucky Graduate Travel Funding
05/2016	University of Kentucky Graduate Travel Funding
11/2014	University of Kentucky Graduate Travel Funding
05/2011	Bachelor of Science in Medical Technology, Magna Cum Laude honors

RESEARCH PRESENTATIONS

2016 **American Society of Hematology (ASH), San Diego, CA**
Growth regulation of B-cell chronic lymphocytic leukemia by Interleukin-10 (Poster Presentation)

2016 **Markey Cancer Center Research day**
Growth regulation of B-cell chronic lymphocytic leukemia by Interleukin-10 (Poster Presentation)

2016 **Microbiology, Immunology, and Molecular Genetics Departmental Retreat**
Growth regulation of B-cell chronic lymphocytic leukemia by Interleukin-10 (Poster Presentation)

2016 **Immunology Conference (AAI), Seattle, WA**
The role of IL-10 in B-cell chronic lymphocytic leukemia cell survival (Poster Presentation)

2015 **Markey Cancer Center Research day**
The role of IL-10 in B-cell chronic lymphocytic leukemia cell survival (Poster Presentation)

2015 **Microbiology, Immunology, and Molecular Genetics Departmental Retreat**
The role of B cell receptor signaling in IL-10 production and the effects of IL-10 on B-1 and B-CLL cell survival (Poster Presentation and 3-minute thesis)

2015 **Immunology Conference (AAI), New Orleans, LA**
Constitutive IL-10 production by normal and malignant B-1 cells is dependent on B-cell receptor signaling (Poster Presentation)

2014 **Autumn Immunology Conference, Chicago, IL**
A role for B cell receptor signaling pathway in constitutive IL-10 production by normal and malignant B-1 cells (Oral and Poster Presentation)

2014 **Merinoff World Congress 2014: B-1 Cell Development and Function**, **Tarrytown, NY**
Novel role of B cell receptor signaling pathway in constitutive IL-10 production by normal and malignant B-1 cells (Poster Presentation)

2014 **Microbiology, Immunology, and Molecular Genetics Departmental Retreat**
The role of B cell receptor signaling pathway in IL-10 production by B-cell chronic lymphocytic leukemia cells (Poster Presentation)

2014 **Markey Cancer Center Research Day**
B-cell chronic lymphocytic leukemia resistance to IL-10 mediated suppressive effects (Poster Presentation)

2013 **Autumn Immunology Conference, Chicago, IL**
Immunosuppressive activities of Withaferin A, a withanolide derived from *Withania somnifera* (Oral and Poster Presentation)

2013 **Microbiology, Immunology, and Molecular Genetics Departmental Retreat**
Immunosuppressive activities of Withaferin A, a withanolide derived from *Withania somnifera* (Poster Presentation)

2013 **Markey Cancer Center Research Day**
Anti-lymphoma and leukemic activity of withaferin A, a withanolide derived from *Withania somnifera* (Poster Presentation)

RESEARCH PUBLICATIONS

Alhakeem, S. Sindhava, V. *et al*. Role of B cell receptor signaling in IL-10 production by normal and malignant B-1 cells. *Ann. N.Y. Acad. Sci.* 2015. DOI: 10.1111/nyas.12802.

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Alhakeem, S. McKenna, M. *et al*. Chronic lymphocytic leukemia derived interleukin-10 suppresses anti-tumor immunity. (Manuscript under review).

McKenna, M. Noothi. S. Alhakeem, S. *et al*. Novel pro-growth role for the tumor suppressor Par-4 in CLL. (Manuscript to be submitted)