EXAMINING THE ROLE OF REGULATORY LYMPHOCYTES IN A MOUSE MODEL OF BCL1 TUMOR DORMANCY

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DEDICATION

to my mother Elda Sargon, and my sister, Arlene D. Bitmansour, Ph.D., who have always been constant sources of love and support

to the memory of Fedra Shahbaz, whose bright light was extinguished too soon

and to the Lord who daily provides me with grace, hope, and love

ACKNOWLEDGEMENTS

First, I would like to thank my mentor Dr. Ellen S. Vitetta for giving me the opportunity to join the lab and providing me a project that helped me learn about cancer, immunology, and scientific thinking, and who always encouraged me to think outside the box.

I would also like to thank my committee members, Drs. Lora Hooper, Jerry Niederkorn, Nancy Street, and Michelle Tallquist for serving as my thesis committee members.

I interacted with many people throughout my time at UTSW. Foremost, I would like to thank the following people in the Cancer Immunobiology Center for helping me at some point during my project: First, and most importantly, I thank Dr. Laurentiu Pop for helping me initially during my rotation project, then helping me get settled in once I joined the lab. Larry was also instrumental in directly contributing to my project. Larry has a brilliant mind, and most specifically, he helped me approach immunology with a clinical aspect. More than this, Larry has constantly supported me both as a student and a friend and I will cherish his friendship beyond the walls of the CIC. Dr. Victor Ghetie, who taught me everything about column chromatography and also has been a constant source of support. Many others at the CIC include: Kelly Mapes,

Dr. Joan Smallshaw, Dr. Radu Marches, Drew Ivey, Linda Berry, Catherine Holloway, and Dr. Jonathan Uhr, all whom enriched my experience at the CIC.

I am also fortunate to have met many different people throughout this process. I collaborated with Dave Bauman on a project and gained valuable molecular biology training and forged a friendship as a result. I am grateful to have developed friendships with many classmates and students in the immunology and other programs. These include: Farr Niere, Amy White, Andre Durudas, Jason Mendoza, Mark Chen, Nore Ojogun, Liliana Carbajal, Kristen Tolson, Ann-Marie Corgan, Arif Jivan, and Sean Murray.

I would also like to thank all my friends from outside the program who have encouraged me through this time: Ben Morgan, Michael Melder, Cindy Melder, Herb Axilrod, Toby Linder, Teresa Linder, George Brewer, Amelie Bargas, Clive Miskin, Bill Shipley, Manoj Massand, Kim Dolmatch, David Schroeder, Greg Stancel, Mike Bartman, Colin Ford, Tim Anderson, Dede Cole, and Christy McFarland.

I would also like to thank my fellow labmates over the years: Elaine Coleman, Kimberly Brooks, Lydia Tsai, Pavitra Chakravarty, Praveena Marconescu, Allison Case, Angela Desmond, and Angela Collins. We

experienced many accomplishments and struggles together and I appreciate their wit and support throughout.

Finally, I acknowledge my mother and sister who encouraged me to take this route and were supportive throughout the entire process especially the final year.

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by

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DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

May, 2012

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The University of Texas Southwestern Medical Center at Dallas, 2012

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Cancer dormancy is a clinical state where residual tumor cells persist for long periods but do not cause detectable disease. However, tumor regrowth can occur and is accompanied by resistance to treatment and high mortality rates. The mechanisms that mediate tumor dormancy have been studied using the mouse B cell lymphoma (BCL1) model of tumor dormancy. However, the events that lead to cancer relapse are not known since the tumor microenvironment consists of many cell types that either facilitate or prevent tumor progression or have a dual role depending on the disease stage. Regulatory T (T_{reg}) cells play a key role in maintaining systemic immune tolerance and have been described to promote cancer progression. The objective of this study was to determine the role of T_{reg} cells in preventing BCL1 tumor dormancy by suppressing the anti-tumor immune responses. Surprisingly, we found that the total numbers of T_{reg}

cell were highest in mice bearing dormant tumor cells, whereas mice with the highest BCL1 tumor burden had the lowest number of T_{reg} cells in their tumor microenvironment. Moreover, we compared the functional differences between T_{reg} cells isolated from mice bearing dormant tumors and those bearing non-dormant tumors. We found that they equally suppressed T cell subsets from their respective tumor microenvironments. T_{reg} cells have also been shown to suppress B cells. Since the BCL1 tumor cells are malignant B cells, we examined the effects of T_{reg} cells on the tumor cells themselves. We found that T_{reg} cells did not suppress the proliferation nor inhibit IgM secretion by the tumor cells. Interestingly, we then found that the BCL1 tumor cells shared features with regulatory B (B_{reg}) cells. Like T_{reg} cells, B_{reg} cells also induce immune tolerance by suppressing effector T cells. The BCL1 tumor cells homogeneously expressed the characteristic phenotype ($CD1d^{hi}CD5^{+}$) and cytokine profile (secretion of high levels of IL-10) of the B10 subset of B_{reg} cells. Moreover, the tumor cells directly induced T cell apoptosis through a cell-contact dependent, caspase-3-mediated pathway. Therefore, the adoption of B_{reg} cell characteristics may be another approach that BCL1 tumor cells employ to evade immune responses.

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APC	Antigen Presenting Cells
APC (FACS)	Allophycocyanin
BCL1	B cell lymphoma 1
BCR	B cell receptor
B_{reg}	Regulatory B cells
CD	Cluster of Differentiation
CFA	Complete Freund's Adjuvant
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CPM	Counts Per Minute
CTL	Cytotoxic T Lymphocytes
CTLA-4	Cytotoxic T lymphocyte-Associated Antigen 4
DC	Dendritic Cells
DISC	Death-inducing signaling complex
EAE	Experimental Autoimmune Encephalomyelitis
EDTA	Ethylene Diamine Tetraacetic Acid
ELISA	Enzyme Linked ImmunoSorbent Assay
FACS	Fluorescence Activated Cell Sorting
FasL	Fas Ligand

FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
Foxp3	Forkhead box P3
g	grams
GITR	Glucocorticoid Inducted TNFR-related protein
HBSS	Hanks Balanced Salt Solution
i.p.	intraperitoneal
Id	idiotype
IFN	Interferon
IL	Interleukin
IPEX	Immunodysregulation Polyendocrinopathy Enteropathy X-linked syndrome
KLH	Keyhole Limpet Hemocyanin
LN	Lymph Nodes
MAPK	Mitogen-activated Protein Kinase
min	minutes
MZ	Marginal Zone
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NK	Natural Killer cells
OD280	Absorbance reading at 280λ

OPD	O-phenylenediamine dihydrochloride
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PDGF	Platelet-derived Growth Factor
PE	Phycoerythrin
PMA	Phorbol Myristate Acetate
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
SCID	Severe Combined Immunodeficiency
SDS	Sodium Dodecyl sulfate
SLE	Systemic Lupus Erythematosis
STAT3	Signal Transducer and Activator of Transcription 3
T2-MZ	Transitional 2 Marginal Zone
TAA	Tumor Associated Antigens
TAM	Tumor Associated Macrophages
TGF-β	Transforming Growth Factor-beta
TIL	Tumor Infiltrating Lymphocytes
TLR	Toll-like Receptor
TNF	Tumor Necrosis Factor
T_{reg}	Regulatory T cells
TRAIL	TNF-related apoptosis-inducing ligand

uPAR	Urokinase Receptor
UTSW	University of Texas Southwestern Medical Center
VEGF	Vascular Endothelial Growth Factor

INTRODUCTION

It is hypothesized that every effector-type immune cell subset has a regulatory-type counterpart. Under normal physiological conditions, both subsets contribute to the maintenance of functional immunity and homeostasis. However, as cancer develops, tumor cells can employ various strategies to reshape their tumor microenvironment for their own benefit, including evading destruction by the immune system through the manipulation of regulatory immune cells. Regulatory T cells (T_{reg} cells) are a well-defined subset of T cells that suppress auto-reactive T cells, and thus play a key role in preventing autoimmunity. Tumor cells can recruit T_{reg} cells to the tumor microenvironment or convert infiltrating effector cells to T_{reg} cells to neutralize anti-tumor immune responses. Similar to T_{reg} cells, regulatory B cells (B_{reg} cells) are a subset of B cells that can also dampen immune responses, and their role in the tumor microenvironment is currently being investigated. Therefore, developing tumor cells not only re-construct their local microenvironment to supplement their growth, they can also negatively regulate the immune responses directed at eliminating them.

A. Tumor development and the tumor microenvironment

The tumor microenvironment is a complex and specialized compartment of cells and soluble factors that favors the expansion of malignant cells. Active tumor cells are effective engineers of their local microenvironment, creating a milieu that is favorable to their survival and in turn inhospitable to the host immune system (1). Tumor cells shape their microenvironment by manipulating the local stromal cells to secrete tumor-promoting growth factors, as well as engineering blood vessels through angiogenesis for the benefit of tumor growth. The idea that tumors are simply a mass of homogeneous cells undergoing unrestrained proliferation has evolved into the recognition of the tumor as an entity composed of heterogeneous cells, each with specialized features that serve to promote the growth and expansion of the tumor. Therefore, the "tumor microenvironment" can be viewed as an autonomous organ within the body that usurps physiological resources for its own benefit.

1. Transformation of malignant cells

a) Cell-intrinsic factors

The sustained proliferation of malignant cells is the most fundamental trait of cancer (2). The transformation of normal cells into

malignant cells is driven through changes in both cell-intrinsic factors and the surrounding microenvironment. The intracellular transformation of normal cells primarily involves genetic mutations that result in the deregulation of checkpoints that normally prevent cancer cell development. These mutations activate proto-oncogenes that stimulate the formation of cancer cells, as well as inactivate tumor suppressor proteins such as the retinoblastoma (RB) protein and p53, to liberate the malignant cells from growth inhibition (reviewed in (3, 4)). Furthermore, changes occur that enable malignant cells to resist cell death by overcoming apoptotic triggers. For example, malignant cells express high levels of the Bcl-2 family of proteins, which have been shown to inhibit apoptosis (5). Developing cancer cells also acquire replicative immortality through maintaining functional telomerase, which continues to add telomere units to the ends of DNA fragments, thus enabling unlimited proliferation (6). Therefore, the incapacitation of growth-inhibitory pathways and activation of growth-promoting machinery allows unrestrained replication of tumor cells.

b) Re-shaping the tumor microenvironment

In addition to intrinsic transformation, the newly malignant “tumor” cells actively reconstruct their surrounding microenvironment to enhance their growth and expansion. A key change is the formation of new vasculature, termed angiogenesis, for the purpose of supplying nourishment to the expanding tumor mass (7). Under normal conditions, the development of vasculature occurs during embryogenesis, after which it becomes largely quiescent (2). During tumor development, tumor cells are thought to trigger the so-called “angiogenic switch,” which results in the sustained generation of new vasculature surrounding the tumor architecture (8). The key regulator for angiogenesis is the family of vascular endothelial growth factors (VEGF). So far, five VEGF ligands (A-E) have been identified that recognize three VEGF receptors (VEGF-R1-3) (9). The VEGF ligands are produced by tumor cells, tumor-associated stromal cells, surrounding perivascular cells, and bone marrow-derived myeloid cells (10). Therefore, the process of tumor-induced angiogenesis involves a complex network of diverse cells that serves a critical task for the advancement of tumor growth.

c) Inflammation and tumor growth

The host inflammatory response triggered by bacterial and viral pathogens is now also known to promote tumor cell initiation and growth (11). The inflammatory environment, especially when chronic, can accelerate mutation rates and cause genomic instability in resident cells. Furthermore, the influx of immune cells to the site of inflammation contributes to tumor promotion since these cells are replete with agents such as reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) that promote genomic instability (12). Pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), which is primarily known for its tumoricidal effects, have been shown to contribute to tumor growth (13). TNF-α and IL-6 are key pro-inflammatory cytokines that activate transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and STAT3 (signal transducer and activator of transcription 3) to induce genes that enhance propagation of tumor cells (14, 15). Activation of the NF-κB pathway initiates a feed-forward loop that produces more cytokines and chemokines, in turn attracting additional pro-inflammatory cells to the tumor site to promote tumor cell growth and subsequent expansion.

B. The anti-tumor immune response

1. Innate and adaptive immune responses

The observation by Rudolf Virchow in 1863 that leucocytes infiltrated tumors was the first indication that the immune system actively targets tumor cells for elimination (16). Leucocytes that are found in the tumor microenvironment include those that mediate both innate and adaptive immunity. Macrophages present in tumors are referred to as tumor-associated macrophages (TAMs). Although TAMs enter the tumor microenvironment to destroy tumor cells, they can be re-programmed by the tumor cells to inhibit lymphocyte function through the release of interleukin (IL)-10 and ROS (17). Tumor-infiltrating lymphocytes (TIL) from the adaptive immune response include both $CD4^+$ helper T cells and $CD8^+$ cytotoxic T lymphocytes (CTL). Many of these infiltrating T cells are specific for tumor-associated antigens (TAA), as demonstrated by their clonal expansion in the presence of such antigens. Although TIL can accumulate in the tumor, they do not appear to be very effective in eliminating tumor cells. It would be reasonable to expect that tumors expressing TAA would be selectively targeted for removal by the immune cells. Unfortunately, studies have not validated this hypothesis. First, it has been proposed that perhaps tumor cells express low levels of TAA,

which causes an inefficient priming of the immune system (18). Second, tumor cells are malignant manifestations of normal cells. The immune system undergoes multiple steps of "educational" checks and balances to remove autoreactive cells. This action is extended into the tumor microenvironment where infiltrating immune cells are "tolerized" against tumor antigens (19). Therefore, the immune response against tumor cells is a complex process involving interactions between multiple cell types.

2. The cancer immunoediting hypothesis

The complex interplay between immune and tumor cells has been addressed by the cancer immunoediting hypothesis, which stresses the dual host protective and tumor-promoting actions of the immune system on developing tumors (20). This hypothesis describes the interactions between tumor cells and the immune response in three phases: elimination, equilibrium, and escape [reviewed in (20)]. During the elimination phase, as neoplastic cells form, intrinsic tumor suppression mechanisms such as DNA repair and apoptosis machinery attempt to control the rapid proliferation of the transformed cells. Surviving tumor cells expressing TAA and other tumor-associated "danger" signals activate the innate and adaptive immune responses. Many transformed cells are

successfully eliminated in this phase before they become clinically apparent. However, it is postulated that a small number of tumor cells, or variants of the original tumor as a result of selection pressure, may escape elimination and enter the equilibrium phase. At this stage, tumor outgrowth is prevented by anti-tumor responses, yet tumor cells are not completely eliminated. The tumor cells in this phase are described to exist in a “dormant” state that can last for long periods without any clinical manifestations. Cancer immunoediting is thought to occur in this equilibrium phase where tumor cell clones that prompt immune activation are continually removed. This constant immune surveillance imposes a selection pressure on genetically unstable tumor cells to generate clones that are not recognized by the immune system. These select clones enter the “escape” phase after having adapted several immunoevasive mechanisms. They can mask their features that were initially recognized by the immune system. Furthermore, they can induce apoptosis of immune cells by contact and also generate an immunosuppressive environment through the secretion of inhibitory soluble factors and the recruitment of immunosuppressive cells that, in turn, inhibit the anti-tumor immune response. Thus, while the immune response may initially keep tumor growth in check, tumor cells can tip the balance to favor their

expansion by manipulating the immune cells that infiltrate the tumor microenvironment.

The transition that tumor cells make from the equilibrium to the escape phase poses a critical turn of events in the development of cancer. Anti-tumor immune responses may initially destroy tumor cells or drive them into a “dormant” state. Unfortunately, this appears to be a temporary state and dormant tumor cells eventually “relapse” or regain their proliferative potential. Therefore, tumor dormancy represents an important component of cancer research since it encompasses the encounter between tumor cells attempting outgrowth, and the anti-tumor immune responses directed against them.

C. Tumor dormancy

1. Manifestation of tumor dormancy

Cancer dormancy is a clinical condition that describes the status of residual tumor cells following the removal of the original tumor mass through resection and/or other therapy. These residual tumor cells can adopt a physiologically “dormant” state, where their numbers do not increase for long periods of time or their proliferation rate is matched by their death rate (21, 22).

For growth to occur, tumor cells must receive sufficient intracellular signaling from their microenvironment. When tumor cells do not receive sufficient intracellular signaling for growth, they can enter the dormant state. One such signal driving tumor cell proliferation is transmitted by the metastasis-associated urokinase receptor (uPAR) to $\alpha 5\beta 1$ integrin on tumor cells (23). This triggers a complex intracellular signaling pathway that ultimately drives tumor cell proliferation. Blocking of the uPAR pathway and concurrent activation of p38 and mitogen-activated protein kinase (MAPK) signaling pathway induces growth arrest, thus inducing tumor dormancy (24, 25).

Tumor cells engage in continuous crosstalk with their microenvironment to determine whether it is permissive for their growth. The high nutritional requirement of the tumor cells induces the restructuring of their microenvironment by increasing the vasculature around the tumor mass. This process of angiogenesis is induced through pro-angiogenic factors such as VEGF and platelet-derived growth factor (PDGF). When tumor growth can no longer be supported by the vasculature or when anti-angiogenic factors inhibit neovascularization, then tumor cells can enter a state of angiogenic dormancy (26).

In contrast to the factors that influence a tumor cell to become dormant, the mechanisms by which tumors overcome dormancy are even

less well understood, and represent a significant clinical problem. The recurrence of cancer after therapy and long periods of remission is frequent (26). This has been observed most prominently in breast cancer patients where after diagnosis and mastectomy, the cancer has been declared to enter into remission, only for it to relapse 10 to 20 years later (27-29). Tumor dormancy is also observed in other cancers such as B cell lymphoma (30), melanoma (31), and prostate carcinoma (32). Treatment protocols for patients with B cell lymphoma have used anti-idiotype antibodies specifically targeted for the IgM antigen receptor on tumor cells (33-35). Although monoclonal anti-idiotype antibody treatment resulted in remissions in patients, relapses were later observed. Examination of the relapsed tumor cells showed that certain clones re-emerged as resistant to the initial anti-idiotype therapy, suggesting that the therapy exerted a strong selective force against tumor cells and caused subclones to appear with changes in their immunoglobulin idiotypes (33). Therefore, when dormant tumor cells relapse, the re-activated cells can be of the original clone or one or multiple new subclones as a result of somatic mutations to their antigen receptors. This finding supports the cancer immunoediting hypothesis, indicating that tumor cells can overcome dormancy and enter the escape phase by altering a feature formerly recognized by the immune system. It also suggests that B cell lymphoma can serve as an important

model for investigating the factors that influence tumor relapse following dormancy.

2. The BCL1 tumor dormancy mouse model

The B cell lymphoma 1 (BCL1) mouse model of tumor dormancy was developed following the initial observation of the spontaneous appearance of malignant B cells in the spleen of an elderly BALB/c mouse (36). The mouse had an enlarged spleen and although it had a high tumor load, it was able to survive for a prolonged period. This tumor resembles the prolymphocytic form of chronic lymphocytic leukemia (CLL) in humans (37), thus making it a clinically relevant tumor model to study.

Early studies showed that the transfer of one million BCL1 tumor cells into healthy BALB/c mice resulted in the enlargement of the spleen by 3 weeks (38). By 12 weeks, the total of tumor cells appeared to plateau at 10^9 cells and represented approximately 50% of the spleen cells. Although BCL1 tumor cells are confined to the spleen early on, they circulate through the blood and lymph nodes at later time-points. The BCL1 tumor cells are also characterized as expressing high levels of MHC class II antigens (IA/IE) (39), the costimulatory markers CD80 and CD86, and the B cell activation marker CD40 (40). However, the key feature of

BCL1 tumor cells that made them an experimentally feasible model to develop and study was that they formed a monoclonal tumor. Thus, their surface IgMλ and IgDλ receptors share a common idiotype, which serves as a tumor-specific marker (41). Therefore, monoclonal anti-idiotype antibody against the tumor IgM receptor could be generated and used for the specific identification and isolation of BLC1 tumor cells from within a heterogeneous cell population (42).

To develop the BCL1 tumor dormancy model, healthy mice were injected subcutaneously with a conjugate of purified BCL1 IgMλ antibody and keyhole limpet hemocyanin (KLH) that was emulsified with an equal volume of Complete Freund's adjuvant (CFA) in three rounds separated by one week. These injections primed the mice to develop antibodies against the BCL1 IgM idiotype (BCL1-Id). As a result, high serum titers of anti-BCL1-Id were reported in mice following this immunization protocol (43, 44). Immunization against the BCL1-Id proved to be protective against the injection of BCL1 tumor cells. Studies showed that approximately 70% of the immunized mice did not develop splenomegaly by 60 days post BCL1 tumor cell challenge (43, 44). These mice were designated as bearing "dormant" BCL1 cells. All mice with pronounced splenomegaly (non-dormant and non-immunized) experienced increased tumor burden and declining health through day 120 following the BCL1

tumor challenge. However, mice bearing dormant tumor cells remained healthy over that time period where no tumor growth was apparent. Although immunization with Id-IgM invoked BCL1 tumor dormancy, it did not eradicate the tumor cells. The dormant BCL1 tumor cells could relapse and develop BCL1 tumor. This re-growth of BCL1 tumor was attributed to a drop in the titer of anti-Id antibody or mutations in the Id signaling pathway that were shown to induce cell cycle arrest in the tumor cells (44).

While these studies demonstrated that immunization with Id-IgM could lead to BCL1 tumor dormancy, further studies using this model provided additional insight into the immune system's influence on the induction of tumor dormancy. Passive transfer of anti-Id antibody was shown to induce BCL1 tumor dormancy in SCID mice (45). Furthermore, although anti-Id antibody was sufficient to induce dormancy, transfer of $CD8^+$ T cells and IFN-γ were also shown to prolong dormancy (46), demonstrating that T cell subsets impact BCL1 tumor dormancy.

Based on the findings of T cell-mediated immunity in BCL1 tumor dormancy, a novel approach to prolonging dormancy was undertaken. Pop *et al.* attempted to neutralize T_{reg} cells by administering anti-CD25 antibody and anti-CTLA-4 antibody to Id-immunized mice before and after challenge with BCL1 tumor cells (47). The depletion of T_{reg} cells was

thought to augment $CD8^+$ T cell function, which would result in prolonged tumor dormancy. However, these treatments did not prolong in tumor dormancy. The authors noted that T_{reg} cells were not completely depleted, therefore, the effects of T_{reg} cells in BCL1 tumor dormancy remained unclear.

D. Regulatory T cells

1. T_{reg} cell discovery and function

T_{reg} cells are a specific subset of T cells that suppress immune responses. The existence of "suppressor T cells" was first reported by Nishizuka and Sakakura (48) and Gershon and Kondo (49). In a series of experiments using thymectomized mice, Nishizuka and Sakakura reported that subsets of thymus-educated T cells existed that individually induced and suppressed autoimmune responses. Similarly, Gershon and Kondo, while examining tissue tolerance, noted that donor spleen cells suppressed antibody responses in recipient mice. Unfortunately, subsequent studies by both groups failed to define the suppressor cells more specifically. The largest obstacle was the lack of a surface marker to specifically identify and isolate the suppressor cells for further study. As a consequence, the concept of suppressor T cells fell out of favor with immunologists for many years.

In a landmark study in 1995, Sakaguchi *et al.* resurrected the notion that suppressor T cells existed when they described a subset of T cells that were enriched within $CD4^+CD25^+$ T cells, and comprised approximately 10% of all $CD4^+$ T cells (50). To escape the stigma associated with the history of suppressor T cells, these cells were named "regulatory T cells." This report armed investigators with the technical knowledge to isolate and examine these cells. Since then, T_{reg} cells have been studied in the context of autoimmunity, transplantation, and cancer. As a result, over 19,000 papers on T_{reg} cells have been published to date.

2. T_{reg} cell characterization

T_{reg} cells contribute to the maintenance of self-tolerance and homeostasis of the immune system. They suppress immune responses to both self and foreign antigens and thus influence both autoreactive and host-defense immune responses. $CD4^+CD25^+$ T_{reg} cells are either generated in the thymus along with effector T cells or in peripheral tissues. Thymus-derived T_{reg} cells are referred to as "natural" T_{reg} cells, and their primary role is to delete autoreactive T cells in the thymus. T_{reg} cells generated in the periphery are thought to destroy those clones of autoreactive effector T cells that have escaped thymic deletion.

The relevance of the $CD4^+CD25^+$ T_{reg} cell subset first described by Sakaguchi and colleagues was further solidified when Fontenot *et al.* reported that the murine subset of T_{reg} cells specifically expressed the transcription factor forkhead box P3 (FoxP3), which plays a critical role in the suppression of immune responses (51). Mutations in the human gene encoding FoxP3 lead to the onset of a genetic disease termed immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), characterized by global immune dysregulation and autoimmunity. Natural T_{reg} cells were thus defined as $CD4^+CD25^+FoxP3^+$ T_{reg} cells, and FoxP3 was shown to control the genes that endow T_{reg} cells with their suppressive capacities (52). Moreover, forced expression of FoxP3 in $CD4^+CD25^-$ T cells resulted in their conversion to $CD4^+CD25^+$ T_{reg} cells bearing suppressive capacity (51, 53). However, there appears to be a major caveat to FoxP3 expression with regard to human and mouse T_{reg} cells. While all mouse T_{reg} cells express FoxP3, it appears that its expression may be temporal in human T_{reg} cells and that FoxP3 expression may be attained by all subsets of T cells, including effector T cells (54). Therefore, extrapolating conclusions from human to mouse T_{reg} cell studies and vice versa must be done with caution.

Other subsets of T_{reg} cells have been reported that lack FoxP3 expression such as Tr1 cells, which secrete IL-10 (55), and Th3 cells,

which secrete transforming growth factor-β (TGF-β) (56). However, due to their lack of specific cell surface markers with which to identify these cells, most reports to date discuss the $CD4^+CD25^+FoxP3^+$ subset of T_{reg} cells.

3. Mechanisms of T_{reg} cell function

The mechanism by which T_{reg} cells suppress immune cells has been extensively examined and is shown to be through both cell contact and secretion of suppressive cytokines. $FoxP3^+$ T_{reg} cells secrete immunosuppressive cytokines such as IL-10 (57), TGF-β (58), and IL-35 (59). IL-10 and TGF-β have been shown to be essential for T_{reg} cell-mediated suppression *in vivo* (60, 61). Additionally, IL-35 was most recently shown to be an inhibitory cytokine that is secreted specifically by T_{reg} cells and contributes to the suppression of conventional T cells *in vitro* (59, 62).

T_{reg} cells can also inhibit effector T cell proliferation by competing for IL-2 *in vitro*. In fact, T_{reg} cells have been referred to as an "IL-2 sink" because they can deny effector T cells of available IL-2 (63). T_{reg} cells can out-compete effector T cells for IL-2 because they express the high affinity IL-2 receptor which is composed of CD25 (IL-2Rα-chain), CD122 (IL-2Rβ-chain), and CD132 (IL-2Rγ-chain) (64). Expression of this high affinity IL-2

receptor is not only essential for T_{reg} cell survival but also for their effector function (65).

Examination of T_{reg} cell-mediated suppression *in vitro* has shown that T_{reg} cells suppress target cells primarily by cell contact (66, 67). Target cells include $CD4^+$ and $CD8^+$ T cells, antigen-presenting cells (APC) such as dendritic cells (DC), macrophages, and B cells, and also natural killer (NK) and NK-T cells. Cell contact inhibition has been demonstrated to be mediated by T_{reg} cells with membrane-bound TGF-β (68), granzyme B (69), and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) (70, 71). T_{reg} cells are the only lymphocyte subsets that have been shown to constitutively express CTLA-4 (70). Since CTLA-4 interacts with CD80 and CD86 on APCs and transmits inhibitory signals, it was thought to be a key immune regulator in the T_{reg} cell arsenal. In fact it has been shown that interaction of CTLA-4 on T_{reg} cells with CD80 and CD86 on DCs blocks further upregulation of these molecules (72). Moreover, T_{reg} cells from mice lacking CTLA-4 are not able to inhibit upregulation of the co-stimulatory molecules on DCs as compared to T_{reg} cells from wild-type mice (73).

Although the mechanisms utilized by T_{reg} cells in mediating immunosuppression remain to be fully understood, it is accepted that T_{reg} cells induce immune tolerance using a variety of membrane-bound and

soluble molecules. Unraveling these mechanisms will enable us to better understand their role, especially in disease.

4. T_{reg} cells in tumor immunity

Initially, T_{reg} cells were examined in a variety of autoimmune disease models. Their role in promoting tumor growth was first demonstrated in a series of studies showing that T_{reg} cell depletion using anti-CD25 antibody prior to tumor challenge eradicated syngeneic tumors (74, 75). Subsequent clinical studies of patients with a variety of cancers associated high numbers of T_{reg} cells with cancer progression (76, 77). The presence of intratumoral T_{reg} cells suggested that they were specifically recruited to the tumor environment from the periphery. T_{reg} cells have been shown to express chemokine receptors and respond to a variety of chemokines (78). For example, it was shown that the ovarian tumor microenvironment secretes the chemokine CCL22, which attracts T_{reg} cells that express CCR4 (77). In addition to the CCL22-CCR4 interaction, other chemokines and receptors have been shown to mediate T_{reg} cell mobilization to tumor sites. Intratumoral T_{reg} cells in pancreatic cancer patients express high levels of CCR5, which was shown to pair with the chemokine CCL5 produced by pancreatic tumor cells (79).

In addition to trafficking, the accumulation of T_{reg} cells in tumors is also attributed to both the expansion of intratumoral T_{reg} cells and their *de novo* generation from $CD4^+CD25^-$ T cells. The tumor microenvironment contains inflammatory and immunosuppressive cytokines, both of which support the growth of T_{reg} cells. Key cytokines such as IL-2 (80), TGF-β (81), and IL-10 (82) mediate the growth of natural T_{reg} cells and also their conversion from naïve $CD4^+CD25^-$ T cells. Studies of tumor-invoked T_{reg} cells have further substantiated the direct role of tumor cells and the tumor microenvironment in both recruiting and generating T_{reg} cells. Zhou *et al.* observed increased numbers of T_{reg} cells in mice following their inoculation with B lymphoma cells (83). Furthermore, the examination of patients with non-Hodgkin's lymphoma has shown that tumor cells induced the conversion of $CD4^+CD25^-$ T cells to $FoxP3^+$ T_{reg} cells (84, 85).

The increased number of intratumoral T_{reg} cells has also been correlated with the decreased numbers or suppressed function of multiple subsets of effector antitumor lymphocytes such as $CD4^+$ helper T cells, $CD8^+$ CTLs, and NK cells. The depletion of T_{reg} cells resulted in the activation of latent subsets of anti-tumor $CD8^+$ CTLs (86, 87). In a study of a transplanted tumor model, CTLs in tumor-bearing mice were able to reject a secondary challenge with tumor cells after the depletion of T_{reg} cells following the initial inoculation (88).

Altogether, these studies suggest that T_{reg} cells, whether recruited by tumor cells or generated at the tumor site, contribute to tumor growth by suppressing anti-tumor immune responses.

E. Regulatory B cells

1. Identification of B_{reg} cells

B_{reg} cells were first described as a subset of B cells that induced immune tolerance and contributed to homeostasis. Their function was initially reported to be restricted to the production of inhibitory antibodies (89). However, their role as immune regulators was expanded in a study reporting that B cell-deficient mice injected with myelin basic protein failed to control experimental autoimmune encephalomyelitis (EAE), unlike their wild-type counterparts (90). This article was the first to ascribe an immunosuppressive role to B cells in autoimmune disease.

Shortly thereafter, Bhan and Mizoguchi were the first to use the term “regulatory B cells” to describe this unique subset of B cells that controlled the development of colitis (91). They later demonstrated that B_{reg} cells were present in mice with chronic intestinal inflammation and could be identified by their upregulation of CD1d and secretion of IL-10 (92). Since then, B_{reg} cells have been described to control immune

pathology in various mouse models of autoimmunity such as diabetes (93), arthritis (94), and lupus (95).

2. B_{reg} cell subsets and origin

B_{reg} cells are phenotypically diverse and appear to derive from different B cell precursors. Various studies have reported several potential cell-surface markers to identify B_{reg} cells (reviewed in (96)). Some markers include CD1d (92, 97), CD5 (98-100), CD21 (101, 102), and CD43 (94, 103). However, the unifying feature of all of these B_{reg} cells subsets is their capacity to secrete IL-10.

Examination of B cell subsets has shown that subsets of $CD5^+$ B1a cells, which arise from fetal liver precursors, and $CD21^+$CD23- marginal zone (MZ) B cells, which are bone marrow-derived, secrete high levels of IL-10 (104, 105). Mauri and colleagues also identified a precursor B cell subset that is derived from transitional-2 MZ (T2-MZ) B cells bearing a phenotype of $CD19^+CD21^{high}CD23^+CD24^{high}IgM^+$, which suppressed autoimmune responses in an IL-10-dependent manner (101). Interestingly, they argued that transfer of T2-MZ B cells but not the MZ B cells prevented arthritis in recipient mice.

More recently, Tedder and colleagues identified a subset of mouse spleen cells with phenotype of $CD19^{+}CD1d^{hi}CD5^{+}$ that produced IL-10 but no other cytokines (100). They referred to this subset as "B10" B_{reg} cells to highlight their production of IL-10. The transfer of B10 B_{reg} cells was shown to reduce inflammation due to contact hypersensitivity (100) and also reduce the severity of EAE in host mice (106).

3. B_{reg} cell activation and mechanisms of suppression

Most studies attempt to activate B_{reg} cells prior to their examination. The requirement for pre-stimulation appears to be two-fold: 1) B_{reg} cells are rare, therefore stimulation induces their expansion to numbers feasible for study; 2) B_{reg} cell stimulation induces their production of IL-10, thus making them functionally competent as immune-suppressor cells.

B_{reg} cell stimulation and expansion has been demonstrated through the use of lipopolysaccharide (LPS), CpG, phorbol myristate acetate (PMA) + ionomycin, and anti-CD40 (94, 99, 100, 107, 108). Mauri *et al.* reported that stimulation of B cells with type II collagen and anti-CD40 generated B_{reg} cells *in vitro.* The transfer of these *ex vivo* activated B_{reg} cells protected mice from arthritis (94). Similarly, Tedder and colleagues have also discussed the requisite activation of B10 B_{reg} cells through LPS

and PMA + ionomycin prior to isolation and study (109). Moreover, toll-like receptor (TLR) signaling appears to activate B_{reg} cells since mice deficient in MyD88 or TLR2/4 have exacerbated EAE (110).

While these studies have shed light on the factors that can activate B_{reg} cells, the exact mechanisms by which B_{reg} cells can suppress immune responses are still unclear. Adoptive transfer studies have shown that B_{reg} cells suppress T cell proliferation and their secretion of pro-inflammatory cytokines (100, 101). Additionally, it has been proposed that B_{reg} cells also have the capacity to convert effector T cells into IL-10 secreting suppressor-type cells (111). IL-10 also appears to be critical for B_{reg} cell-mediated suppression since it has been shown that IL-10-deficiency enhances the severity of EAE and contact-induced autoreactivity (112-114). Yet, the direct cellular mechanism by which B_{reg} cells suppress effector T cells has not been reported.

4. B_{reg} cells in cancer

The evolution in the study of B_{reg} cells has resembled that of T_{reg} cells in that their function was initially elucidated using autoimmune disease models. This led investigators to speculate about their possible role in cancer. Since B_{reg} cells suppress autoreactive T cells, it was

conceivable that they may also regulate anti-tumor immunity. Indeed, these speculations were based on the examination of the role of B cells in mouse models of cancer. For example, two independent studies showed that B cell-deficient mice inoculated with tumor cells were not able to control tumor growth in contrast to wild-type mice (115, 116). Moreover, the depletion of B cells using anti-CD20 antibody was shown to exacerbate the growth and metastasis of B16 melanoma cells in mice (117). These studies suggested that the B cell component in immunity, like T cells, contains both effector and immune-inhibitory subsets that contribute to the balance between immune activation and tolerance.

Due to increased understanding of the function of B_{reg} cells in regulating immune responses, research is now being focused on examining their role in cancer. A recent study by Olkhanud *et al.* described a network of immunoregulatory events in the tumor microenvironment that led to the pulmonary metastasis of breast cancer cells (118). In this setting, tumor cells activate B_{reg} cells (termed "tumor-evoked"), which in turn convert conventional $CD4^+$ T cells to T_{reg} cells in the tumor microenvironment through a TGF-β-dependent pathway. The authors conclude that B_{reg} cells are a critical component for the growth and expansion of tumor cells. Taken together, the study summarized here makes a case for the contribution of B_{reg} cells towards tumor cell evasion

of anti-tumor immune responses as well as their ability to metastasize, which is key to tumor growth. However, the understanding of the role of B_{reg} cells in cancer is still in its infancy and their contribution to tumor growth is incompletely understood.

5. Evidence for human B_{reg} cells

The increasing relevance of B_{reg} cells in mediating immune tolerance has led to the search for the human counterparts to mouse B_{reg} cells. Blair *et al.* have identified a subset of B cells in the peripheral blood with a phenotype of $CD19^{+}CD24^{hi}CD38^{hi}$ that can develop into B_{reg} cells with regulatory function (119). Interestingly, this subset is phenotypically similar to the B10 B_{reg} cells, since they also express CD1d and CD5. They can also suppress the proliferation of effector T cells in an IL-10-dependent manner. The authors also demonstrated that this subset of B_{reg} cells was dysfunctional in patients with systemic lupus erythematosis (SLE), and did not suppress cytokine production by $CD4^{+}$ T cells. This finding expands the study of B_{reg} cells towards clinical relevancy, yet it has not categorically defined a human subset of B_{reg} cells. The phenotype presented is not exclusive to B_{reg} cells, but encompasses transitional B cells also. Moreover, the exact function of these human B_{reg} cells has yet

to be formally examined. Nevertheless, as our understanding of the role of B_{reg} cells grows, it is becoming apparent that these cells are contributors to the balance between health and disease.

F. Study Objectives

For this project, the BCL1 tumor dormancy mouse model was used in an effort to assess how T_{reg} cells affected the factors that mediate tumor dormancy or relapse. BCL1 tumor dormancy is imposed by both humoral and cell-mediated immune responses following immunization using BCL1-Id antibody. One way which the BCL1 tumor cells may overcome dormancy is by the recruitment of T_{reg} cells to neutralize the anti-tumor immune responses. Therefore, we sought to compare the number and function of T_{reg} cells in mice bearing dormant vs. those bearing non-dormant BCL1 tumor cells.

Tumor cells are also known to be capable of immunosuppression. Since BCL1 cells are malignant B cells, they were examined for features of B_{reg} cells to better understand how the tumor cells directly affect immune suppression in the tumor microenvironment.

METHODS AND MATERIALS

A. Mice

BALB/C.cum (breeders purchased from Cumberland Farms, TN) were bred and maintained in the UTSW K building mouse colony in compliance with the National Institutes of Health guidelines and the UTSW Animal Resources Center. Adult females (>10 weeks) were used for all experiments.

B. Purification of BCL1-idiotype IgM antibody

1. Generation of BCL1-idiotype hybridoma

A BCL1 hybridoma cell line (BCL1xx63) that secretes idiotype-IgM was used for generating and purifying antibody. The generation of this cell line has been described (120). Briefly, lipopolysaccharide (LPS)-activated BCL1 tumor cells were fused to P3/X63-Ag8 myeloma cells in culture. Hybridoma clones secreting IgM μ heavy chain were identified on plate wells coated with Rabbit anti-mouse μ heavy chain followed by visualization with ^{125}-I-rabbit anti-mouse λ light chain for detection of IgM-λ light chain antibody. IgM-λ-positive clones were adapted to growth in

culture and the secretion of BCL1 IgM-Id was analyzed by immunoprecipitation of ^{3}H-leucine labeled secreted antibody.

2. Growth and expansion of BCL1xx63 hybridoma

For purification of BCL1-idiotype (BCL1-Id) IgM, the BCL1xx63 cell line was expanded in growth medium (Dulbecco's Modified Eagle's Medium (DMEM) [SIGMA, #D5796] supplemented with10% Fetal Bovine serum [HYCLONE] (heat inactivated at 56°C, 30 min) in a standard incubator (37°C, 5%CO_2) in 75 cm^2 and 175 cm^2 flasks (CORNING #430641 and #431079). Cells were monitored and split when reaching 70% confluency. Following cell expansion, cells were transferred to 2 liter roller bottles (CORNING #430849) at a concentration of 50 milliliters (ml) cell volume per 450 ml growth medium per bottle. The roller bottle caps were vented and the bottles stored in a standard incubator overnight to allow for CO_2 accumulation in the bottles. The following day, caps were tightened and the bottles were transferred to a rotating rack in a warm room (37°C). The bottles were allowed to incubate for 5-7 days. Following the incubation period, the cell solution from each roller bottle (500 ml) was transferred to a conical 500 ml conical tube (CORNING #431123) and centrifuged at 600*g* for 30 min. at 4°C. The supernatants

were carefully removed, as not to disturb the pellet, and combined into 1 L glass Erlenmeyer flasks.

3. Precipitation of total protein from BCL1xx63 supernatant

A super-saturated ammonium sulfate solution (SIGMA #A4915) was made by dissolving 762 g of powder per 1L of Millipore water. The cell supernatant and super-saturated ammonium sulfate solution were combined to achieve a final concentration of 30% ammonium sulfate (*i.e*. 700 ml supernatant solution + 300 ml super-saturated ammonium sulfate). The mixture was stored overnight at 4°C to allow for protein precipitation. The following day, the solution mixture was transferred to 1L high-speed centrifuge bottles (NALGENE #3120-1000) and centrifuged for 30 min at 900*g*. The supernatant was carefully removed and the pellet was dissolved in Millipore water to give a consistency color of “hot tea”. The resulting protein solution was transferred to dialysis membrane tubing, MWCO 12-14,000 (SPECTRUM #S432700) and dialyzed against a 10X volume 1X phosphate buffered saline (PBS), pH 7.4 initially for 2 hours (h), then overnight following replacement with fresh 1X PBS. The dialyzed protein solution was retrieved and sterile-filtered through a 2 µm filter (MILLIPORE # SLGP033RS). A sample of the dialyzed solution was

measured for total protein concentration by UV spectrophotometry (ODU_{280}). The sample was adjusted to 2 mg/ml and evaluated on an SDS-PAGE PhastGel (Gradient 4-15%) system (GE Healthcare #17067801) to validate the presence of intact IgM in the precipitated solution.

4. Preparation of an Ultrogel size exclusion chromatography column

To purify the BCL1-Id from other precipitated protein, a chromatography column was prepared using a bead solution containing polyacrylamide and agarose gel matrix [Ultrogel AcA 34] (PALL Life Sciences #23015-019/SIGMA #U8878). One liter of the bead solution was transferred into a 2 L Erlenmeyer flask. The bead storage solution was gently decanted. The beads were rinsed three times with Millipore water to remove the residual storage solution. “Packing buffer” (0.5 M Tris+1.7 M NaCl + HCl, pH=7.4) was prepared and de-gassed for 1 h in a 2 L vacuum flask. After the final rinse, the beads were resuspended with packing buffer (2 parts buffer, 1 part beads) to generate a slurry. A glass chromatography column [1.5 cm x 120 cm] (BIORAD #737-1593) was rinsed with Millipore water with 5 equivalent column volumes. De-gassed packing buffer was pumped through the column bottom to remove

possible air bubbles from the frit. The bottom outlet of the column was capped and a 5-inch height of degassed buffer was poured into the column. The bead slurry was gently poured into the column while the bottom outlet was opened to allow for a slow flow of the bead slurry and to generate even packing with minimum plating. The bead slurry was added until it was packed at approximately 5 inches from the top of the column.

5. BCL1 IgM purification through size exclusion column chromatography

The Ultrogel column was equilibrated with “column buffer” (0.05 M Tris-HCl + 0.17M NaCl, pH 7.4). The precipitated protein solution was poured over the column, allowed to diffuse, and followed with column buffer at a continual gravity-rate flow. A Spectra/Chrom UV monitor (SPECTRUM) and chart recorder (Ross Recorders) were connected to the column to assess the time and rate of the protein peak yields. According to this setup, the first peak recorded was approximately 90 min. from the start and corresponded to the heavier IgM protein. The subsequent peak appeared 60 min. after the first peak and corresponded with lighter weight protein such as albumin. The purity of the peaks was assessed by SDS-

PAGE PhastGel (Gradient 4-15%). The IgM solution was filter-sterilized (0.2 μM) and its concentration was measured by a UV spectrophotometer.

6. Evaluating purified BCL1-Id IgM by radioimmunoassay

Rat anti-mouse BCL1-Id (clone: 6A5) "coating" antibody was diluted to 10 μg/ml with 1X PBS + 1% FBS + 0.04% NaN_3 solution and added in triplicates (100 μL/well) in a 96-well, u-bottom, vinyl plate (THERMO SCIENTIFIC #2401). The plate was covered with parafilm and incubated at room temperature (RT) for 2 h or overnight at 4°C. The plate was washed 5X with deionized water (DIH_2O) and blotted dry on paper towels. "Blocking" solution (1X PBS + 10% FBS + 0.04% NaN_3) was added to each coated well (200 μL/well) and the plate was covered with parafilm and incubated overnight at 4°C. Dilutions of both the standard curve (using a functional BCL1-Id IgM antibody from our lab stocks) and the purified BCL1 IgM to be tested were made for the binding assay. For the standard curve, two-fold dilutions ranged from 5 μg/ml-0.05 g/ml. The purified BCL1-Id IgM dilutions ranged from 1:50-1:10,000. After blotting out the blocking solution, the binding antibody dilutions were plated at 100 μL/well and incubated at 37°C for 1.5 h. Concurrently, the "detection"

antibody (Rat anti-μ chain, clone: Bet-2) was radiolabeled with ^{125}I-Na solution.

7. ^{125}I-Na labeling of rat anti-mouse μ-chain antibody

One hundred μg of the detection antibody (clone: Bet-2) was placed in ice-cold IodoGen tubes (PIERCE #28600). Immediately, 1.0 μCi of ^{125}I-Na (AMERSHAM #IMS-30) was added and incubated for 20 min on ice. MicroSpin G-25 columns (PHARMACIA #27-5325-1) were prepared by washing first with bovine serum albumin solution [100 μg/ml] (SIGMA #85040C), then 1X PBS by centrifuging (734*g*, 1 min). The radiolabeled antibody was loaded onto the column (fitted into a clean microcentrifuge tube) and centrifuged for 2 min. (734*g*). To check for free ^{125}I-Na, the solution was precipitated with 10% trichloroacetic acid [TCA] (SIGMA #T6399) by combining 495 μL BSA (10 mg/ml) with 5 μL radiolabeled antibody in a borosilicate glass tube (VWR #47729-570) and 500 μL 10% TCA. The mixture was incubated at RT for 1 minute, and then centrifuged (2500*g*, 6 min). Five hundred microliter of the supernatant were transferred to a new tube and the radioactivity of the tube containing the precipitate and supernatant and the tube containing supernatant only was measured on a γ-counter.

C. Conjugation of BCL1-IgM to Keyhole Limpet Hemocyanin (KLH)

To prepare for immunization, the BCL1-Id IgM was conjugated with Keyhole KLH (CALBIOCHEM #B61840) at a 1:1 ratio. An amount equivalent to the BCL1-IgM Id of KLH was measured and dissolved in 1X PBS to a concentration of 1 mg/ml. The BCL1-IgM and KLH solutions were combined along with 1.25% Glutaraldehyde (FLUKA #013430/1). The mixture tube was covered with aluminum foil and allowed to incubate for 4 h on a rotator (NUTATOR). After the incubation period, the solution turned a light yellow color indicating that conjugation had occurred. The conjugated solution was dialyzed with a 10X volume of 1X PBS for 2 h, then overnight. Immunization of mice required 50 μg/mouse of BCL1-Id/KLH per injection for 3 rounds for a total of 150 μg/mouse/experiment. The dialyzed solution was divided into 2.5 ml aliquots (sufficient for 16 mice/experiment) into 15-ml conical polypropylene tubes (BD FALCON #352097) and stored at -80°C.

D. Preparation of BCL1-Id/KLH/CFA and immunization of mice

An aliquot of the BCL1-Id-KLH conjugate was thawed and combined with an equal volume of Freund's complete adjuvant (CFA)

(SIGMA #F5881) in a ¾" x 2" polystyrene vial (SPEX #6133). The tube was capped tightly, sealed with parafilm and the solution was emulsified for 5 min in an SPEX mixer (SPEX #5100). To immunize mice, 200 µl/mouse was drawn into a 1cc glass tuberculin syringe (BD #2004) and fitted with a 25 gauge x 5/8" needle (BD #305122). Female BALB/C.cum mice (12-14 weeks) were injected subcutaneously. The first and second injections (D+1 and D+7) were administered in the lower and upper flanks (100 µL/side), while the final immunization (D+17) was administered under the scruff of the neck (200 µl).

E. Generation of in vivo BCL1 tumor cells

A frozen stock of BCL1 "in vivo" tumor cells was obtained from the Cancer Immunobiology Center. These cells were whole splenocytes from a BCL1+ BALB/C.cum mouse. The cells were thawed, counted and resuspended in sterile 1X PBS and $1x10^6$ cells were injected intraperitoneally into healthy female BALB/C.cum mice. At D+30, mice were checked for BLC1 cell expansion by palpation of their spleen. As described previously (44) a spleen index (SI) of 2.5+, when the spleen expands beyond the mid-sagittal region, indicates full-blown disease. The spleens of mice with an SI > 2 were harvested and homogenized into

single cells using sterile technique in a ventilated hood. Each spleen harvested was placed into a sterile petri dish (BD FALCON #353002) containing 1X Hank's Balanced Buffer Solution (HBSS) (GIBCO #14025) + 2% heat-inactivated FBS (wash buffer). The spleen was cut into 2-4 sections and each section was transferred onto a 70 μm cell strainer (BD FALCON #352350) and fitted onto a 50-ml sterile conical centrifuge tube (BD FALCON #352098). The plunger of a 10 cc syringe (BD FALCON #309604) was used to simultaneously homogenize and filter the spleen sections. The homogenized spleen cells were transferred to a 15-ml conical centrifuge tube (BD FALCON #302097) and centrifuged for 6 min. at 300*g* in a refrigerated centrifuge (SORVAL RC3C). The supernatant was discarded and cells were resuspended in wash buffer and counted by a hemocytometer. The cell concentration was adjusted to 10^7 cells/ml in "freezing medium" composed of 90% FBS + 10% DMSO (SIGMA #2650). Cells were transferred into cryotubes (CORNING #430488) in 1-ml aliquots and stored at -80°C.

G. Injection of mice with BCL1 tumor cells

Thirty days after the third round of immunization, mice were injected with $5x10^4$ cells of BCL1 "in vivo" tumor cells. A vial of frozen

tumor cells (see section V) was thawed by hand in a tissue culture hood and transferred to a sterile 15-ml conical tube (BD FALCON #302097) containing 10 ml 1X HBSS (GIBCO #14025). The tube was centrifuged for 6 min at 300*g* in a refrigerated centrifuge (SORVAL RC3C) and the supernatant was discarded and the pellet was resuspended in 1ml 1X HBSS. The cells were counted on a hemocytometer using 0.4% Trypan-Blue solution (SIGMA #T8154). The cell concentration was adjusted to the number of mice to be injected at $5x10^4$ cells/100 μL volume of 1XHBSS. For injections, the BCL1 *in vivo* cells were drawn in ½ cc insulin syringes fitted with a 28 gauge x ½" needle (SHERWOOD MEDICAL #500014). The lower right abdominal region of each mouse was wiped with an alcohol pad and 100 μL was injected in the peritoneum.

H. Examination of mice for dormancy or splenomegaly

Beginning at D+28 post-injection of BCL1 *in vivo* tumor cells, mice were examined for splenomegaly as described in section E. To examine for dormancy, mice were palpated twice weekly and by D+60, those mice that had an SI ≤ 2 were considered to bear dormant tumors.

I. Phenotypic analysis of leucocyte subsets by flow cytometry

The spleens and inguinal and mesenteric lymph nodes of the experimental mice were harvested and homogenized into single cell suspensions in 1X HBSS + 2% FBS (stain buffer). This was done by placing each organ between the frosted-end of microscope slides (FISHER #12-550-33) and gently rubbing the slides against each other. Each cell suspension was filtered through an 80 μm nylon mesh (SMALL PARTS INC. #CMN-OO74) into 15-ml conical tubes (BD FALCON #302097). The cells were centrifuged (300*g*, 6 min, refrigerated centrifuge) and the lymph node samples were resuspended in ice-cold stain buffer. The spleen cell pellets were resuspended in ammonium chloride lysing solution and incubated at RT for 5 min. The lysis was stopped by adding stain buffer and followed by centrifugation. The spleen cell pellets were resuspended in ice-cold stain buffer. All cells were counted using a hemocytometer or Countess Cell Counter (INVITROGEN). For each staining combination, $1x10^6$ cells were distributed into wells of a 96-well conical plate (NUNC #249662) containing normal rat serum (CALTAG #10710C) as blocking solution. The following antibodies were used for staining: anti-CD1d (BD Pharmingen, clone: 1D1), anti-CD3 (BD Pharmingen, clone: 145-2C11), anti-CD4 (EBIOSCIENCE, clone: GK1.5) anti-CD5 (BD Pharmingen,

clone: 53-7.3), anti-CD8 (EBIOSCIENCE, clone: 53-6.7), anti-CD11b (EBIOSCIENCE, clone: M1/70), anti-CD19 (Caltag; clone: 6D5), anti-CD25 (BD Pharmingen, clone: PC61), anti-Ter119 (EBIOSCIENCE, clone: TER-119), anti-B220 (BD Pharmingen, clone: RA3-6B5), anti-DX5 (EBIOSCIENCE, clone: DX5), anti-Gr-1 (EBIOSCIENCE, clone: RB6-8C5) and anti-FoxP3 (EBIOSCIENCE, clone: FJK-16s). All antibody lots were first titered to their optimum dilutions (maximum separation of positive and negative signals). Staining tubes (BD FALCON #352008) containing $1x10^6$ erythrocyte-lysed spleen cells from a healthy BALB/C.cum mouse were pre-labeled. Antibodies were diluted 100- to 3200-fold through serial dilutions and each added at 100 μL/tube, mixed well by vortexing, and incubated on ice for 20 min. The tubes were washed by adding 1 ml stain buffer/tube and centrifuged. Pellets were resuspended in wash buffer (200 μL/tube) and analyzed on a flow cytometer (BD Biosciences, FACSVantage equipped with dual [488nm and 635nm] lasers).

1. Cell surface staining

For surface staining, antibody combinations were prepared according to their optimum dilutions and added to cells ($1x10^6$ cells/sample), mixed well, and incubated on ice for 20 min. Each sample was washed and

resuspended in cold Propidium Iodide (PI) buffer (SIGMA #P4170) at a final dilution of 1 μg/ml.

2. Intracellular staining

For analysis of FoxP3, samples were first stained by the "surface staining" method, washed, and resuspended in Fix/Perm Buffer (EBIOSCIENCE #00-5523) at 1 ml/sample and incubated on ice for 30 min. Samples were washed with Perm Buffer (EBIOSCIENCE), resuspended with FoxP3 antibody dilution prepared in Perm Buffer, and incubated on ice for 20 min. Samples were washed in Perm Buffer and resuspended in wash buffer.

J. FITC conjugation of anti-BCL1-Id antibody

An aliquot of a purified Rat anti-BCL1-IgM Id antibody (clone: Mc10-6A5) was acquired from the freezer stocks of the Cancer Immunobiology Center. It was first assessed for purity by SDS-PAGE then prepared for dialysis by transferring the antibody volume into dialysis membrane tubing, MWCO 12-14,000 (SPECTRUM #S432700). Freshly made sodium carbonate solution (0.1 M Na_2CO_3, pH = 11) was used to titer sodium bicarbonate (0.1M $NaHCO_3$ pH = 8.2) to pH = 9.0. A 10X volume of the

0.1 M Na_2CO_3, pH = 9.0 buffer was used to dialyze the antibody solution overnight at 4°C. Following dialysis, the antibody solution was concentrated to 2-5 mg/ml using concentration tubes, MWCO = 5,000 (Amicon Ultra #UFC8000524). Fluorescein (FITC) powder (PIERCE #46425) was dissolved in the carbonate-bicarbonate buffer, pH = 9 to make a known concentration solution (3 mg/ml). The FITC solution was combined with a 5-fold excess of antibody in a light-protected tube and mixed at a constant speed (NUTATOR) at room temperature for 2 h. To purify the FITC-conjugated antibody, a gel (G-25 Sephadex, GE Healthcare #17-0033-01) filtration column was prepared (20-cm, BIORAD). The antibody-FITC conjugate solution was poured gently through the column and washed with 1XPBS, pH = 7.4. The FITC "colored" solution appeared in two phases. The first phase, the heavier antibody-FITC conjugate, was collected in 0.5 ml fractions. The second phase, which was the unbound FITC, was not collected. The absorbance of the FITC-conjugated antibody (ODU_{280} and ODU_{495}) was measured and the concentration determined by the following equation: $[ODU_{280}-(0.3x)ODU_{495}]/1.4$. To determine the F/P ratio (the number of fluorochrome molecules per protein) the following equation was used: $(ODU_{495} \times 150{,}000)/\text{Concentration} \times 68{,}000$. The optimal F/P range is 3-9.

K. Purification of regulatory T cells from donor mice

1. Pre-enrichment for $CD4^+$ cells

Spleens from donor mice were harvested and homogenized in single cell suspensions. After removal of erythrocytes by lysis, cells were counted and adjusted to $1x10^8$ cells/ml in ice-cold wash buffer (1X HBSS + 2% FBS) containing 5% normal rat serum (STEMCELL #19772). "$CD4^+$ T cell enrichment cocktail" (STEMCELL #19772) was added to the cells at 50 μL/ml and cells were incubated at 4°C for 15 min. Immediately after, "Biotin Selection Cocktail" (STEMCELL #19772) was added at 100 μL/ml and cells were incubated at 4°C for 15 min. Finally, "Magnetic Nanoparticles" (STEMCELL #19772) were added at 50 μL/ml and cells were incubated at 4°C for 15 min. The tube containing the cells was placed in an EasySep magnet (STEMCELL #18000) for 5 min. at room temperature. The cell solution was gently poured into a new tube and placed into the magnet for 5 min. Finally, the cell solution was poured into a new 5-ml sterile conical tube (VWR #60818-102) and centrifuged (5 min, 300*g*, 4°C). The pellet was resuspended in wash buffer and the CD4-enriched cells were counted.

2. Staining and sorting $CD4^+CD25^+$ regulatory T cells

The cell concentration was adjusted to $1x10^8$ cells/ml in ice-cold wash buffer and mixed with "T_{reg} cell staining cocktail" (CD4 PE, CD25 APC, Lineage [CD11b, B220, Ter119]) at $1x10^8$ cells/ml of cocktail. Cells were incubated on ice for 20 min. then washed with wash buffer and resuspended in PI medium ($1x10^8$ cells/ml). T_{reg} cells ($CD4^+CD25^+Lin^-$) and naïve $CD4^+$ cells ($CD4^+CD25^-Lin^-$) were sorted on a FACSAria cell sorter (BD Biosciences) at the UT Southwestern Core Flow Cytometry Facility.

L. Purification of T cell subsets from naive mice

1. Purification of $CD4^+$ cells

$CD4^+$ cells from spleens of healthy BALB/c.cum mice (10-12 weeks) were isolated by the same method outlined in Section K.

2. Purification of $CD8^+$ cells

Spleens from healthy BALB/c.cum mice (10-12 weeks) were depleted of erythrocytes, and put into single-cell suspensions and

counted. The cell concentration was adjusted to $1x10^8$ cells/ml in ice-cold wash buffer (1X HBSS + 2% FBS). A cocktail of biotinylated antibodies against non-CD8 cell markers (CD4 [clone: RM 4.4], CD11b, B220, CD19, DX5, I-A/IE) was prepared in wash buffer and mixed with the spleen leucocytes at 1ml antibody cocktail/$1x10^8$ cells and incubated on ice for 20 min. Wash buffer was added to the cell mixture and centrifuged (5 min, 300*g*, 4°C). Cells were resuspended in wash buffer at 80µL buffer per 10^7 cells. Streptavidin-microbeads (MILTENYI BIOTEC #130-048-101) were added at 20µL buffer per 10^7 cells and the cell mixture was incubated at 4°C for 15 min. Cells were washed and filtered (80 µm mesh) and resuspended in wash buffer (0.5 ml/10^7 cells). To remove the non-CD8 cells, the sample was run through the DEPLETE program on an autoMACS cell separator (MILTENYI BIOTEC).

M. In vitro T_{reg} suppression assay and analysis by [^{3}H]thymidine incorporation

To examine the capacity of T_{reg} cells to suppress normal T cell subsets, co-cultures of normal purified $CD4^+$ T cells with various doses of T_{reg} cells were prepared in 96-well tissue culture plates (BD BIOSCIENCES #353072). To induce T cell proliferation, irradiated (50

Gy, Cs-source irradiator) whole splenocytes from the same donor as the normal T cells were co-cultured in a 2:1 ratio to the $CD4^+$ T cells along with 4 µg/ml anti-CD3 (clone: 145-2C11, BIOLEGEND #100314). All *in vitro* preparations were performed under strict sterile conditions in a biological safety cabinet using "complete media" containing RPMI 1640 (R8758)/10% FBS/L-glutamine (SIGMA #G7513)/Non-essential amino acids (GIBCO #11140)/sodium pyruvate (GIBCO #11360)/2-mercaptoethanol [50µM] (SIGMA #M7522). Starting cell numbers were $5x10^4$/well of $CD4^+$ T cells, $5x10^4$/well T_{reg} cells (1:1), and $1x10^5$/well irradiated splenocytes. The T_{reg} cell doses were: 1:1, 0.5:1, and 0.25:1. The total media volume per well was 0.2 ml and the plates were placed in a 37°C, 5% CO_2 incubator. Three days later, each well was pulsed with 1 µCi [^{3}H]thymidine (PERKIN ELMER) and 17 h later all wells were harvested by a semi-automatic cell harvester (SKATRON) onto filter paper (FilterMAT; SKATRON #11731). Each filter paper was allowed to dry completely (6 h overnight) then individual filter discs were each transferred into a scintillation vial (RPI #125516) containing 2ml scintillation cocktail (UniverSol #882480). [^{3}H]thymidine incorporation was measured with a β-scintillation counter.

N. Measurement of in vitro T cell proliferation by CFSE dilution

T_{reg} suppression of normal T cell subsets was also measured by the carboxyfluorescein succinimidyl ester (CFSE) dilution assay. T cells ($CD4^+$ or $CD8^+$) were purified by magnetic enrichment from normal BALB/c mice and were resuspended in sterile 1X HBSS without calcium or magnesium (GIBCO #14185) at $2x10^7$ cells/ml in a sterile 5-ml conical tube (VWR #60818). CFSE stock [5mM] (MOLECULAR PROBES #V12883) was diluted to 10 μM with 1X HBSS without calcium and magnesium and mixed 1:1 with the cell volume of purified T cells. The tube was covered with aluminum foil and mixed on a rotator at room temperature for 8 min. CFSE labeling was stopped by adding an equal volume of whole FBS and mixing the tube by inversion. The tube was incubated at room temperature for 5 min, and centrifuged (300*g*, 5 min.). The supernatant was carefully discarded and the pellet was resuspended in RPMI + 10% FBS media and mixed well. The tube was incubated at RT for 5 min, and centrifuged (300*g*, 5 min.). The wash step was repeated once more. The pellet was resuspended in "whole media" and the cells were counted by a hemocytometer. CFSE-labeled cells were plated alone or with various ratios of T_{reg} cells (1:1, 0.5:1, and 0.25:1) in 24-well flat-bottom plates (BD BIOSCIENCES #353047). To induce T cell proliferation, irradiated (50 Gy, Cs-source irradiator) whole splenocytes from the same donor as the

naïve T cells were co-cultured in a 2:1 ratio to the naïve T cells along with 4 µg/ml anti-CD3 [clone: 145-2C11] (BIOLEGEND #100314). Starting cell numbers were $5x10^5$/well CFSE-labeled T cells, $5x10^5$/well T_{reg} cells (1:1), and $1x10^6$/well irradiated splenocytes. In studies where BCL1.3B3 cells were the candidate "regulatory" cell population, they were co-cultured with CFSE-labeled T cells subsets. To study the effects of cell-cell contact, either T_{reg} cells or BCL1.3B3 cells were placed into 0.4 µm cell culture inserts (BD BIOSCIENCES #353095) to separate them from the CFSE-labeled naïve T cell subsets and the irradiated splenocytes. To examine the effects of inhibitory cytokines, antibodies against IL-10 [clone: JES5-2A5] (BIOLEGEND #504904) and TGF-β [clone: 1D11) (R&D SYSTEMS #MAB1835) were added. The plates were placed in a 37°C, 5% CO_2 incubator for 6 days. Afterwards, cells were harvested from each well using 3 ml transfer pipets (BD FALCON #357524) and transferred into 5 ml round-bottom tubes (BD FALCON #352008). Immunophenotyping was performed using CD4 or CD8 PE, CD25 APC, and B220/CD11b/Ter119 Cy5PE. Samples were acquired on a FACSCaliber flow cytometer and analyzed by FlowJo software (TreeStar Inc.; Ashland, OR).

O. Analysis of BCL1-IgM Id secretion by BCL1.3B3 cells by ELISA

The "capture" antibody, rat anti-BCL1-IgM Id (clone: 6A5) was diluted to 4 μg/ml with 0.1 M $Na_2CO_3/NaHCO_3$, pH = 9.6 solution. Duplicate or triplicate wells of a 96-well, flat-bottom ELISA plate (BD FALCON #353279) were coated with 50 μl/well of the capture antibody. The plate was covered with saran wrap and incubated at 37°C for 2 h or overnight at 4°C. Following incubation, the plate was washed three times with wash buffer (1XPBS + 0.01% Tween 20 [SIGMA #P1379]) using 200 μl/well and blotting the plate over paper towels between each wash. A blocking solution of 1X PBS + 2% BSA (SIGMA #L8384) was prepared and plated at 200 μl/well. The plate was incubated for 30 min at 37°C or overnight at 4°C. Following the incubation, the blocking solution was removed and the wells washed three times with wash buffer. Pre-frozen (-20°C) samples of tissue culture supernatants of BCL1.3B3 cells were thawed and used undiluted and also diluted 1:4 in wash buffer. A positive control BCL1-IgM Id (lot: AB 2005, 070708) was serially diluted (5, 1, 0.5, 0.1, 0.05, 0.01, 0.005 μg/ml) and used to generate the standard curve. An IgM of irrelevant specificity (MOPC104E; SIGMA #M5909) was used as the negative control. All samples were added to the coated well (30 μL/well) and incubated at 37°C for 1 h, or 2 h at RT. The plate was washed three times with wash buffer. The detection antibody, rabbit anti-

mouse IgM μ-chain-HRP (JACKSON IMMUNO #315-035-049) was diluted 1:15,000 in wash buffer and added to the plate (75 μL/well) and incubated at 37°C for 0.5 h or 1 h at RT. The plate was washed 3X with wash buffer. The O-phenylenediamine dihydrochloride (OPD) substrate (PIERCE #34006) detection solution was prepared freshly, added to the plate (100 μL/well), and allowed to develop for 15-20 min. at RT. The reaction was stopped by adding 2N H_2SO_4 solution (100 μL/well). The plate was read immediately on an ELISA plate reader (MOLECULAR DEVICES).

P. Analysis of soluble IL-10 by ELISA

The "capture" antibody, rat anti-mouse IL-10 (EBIOSCIENCE #14-7101-81, clone: JES5-16E3), was diluted to 1 μg/ml with 1X PBS, pH 7.4. Duplicate or triplicate wells of a 96-well, flat-bottom ELISA plate (BD FALCON #353279) were coated with 50 μl/well of the capture antibody. The plate was covered with parafilm and incubated at 37°C for 2 h. Following incubation, the plate was washed three times with wash buffer (1X PBS + 0.01% Tween 20 [SIGMA #P1379]) using 200 μl/well and blotting the plate over paper towels between each wash. A blocking solution of 1X PBS + 2% BSA (SIGMA #L8384) was prepared and plated at 150 μl/well. The plate was incubated for 1 h at RT. The plate was

washed three times with wash buffer. The “standard,” recombinant mouse IL-10 (EBIOSCIENCE, #14-8101-62) was serially diluted (10,000, 5000, 2500, 1,250, 625, 312.5, 156.2, and 78.1 pg/ml) using 1X PBS + 0.5% BSA and plated at 25 μL/well. Pre-frozen (-20°C) samples of tissue culture supernatants of BCL1.3B3 cells were thawed and used undiluted and also diluted 1:4 in wash buffer and plated at 25 μL/well. The plate was incubated for 2 h at RT then washed three times with wash buffer. The “detection” antibody, biotin rat anti-mouse IL-10 [clone: JES5-2A5] (EBIOSCIENCE #13-7102-8), was diluted to 0.5 μg/ml using 1XPBS + 0.5% BSA and added to the plate at 50 μL/well. The plate was incubated for 1 h at RT. The plate was washed three times with wash buffer. To each well, 75 μL of a 1 mg/ml solution of streptavidin-HRP (SIGMA, #S5512) was added. The plate was incubated for 0.5 h at RT. The plate was washed three times with wash buffer. The OPD substrate (PIERCE #34006) detection solution was prepared freshly, added to the plate (100 μL/well), and allowed to develop for 15-20 min. at RT. The reaction was stopped by adding 2N H_2SO_4 solution (100 μL/well), and the plate was read immediately on an ELISA plate reader (MOLECULAR DEVICES). The cytokines IL-1β, IL-4, IL-6, IL-10, TNF-α, and IFN-γ (all from R&D

Systems Inc.), and TGF-β (eBioscience) were also quantified by sandwich ELISA according to the manufacturer's protocol.

Q. Analysis of caspase-3 Activation

$CD4^+$ T cells were purified from the spleens of BALB/c mice using magnetic beads according to the manufacturer's specifications (StemCell Technologies) and cultured with irradiated splenocytes, anti-CD3 (4 μg/ml) (BioLegend), and graded doses of BCL1.3B3 tumor cells in RPMI media (Gibco) for 48 h in 48-well plates (Corning). Anti-FasL (CD178, BioLegend) was added at 10 μg/ml as indicated. Cells were harvested and stained with fluorochrome-conjugated anti-CD4 (eBioscience) and anti-B220 (BD Biosciences) antibodies. Cells were then stained for intracellular caspase-3 (BD Pharmingen) according to the manufacturer's specifications. Samples were acquired on a FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo software (TreeStar Inc.).

RESULTS

CHAPTER I: QUANTIFICATION OF BCL1 TUMOR CELLS, REGULATORY T CELLS, AND CD4$^+$ AND CD8$^+$ T CELLS IN LYMPHOID ORGANS OF MICE IMMUNIZED WITH BCL1-IDIOTYPE IMMUNOGLUBULIN

A. Objective

Previous reports have described the development and study of the BCL1 tumor dormancy model to gain insight into the interactions between cancer and the immune response (44, 45, 120, 121). Following immunization with BCL1-Id IgM and injection of BCL1 tumor cells, approximately 50-70% of mice develop tumor dormancy. BCL1 tumor cells exist in these mice, but there is no outgrowth of their numbers and mice can remain disease-free for long periods and up to their lifetime. In mice where tumor dormancy is not established, the BCL1 tumor cells continue to increase in numbers, leading to overt splenomegaly and ultimately death. These mice are considered to bear “non-dormant” tumor cells. The role of anti-BCL1-Id antibody was shown to be critical to the establishment of dormancy (44). Moreover, CD8$^+$ T cells and IFN-γ were

later shown to contribute to the maintenance of the dormant state in this model of BCL1 lymphoma (46).

Our aim was to examine the role of T_{reg} cells in BCL1 tumor dormancy. The (re-)emergence of T_{reg} cells as a bona fide subset of T cells that counter immune activation led us to investigate their possible involvement in defining the balance between the dormant and non-dormant states. Many studies have associated cancer progression with increases in the number of circulating and tumor-associated T_{reg} cells (76, 77, 122, 123). Since the defining function of T_{reg} cells is to suppress immune responses, we predicted that they would contribute to: 1) Preventing the establishment of a dormant state; 2) Promoting cancer relapse from a dormant state. We hypothesized that BCL1 tumor cells would recruit T_{reg} cells as well as convert infiltrating $CD4^+$ effector T cells into T_{reg} cells in the tumor microenvironment to suppress the specific anti-BCL1 immune responses that lead to the establishment of tumor dormancy. Furthermore, the accumulation of T_{reg} cells in the tumor microenvironment over time would tip the immunomodulatory balance towards immune suppression, thus allowing the tumor cells to re-enter a highly proliferative state. Since T_{reg} cells can contribute to immune suppression, we also hypothesized that a higher BCL1 tumor cell burden would be associated with higher numbers of T_{reg} cells and conversely, a

lower tumor cell burden with relatively fewer T_{reg} cells. We therefore quantified T_{reg} cells in the spleen (the primary BCL1 tumor site) and draining lymph nodes from the dormant, non-dormant, and related control groups to correlate T_{reg} cell numbers to the status of the BCL1 tumor burden. We also quantified the $CD4^+$ and $CD8^+$ T cell subsets to examine and compare their numbers with T_{reg} cells in the model of tumor dormancy.

B. BCL1 tumor cells proliferate rapidly in the spleen.

BCL1 tumor cells are malignant B cells that were originally harvested from the spleen of a 24-month old female BALB/c mouse that had spontaneously developed splenomegaly (36). Upon isolation and examination of the spleen cells, the lymphocytes were shown to respond to B cell but not T cell mitogens (36). Furthermore, the major lymphocyte subset was shown to be a B cell with cell surface IgMλ and IgDλ, and the idiotype (Id) proved to be a tumor-specific marker (39, 124, 125). The transfer of BCL1 tumor cells to normal BALB/c mice produced

splenomegaly and high tumor burden, demonstrating the potency and highly proliferative nature of the BCL1 tumor cells (44, 126).

To examine the correlation between BCL1 tumor cell expansion and the recruitment of T_{reg} cells, we first quantified BCL1 cells in the spleen and draining lymph nodes of mice with dormant BCL1 tumor cells, non-dormant tumor cells, and non-immunized mice with high BCL1 tumor burden. Groups of female BALB/c mice (10-12 weeks) were immunized with BCL1-Id IgM-KLH conjugate emulsified with CFA (50 μg Id-IgM/injection) and injected subcutaneously on days 0, 7, and 17. Thirty days after the final immunization, mice were injected with $5x10^4$ BCL1 spleen cells in their peritoneum (Figure 1). As controls, groups of mice were immunized only, or injected with BCL1 tumor cells only. Mice were monitored weekly for swelling of the injection site, or irritation caused by CFA. Mice that were injected with BCL1 tumor cells were examined for splenomegaly up to D+60, which was the time-point that initially defined “dormancy” by lack of splenomegaly (38). All analyses were performed on or after D+60 post-BCL1 tumor cell injection, unless otherwise indicated.

1. Mice receiving BCL1 tumor cells only

Injection of $5x10^4$ cells of BCL1 spleen cells (see "Generation of *in vivo* BCL1 tumor cells" in Methods section) induced a palpable spleen by D+35 indicating expansion of the BCL1 tumor cells. The continuing proliferation of the BCL1 tumor cells in the spleen resulted in the increase in size, weight, and total cell number. By D+60, the average weight of a spleen from a BCL1-bearing mouse was 3.5 ± 0.3 g, approximately 20-fold higher than the weight of a normal spleen (0.1-0.2 g). The enlargement of the BCL1 tumor-bearing spleen was due to an increase in the total spleen cells, which by D+60 averaged $9.5x10^8$ cells, an 8-fold increase over normal spleen cell numbers ($1.2x10^8$, $p = 0.0004$) (Figure 2). The total number of inguinal and mesenteric lymph node cells in BCL1 tumor-bearing mice also increased by D+60 ($2.7x10^7$ cells vs. $5.4x10^6$ cells in normal mice, $p > 0.05$) but not as markedly as in the spleen. Moreover, the mean number of BCL1 tumor cells in the spleen was $2.4x10^8 \pm 7x10^7$ cells, which accounted for approximately 45% of the total spleen cells (Figure 3). The total number of BCL1 cells in the spleen was 25- and 100-fold higher than in the inguinal and mesenteric lymph nodes, respectively. These data confirm earlier reports that the BCL1 tumor cells reside primarily in the spleen but over time can spread to other lymphoid organs (22, 127).

2. Mice receiving BCL1 Id-IgM immunization followed by BCL1 tumor cell injection

Sixty days after challenge with BCL1 tumor cells, mice that were initially immunized were divided into two categories based on the enlargement of their spleens as measured by palpation: 1) mice with a spleen index ≤ 2.5, indicating only a modest increase in spleen size, were considered to harbor "dormant" tumor cells; 2) mice with a spleen index ≥ 2.5 had palpable splenomegaly and were defined as bearing "non-dormant" tumor cells. Tumor dormancy was also reflected in the spleen weight, total leucocytes, and total BCL1 cell numbers. Mice bearing dormant BCL1 tumor cells had an average spleen weight of 0.26 ± 0.04 g, which was over 4-fold less than the average spleen weight of mice harboring non-dormant BCL1 tumor cells (1.13 ± 0.16 g). Moreover, focal lesions, indicative of tumor-induced necrosis, were grossly apparent in the spleens of mice bearing non-dormant tumor cells but not in mice with dormant tumor cells. The total spleen cell numbers reflected the differences in the spleen size between the dormant and the non-dormant groups. The average total number of spleen cells in the mice with dormant tumor was $3.2 \times 10^8 \pm 4.2 \times 10^7$ cells. In comparison, mice bearing

non-dormant tumor cells had a significantly greater number of spleen cells ($7.0x10^8 \pm 1.4x10^8$, $p = 0.003$) (Figure 2). Both groups had higher total spleen cell numbers than mice that were immunized against BCL1 Id-IgM but not challenged with BCL1 tumor cells ($2.0x10^8 \pm 1.0x10^7$), although the difference was significant only in mice bearing non-dormant tumor cells ($p = 0.004$) (Figure 2). These results suggest the BCL1 tumor cell expansion was the primary cause for the increase in total spleen cell numbers.

Next, we examined the total number and percentage of BCL1 tumor cells in the spleens of all mice. As mentioned earlier, the BCL1 Id-IgM serves as a tumor-specific marker. We used a monoclonal antibody raised against the BCL1 Id (Rat anti-mouse IgG_{2a}; clone 6A5) to specifically identify BCL1 tumor cells in our analyses (128). Moreover, the BCL1 tumor cells can also be prospectively identified using anti-lambda light-chain antibodies since the majority of germ-line light chain rearrangements in mice (>95%) utilize the kappa light-chain. Therefore, lambda-chain$^+$ staining would also indicate of BCL1 tumor cells. Mice bearing non-dormant BCL1 tumor cells had an average of $9.8x10^7 \pm 3.3x10^7$ BCL1 tumor cells, which represented 19.9% of the total spleen cells. However, the average number of BCL1 tumor cells present in the spleens of mice bearing dormant BCL1 tumor cells was 12-fold lower

($8.6x10^6 \pm 3.1x10^6$), and constituted only 3.8% of the total spleen cells (Figure 3). Overall, mice harboring non-dormant tumor cells had significantly higher numbers of BCL1 tumor cells than those bearing dormant tumor cells. In comparison, both groups had significantly lower numbers of BCL1 tumor cells in their spleens than non-immunized mice. Additionally, on a technical note, the tumor cell quantification by absolute counts of total spleen cells and flow cytometry for identification of BCL1 tumor cells confirm the accuracy of the subjective method of palpation to determine the tumor dormancy state in mice.

C. T lymphocyte analysis in the dormant and non-dormant groups

1. Analysis of T_{reg} cells

The spleen cells and inguinal and mesenteric lymph node cells from normal BALB/c mice were analyzed by flow cytometry to determine baseline values of T_{reg} cells, and $CD4^+$ and $CD8^+$ T cells. T_{reg} cells were defined as being $CD4^+FoxP3^+$ and expressing the activation marker CD25, but lacking the non-T cell markers CD45R/B220 (B cell marker), CD11b (myeloid marker), and Ter119 (erythrocyte marker). In normal BALB/c mice, as in most mouse strains, T_{reg} cells constituted 8-15%

(average of 14.4%) of the $CD4^+$ T cell subset and ~10% of all lymphocytes (Figure 4).

In our experiments, we first examined how immunization alone affected T_{reg} cell numbers. Analysis of spleen cells 90 days after immunization showed a significant increase in the total numbers of T_{reg} cells as compared to normal controls ($6.5x10^6$ *vs.* $4.1x10^6$, $p = 0.04$) (Figure 5) but not in their percentage (Figure 6). The increase in T_{reg} cells can be attributed to an increase in the total spleen cell numbers following immunization with BCL1 Id-IgM as compared to normal controls (Figure 2). However, immunization did not enhance total cell numbers in the inguinal and mesenteric lymph nodes. This was reflected in the numbers of T_{reg} cells since they were not significantly higher than in the normal control group (Figure 5). Immunization with antigen potentiates the acquired immune system and induces the expansion of the B and T cell clones that are specific for the antigen. Moreover, in this model, the BCL1 Id-IgM was emulsified with CFA in preparation for injections. CFA is an adjuvant that is meant to prime the innate immune system and cause a slow leakage of antigen into the tissues. Increases in the numbers of T_{reg} cells have been reported following immunization protocols that use adjuvants such as CFA or aluminum hydroxide (129, 130). CFA contains *Mycobacterium tuberculosis* with cell wall components that bind to

mannose receptors on dendritic cells, preventing their maturation and IL-12 production (131). Immature dendritic cells engage T_{reg} cells and together, they promote immune tolerance (132). Moreover, the mycobacterium species can also induce prostaglandin E2 production, which elicits the expansion of T_{reg} cells (133). These studies should not preclude the primary purpose of CFA, which is the activation of the innate and adaptive immune responses. However, it may reflect the possible dual nature of a prolonged immune activation where the accumulation of apoptotic cells promotes dendritic cell-driven immune tolerance (134).

The CFA used in our immunization protocol may have contributed to the increase in the total number of T_{reg} cells in addition to inducing immune activation. It is also important to note that immunization, in this model of BCL1 lymphoma, did not preferentially expand the percentage of T_{reg} cells since the ratio of $FoxP3^+$ cells as a fraction of total $CD4^+$ cells remained similar to normal controls (Figure 6).

Although immunization against BCL1 Id-IgM increased the total T_{reg} cell numbers, the subsequent challenge with BCL1 tumor cells resulted in a reduction in the number of T_{reg} cells. Analysis of the spleen cells 60 days after challenge showed that mice bearing dormant or non-dormant BCL1 tumor cells had lower T_{reg} cell counts and a significantly lower percentage of T_{reg} cells than mice that were immunized but not challenged

with BCL1 tumor cells (Figure 5 and 6). However, mice bearing non-dormant BCL1 tumor cells experienced a significantly greater decrease in T_{reg} cell percentage ($3.2x10^6$ cells, $p < 0.0001$) than those with dormant BCL1 tumor cells ($4.2x10^6$ cells, $p = 0.003$) (Figure 6). Therefore, mice with lower BCL1 tumor cell numbers (dormant) had higher numbers of T_{reg} cells than their immunized counterparts with higher BCL1 tumor cell numbers (non-dormant). The difference in T_{reg} cell numbers between these two groups may be due to the difference in the proliferative state of the BCL1 tumor cells. As discussed in section IB, mice harboring non-dormant BCL1 tumor cells had significantly greater numbers of tumor cells than their dormant counterparts (Figure 3). Therefore, T_{reg} cells may be the casualties of the rapid proliferation of BCL1 tumor cells in the non-dormant group more so than the dormant group.

To examine fully the consequences of BCL1 tumor cell proliferation on T_{reg} cells in the tumor microenvironment, T_{reg} cell numbers were examined in the spleens of mice that were challenged with BCL1 tumor cells without prior immunization. These mice (BCL1 cell control) underwent the greatest loss of T_{reg} cells as compared to the dormant and non-dormant groups (Figures 5 and 6). In non-immunized mice with rapidly growing BCL1 tumor cells, T_{reg} cells in the spleen constituted only 0.8% of the total lymphocytes compared to 9.9% in the normal controls, a

12-fold reduction (Figure 6). The apparent decrease in the numbers of T_{reg} cells was also evident by the decrease in the $CD4^+CD25^+$ subset in the spleen, which is enriched in T_{reg} cells. However, the dynamics of T_{reg} cells were different in the lymph nodes. No significant difference was seen in the T_{reg} cell subset of the BCL1 cell control group and the normal controls either in total numbers ($5x10^5$ cells vs. $2.4x10^5$ cells) (Figure 5) or percentage (5.5% vs. 7.4%) (Figure 6). These results suggest that the deleterious effects on T_{reg} cells due to BCL1 tumor cell growth were contained within the spleen and did not extend to the lymph nodes at this time-point.

The finding that the most significant loss of T_{reg} cells was in the non-immunized group was contrary to our hypothesis and the current dogma of T_{reg} cell biology. We had hypothesized that the group with the highest numbers of BCL1 tumor cells would also have the highest numbers of T_{reg} cells. In the context of tumor immunology, T_{reg} cells are recruited, or converted from T effector cells by tumor cells to suppress anti-tumor responses (135, 136). Many reports in both mouse models of cancer and studies of patient samples correlate disease progression with an increase in T_{reg} cells (76, 77, 123, 137). However, in our studies, both the dormant and non-dormant groups had significantly higher T_{reg} cells (in numbers and percentage) than the non-immunized mice (Figures 5 and

6). Although there was no significant difference in the total numbers of T_{reg} cells in the spleen between the dormant and the non-dormant groups, the non-dormant group experienced a greater loss in the percentage of their T_{reg} cells than the dormant group (76% vs. 42%, respectively) when compared to mice that were immunized only. Altogether, our examination of T_{reg} cells in this model of tumor dormancy shows that BCL1 tumor cell expansion led to a decrease in the numbers of T_{reg} cells in the tumor microenvironment.

2. Analysis of $CD4^+$ T cells

We next determined whether the BCL1 tumor cell expansion also impacted other T cell subsets. Since the $FoxP3^+CD4^+$ T_{reg} cells are a subset of $CD4^+$ T cells, we analyzed absolute cell numbers and the percentage of $CD4^+$ T cells in all experiment groups. If BCL1-induced cell loss affected all lymphocytes in the tumor site, then $CD4^+$ T cells as a whole would experience a reduction. However, if total $CD4^+$ cell counts remained stable, this would suggest that the T_{reg} cells were specifically reduced in the tumor microenvironment.

Following immunization with the BCL1 Id-IgM, $CD4^+$ T cells experienced a significant increase in absolute cell counts but not in

percentage when compared to the normal controls (Figures 7 and 8). The increase in the number of $CD4^+$ cells was likely due to the increase in total leucocyte counts, which occurred following immunization (Figure 2). In contrast, injection with BCL1 tumor cells did not result in significant changes to the total number of $CD4^+$ cells. Therefore, although immunization enhanced the total $CD4^+$ T cell numbers, a subsequent challenge with BCL1 tumor cells did not cause a significant reduction in their numbers as was evident with the T_{reg} cell subset.

The BCL1-induced reduction of $CD4^+$ T cells was more evident when analyzing the population percentage. Interestingly, immunization did not cause a significant increase in the percentage of the $CD4^+$ T cell subset. However, BCL1 tumor cell injections induced a significant reduction in $CD4^+$ T cells in a manner that correlated to tumor burden. Mice in the BCL1 control group experienced the greatest loss of $CD4^+$ T cells, followed by mice bearing non-dormant tumor cells (Figure 8). Altogether, increasing tumor burden was deleterious to the $CD4^+$ T cell subset.

3. Analysis of $CD8^+$ T cells

We examined the absolute counts and percentage of $CD8^+$ T cells in the spleen in all experiment groups. The total numbers of $CD8^+$ T cells also increased following immunization (Figure 7), although they were not significantly different from the normal controls ($1.4x10^7$ vs. $9.3x10^6$ cells, $p = 0.09$). Moreover, the percentage of the $CD8^+$ T cell subset was not increased following immunization (Figure 8). Also, like $CD4^+$ T cells and T_{reg} cells, BCL1 tumor cells negatively affected the numbers and percentage of $CD8^+$ T cells. However, the greatest loss of $CD8^+$ T cells was seen in the spleens but not lymph nodes of the BCL1 cell control group (Figure 9). In the absence of immunization, $CD8^+$ T cells were vastly reduced in the tumor microenvironment. Mice in the BCL1 control group experienced a 33-fold reduction in the percentage and an almost 10-fold reduction in the total numbers of $CD8^+$ T cells when compared to the normal controls (Figures 7 and 8). This resulted in an almost complete elimination of the $CD8^+$ T cell subset in the spleen. In comparison, the reduction in the percentage of T_{reg} cells (12-fold) and $CD4^+$ T cells (4-fold) was much less severe than the $CD8^+$ T cell subset. Therefore, BCL1 tumor burden appeared to negatively impact the $CD8^+$ T cell subset to a greater degree than the $CD4^+$ T cell subset and the T_{reg} cell subset.

SUMMARY

Our goal was to examine the role of T_{reg} cells in our model of BCL1 dormancy and whether T_{reg} cells contributed to tumor dormancy or relapse from the dormant state. T_{reg} cells were quantified in the tumor microenvironment (spleen) and the draining lymph nodes (inguinal and mesenteric) to study the association of T_{reg} cells with disease state. Contrary to our hypothesis, and current dogma, the mice with the highest numbers of T_{reg} cells had the lowest tumor burden (dormant group), and those with the lowest T_{reg} cell numbers had the highest tumor burden (BCL1 control group). Moreover, this trend was observed with the $CD8^+$ T cell subset but not with total $CD4^+$ T cells. Our results suggest that tumor cell expansion was at the expense of T_{reg} cells and $CD8^+$ T cells.

Overall, these results show that T_{reg} cells did not appear to contribute to the development of disease state nor the relapse from the dormant state. This model of tumor dormancy has demonstrated that a robust and specific humoral anti-tumor response elicited through immunization protects the mice against BCL1 tumor challenge and induces the tumor cells to enter into a dormant state (22, 43, 44). Here, we have shown that the process of immunization also induces an increase in the total number of T_{reg} cells. However, the suppressive effects of the T_{reg} cells do not appear to overcome the antibody-mediated anti-tumor

immunity. Moreover, T_{reg} cells do not contribute to the establishment of tumor dormancy since there were no significant differences in their numbers among mice bearing dormant or non-dormant BCL1 tumor cells.

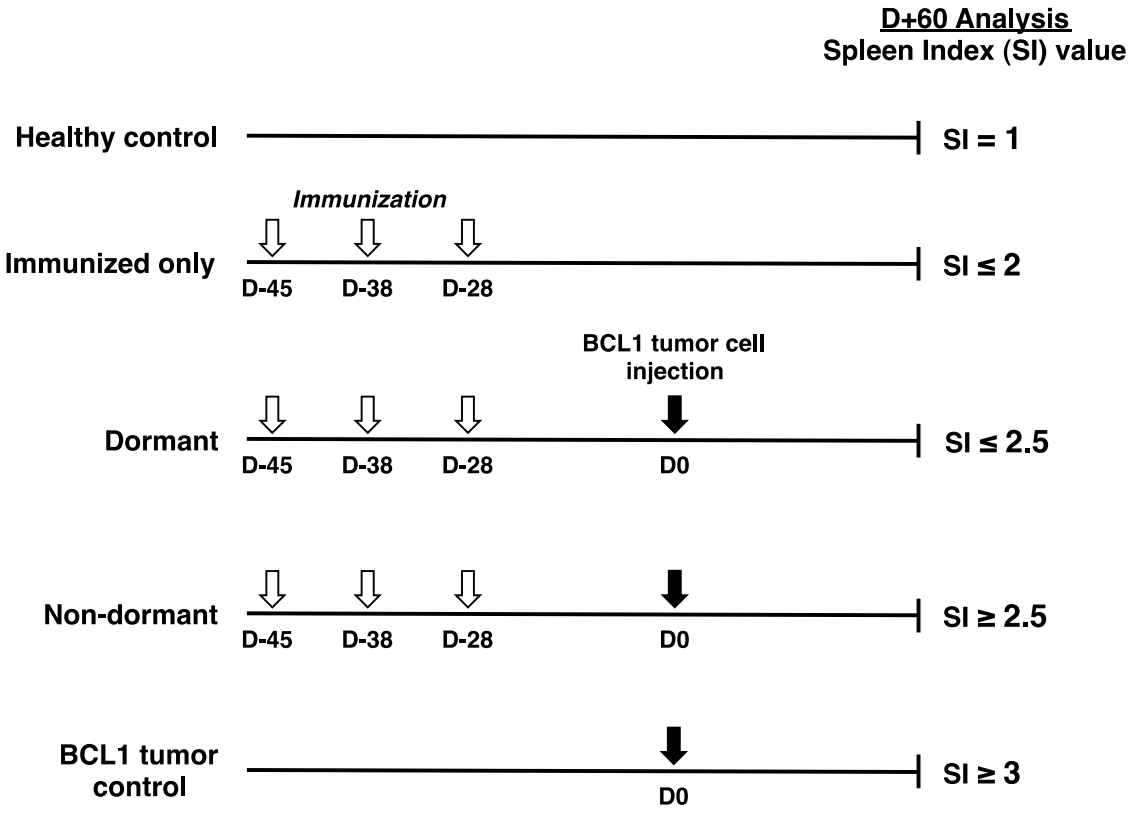

Figure 1. The experimental time-line of the BCL1 tumor dormancy model.

Ten-12 week old female BALB/c mice were immunized with the BCL1 Id-IgM. For each injection, an aliquot of Id-IgM/KLH was thawed and emulsified with an equal volume of CFA. Mice were injected subcutaneously (50 μg Id-IgM/mouse) in three rounds (D-45, D-38, D-28) in the lower abdominal flanks, upper abdominal flanks, and in the neck scruff, respectively. On D0, mice were injected with BCL1 tumor cells ($5x10^4$/mouse) in the peritoneum. All mice were monitored for the development of splenomegaly by palpation of the abdominal region to monitor spleen size. Non-immunized mice (BCL1 tumor control group) routinely began to develop splenomegaly by D+35 post tumor challenge. The spleen index (SI) scale of 1-4 determined the degree of splenomegaly with 1 designating a normal spleen and 4 indicating severe splenomegaly. Immunization alone induced a slight increase in the spleen size (SI ≤ 2). Mice that were immunized then challenged with BCL1 tumor cells underwent more significant changes in their spleen size. Therefore, D+60 was designated as the time-point to distinguish mice with or without

dormant tumor cells. Mice with an SI ≤ 2.5 were placed in the dormant group, whereas mice that had an SI ≥ 2.5 were placed in the non-dormant group. The non-immunized mice had experienced more advanced splenomegaly (SI ≥ 3) by D+60.

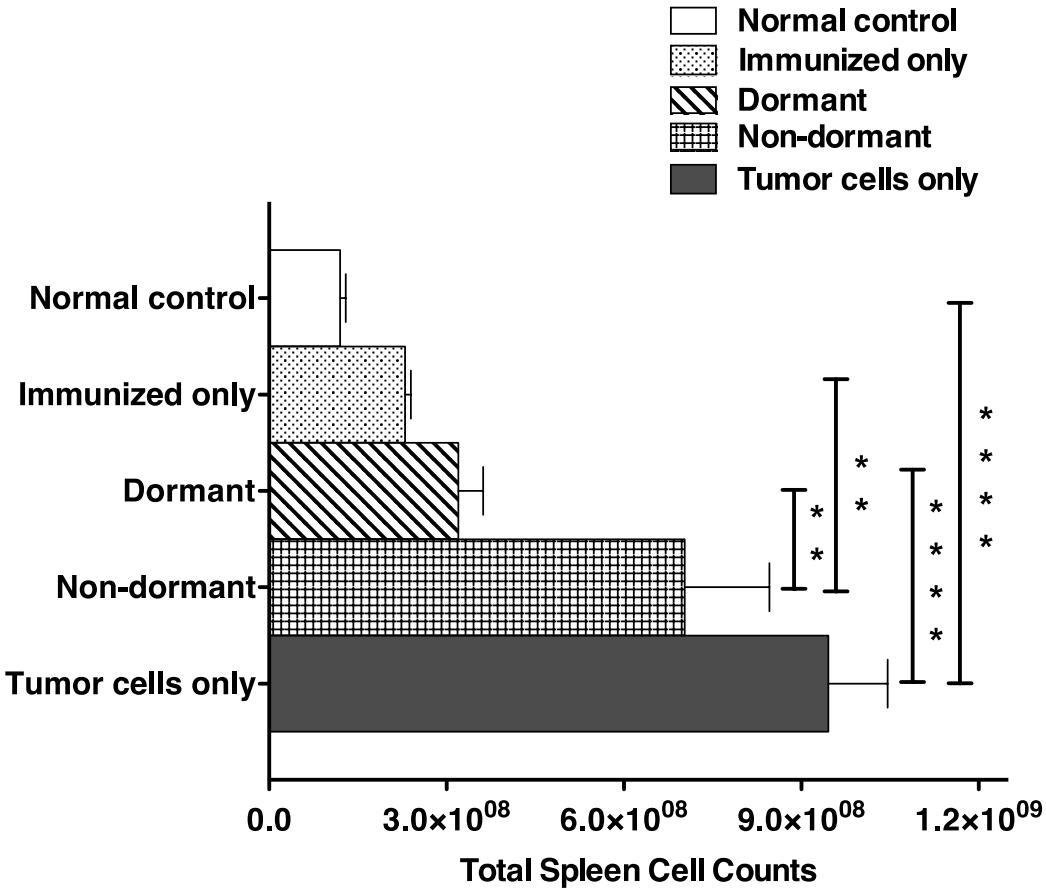

Figure 2. Total numbers of spleen cells increase following injection with BCL1 tumor cells.

The primary site for homing and expansion of the BCL1 tumor cells is the spleen. On D+60 post-BCL1 tumor cell injections, the absolute number of spleen cells was compared among the different experiment groups. Immunization alone induced a slight but not significant increase in total cells counts as compared to controls. Injection with BCL1 tumor cells resulted in an increase in the total number of spleen cells, due primarily to the expansion of the tumor cells in the spleen. Mice bearing dormant tumor cells did not experience a significant increase in total spleen cell as compared to the mice in the immunized and healthy control groups. However, mice with overt splenomegaly (non-dormant and tumor cells only) had on average 2.5-fold (n = 5) and 6.9-fold (n = 6) more

splenocytes than the dormant or healthy control mice, respectively. Normal control, n = 4; Immunized only, n = 7, Dormant, n = 14. Data are shown as the mean ± SEM (* $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$, **** $P < 0.00005$; Students t-test).

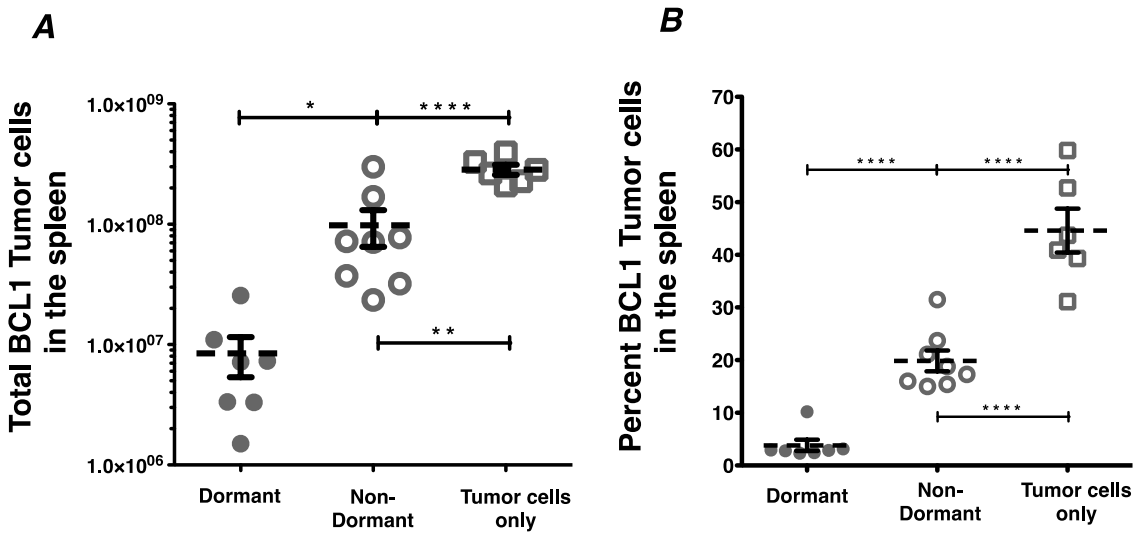

Figure 3. The expansion of BCL1 tumor cells in the spleens of mice following injection with BCL1 tumor cells.
The total number of BCL1 tumor cells in the spleen was examined 60 days after receiving injections of BCL1 tumor cells. Single cell suspensions of spleen cells were stained with anti-BCL1-Id and anti-IgM lambda light-chain antibodies, which serve as specific markers for BCL1 tumor cells. *A,* Mice bearing dormant tumor cells had an average 10-fold (n = 6; $P < 0.005$) and 29-fold (n = 6; $P < 0.005$) fewer tumor cells than the non-dormant (n = 8) and tumor only groups, respectively. *B,* These numbers were reflected in the total percentage of spleen cells also as the total tumor cells in the dormant group constituted less than 4% of total spleen cells, whereas the tumor cells in the non-dormant and tumor only groups were 20% and 45% of total cells, respectively. Data are shown as the mean ± SEM; Students t-test.

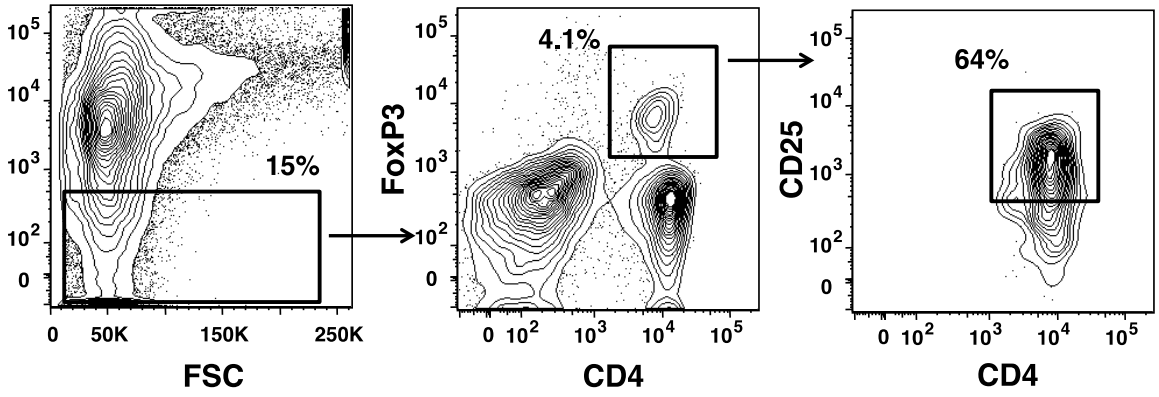

Figure 4. Gating strategy using flow cytometry analysis software for the characterization of the $CD4^+FoxP3^+$ T_{reg} cell subset.
The spleen and lymph node cells (inguinal and mesenteric) were analyzed by flow cytometry to determine the percentage of the T_{reg} cell subset within the corresponding tissues. To identify T_{reg} cells, total cells were stained with a cocktail of "non-T cell" antibodies (anti-B220, anti-CD11b, and anti-Ter119) and the T_{reg} cell markers, CD4, CD25, and FoxP3. Cells that did not express the non-T cell markers but co-expressed both CD4 and FoxP3 were considered to be T_{reg} cells. Greater than 60% of $CD4^+FoxP3^+$ cells also expressed CD25.

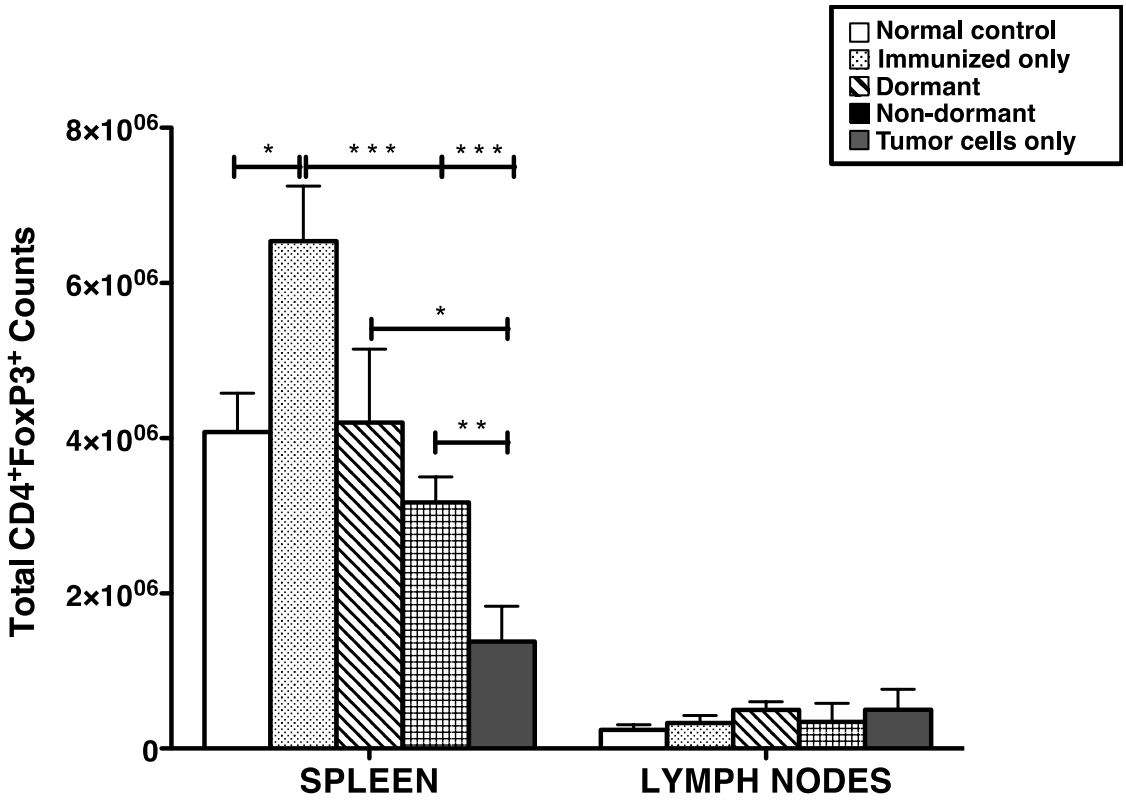

Figure 5. Total numbers of the $CD4^+FoxP3^+$ T_{reg} cell subset examined on D+60 post-BCL1 tumor cell challenge.

The total numbers of T_{reg} cells were in the spleen and lymph calculated based on the percentages of T_{reg} cells and the total organ cell counts. In the spleen, BCL1 Id-IgM immunization resulted in a significant increase in T_{reg} cell numbers (n =7) relative to normal controls (n = 4). However, following challenge with BCL1 tumor cells, pre-immunized mice both in mice bearing dormant tumors (n = 14) and mice with non-dormant tumors (n = 5), experienced a significant reduction in T_{reg} cells. BCL1 tumor-challenged control mice (*n* = 6) experienced the most significant reduction in T_{reg} cells in the spleen. In comparison, T_{reg} cell numbers remained stable in the lymph nodes despite immunization and BCL1 tumor cell challenge. Data are shown as the mean ± SEM (* $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$, ****$P < 0.00005$; Students t-test).

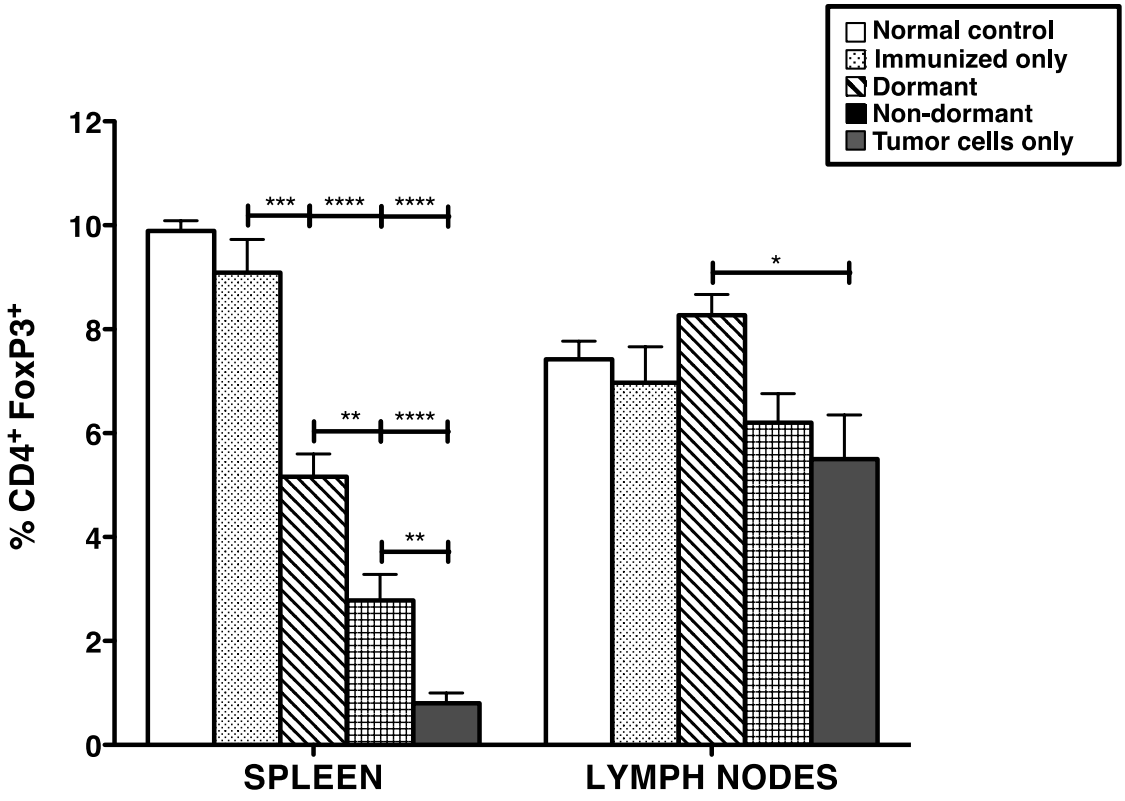

Figure 6. The percentage of $CD4^+FoxP3^+$ T_{reg} cells analyzed on D+60 post-BCL1 tumor cell challenge.

The spleen and lymph node cells (inguinal and mesenteric) were analyzed by flow cytometry to determine the percentage of the T_{reg} cell subset within the corresponding tissues. Immunization with BCL1 Id-IgM did not result in an increase of the percentage of T_{reg} cells in either the spleens or lymph nodes. However, a challenge with BCL1 tumor cells resulted in a significant reduction in the percentage of the T_{reg} cell subset in the spleens but not lymph nodes of mice. Normal controls (n = 4); immunized only (n = 7); dormant (n = 14); non-dormant (n = 5); tumor cells only (n = 6). Data are shown as the mean ± SEM (* $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$, ****$P < 0.00005$; Students t-test).

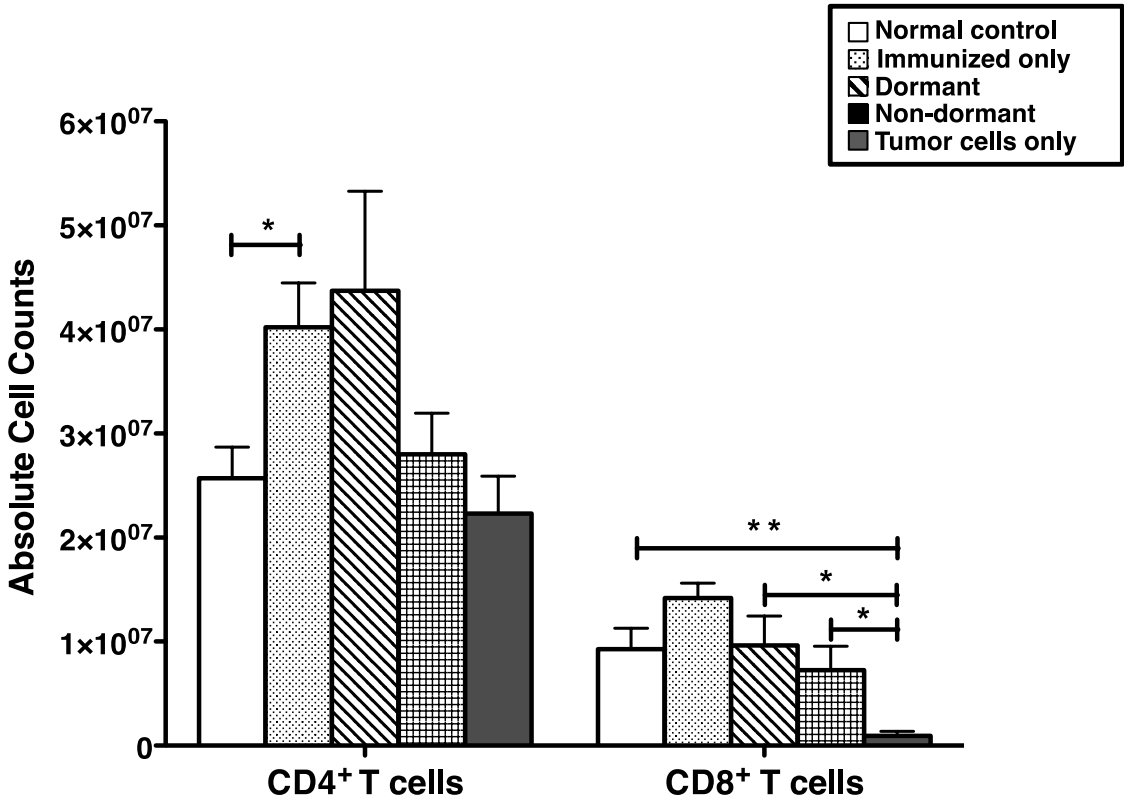

Figure 7. The total numbers of $CD4^+$ and $CD8^+$ T cells in the spleen at D+60 post-BCL1 tumor cell challenge.

Spleen cells were analyzed by flow cytometry to determine the total numbers of $CD4^+$ and $CD8^+$ T cells in all experiment groups. Immunization and BCL1 tumor cell challenge did not significantly affect the total numbers of $CD4^+$ and $CD8^+$ T cells. However, non-immunized mice that were challenged with BCL1 tumor cells (tumor cell only group, n= 6) experienced an almost complete ablation of their $CD8^+$ T cell subset. Normal controls (n = 4); immunized only (n = 4); dormant (n = 8); non-dormant (n = 5); tumor cells only (n = 6). Data are shown as the mean ± SEM (** $P < 0.005$, *** $P < 0.0005$, **** $P < 0.00005$; Students t-test).

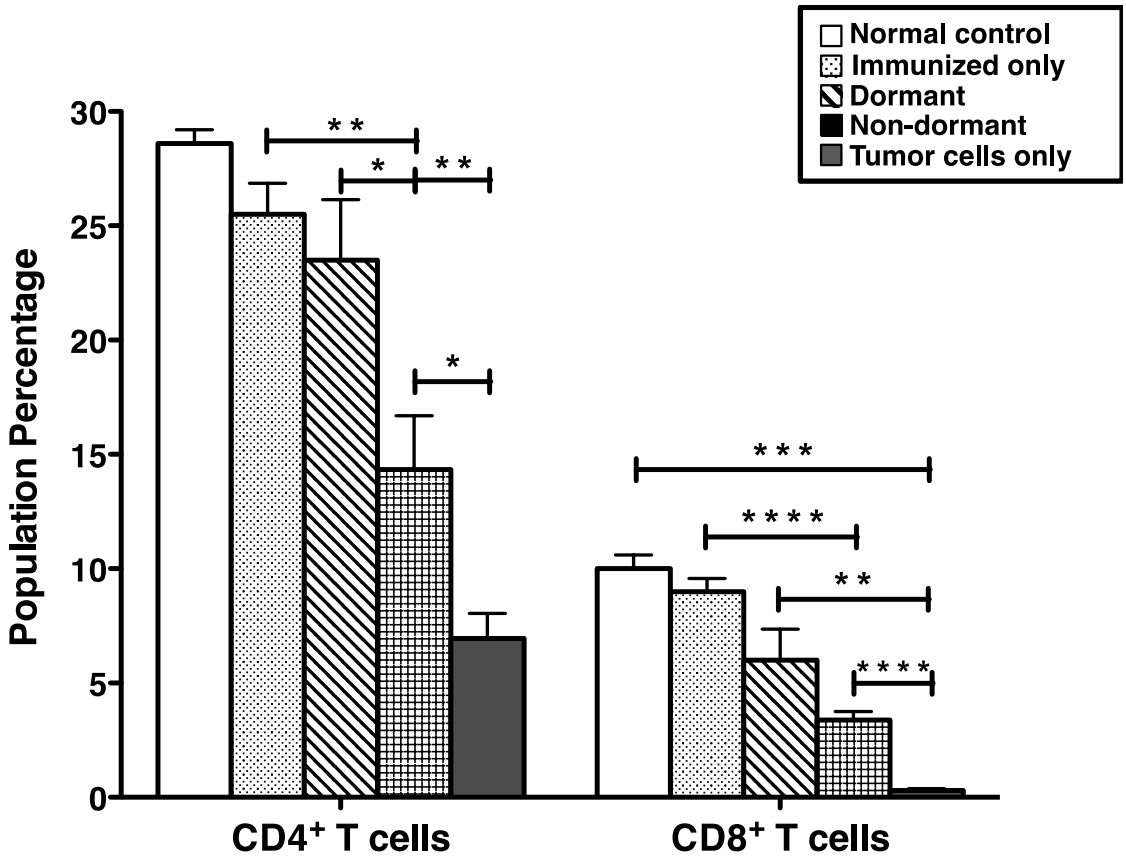

Figure 8. The percentage of splenic $CD4^+$ and $CD8^+$ T cells examined on D+60 post-BCL1 tumor cell challenge.

Spleen cells were analyzed by flow cytometry to determine the percentage of $CD4^+$ and $CD8^+$ T cells in all experiment groups. Increased tumor cell burden as observed in the non-dormant group (n = 5) and the tumor cell only group (n = 4) resulted in the reduction of the $CD4^+$ T cell subset, although not statistically significant. Conversely, the increased tumor burden of mice that were not pre-immunized (n = 6) resulted in an over 30-fold reduction of the $CD8^+$ T cell subset as compared to the normal controls. Normal controls (n = 4); immunized only (n = 4); dormant (n = 8); non-dormant (n = 5); tumor cells only (n = 6). Data are shown as mean ± SEM (* $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$, **** $P < 0.00005$; Students t-test).

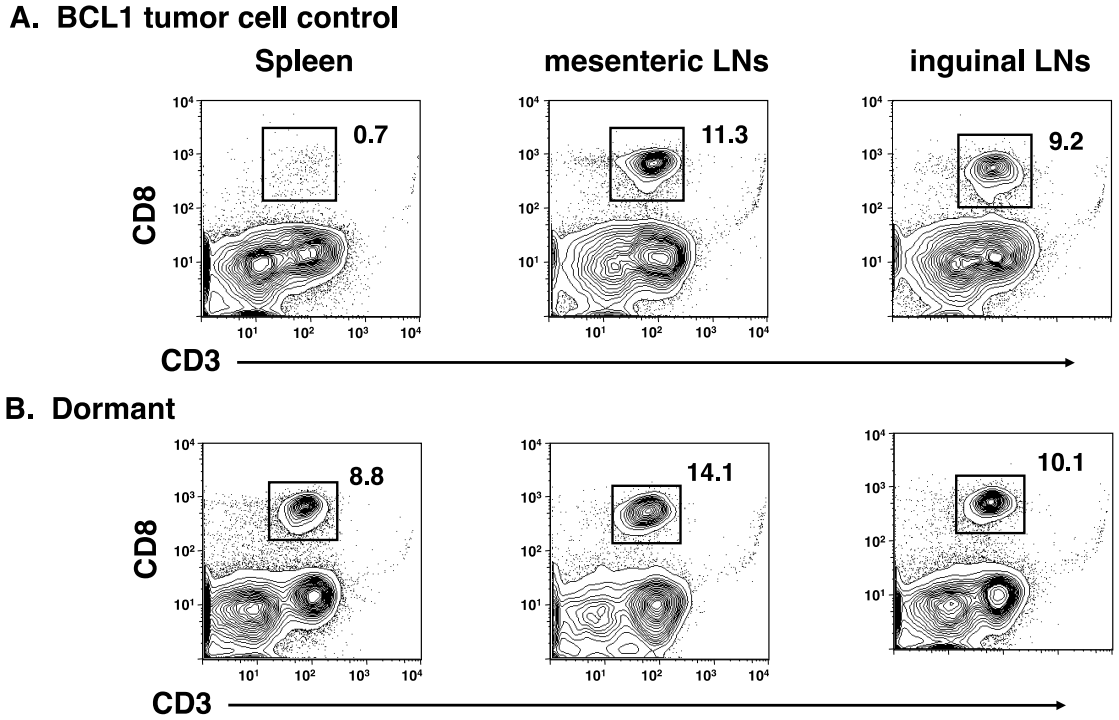

Figure 9. BCL1 tumor cell expansion results in a complete ablation of CD8$^+$ T cells in the absence of immunization.
The spleens and inguinal and mesenteric lymph nodes from mice challenged with BCL1 tumor cells only and mice bearing dormant BCL1 tumor cells were harvested 60 days after challenge with BCL1 tumor cells. Cells were stained with anti-CD3, anti-CD8 and antibodies against non-T cells (anti-B220 and anti-CD11b) and analyzed by flow cytometry. *A,* The CD8$^+$ T cells were completely absent from the spleens but remained unaffected in the draining lymph nodes from unimmunized mice that were challenged with BCL1 tumor cells. *B,* The spleens and lymph nodes from mice bearing dormant tumors showed no significant loss of the CD8$^+$ T cells following challenge with BCL1 tumor cells.

CHAPTER II: EXAMINING THE SUPPRESSIVE CAPACITY OF REGULATORY T CELLS ISOLATED FROM MICE BEARING DORMANT AND NON-DORMANT BCL1 TUMOR CELLS

A. Objective

Our aim in these studies was to examine the role of T_{reg} cells in the BCL1 tumor dormancy model. First, we quantified T_{reg} cells in the tumor microenvironment (spleen) of mice bearing dormant tumor cells and compared their numbers with mice that harbored non-dormant tumor cells, as well as non-immunized mice with profound splenomegaly. Our results showed that mice with the lowest tumor burden (dormant group) had the highest number of T_{reg} cells, whereas mice with the greatest tumor burden (non-immunized group) had the fewest T_{reg} cells in their spleen.

These results were contrary to our hypothesis. Based on the current understanding of T_{reg} cell function and their ability to suppress immune responses, we had predicted that mice with the greatest tumor burden would harbor the highest numbers of T_{reg} cells in their tumor microenvironment. Although the exact role of T_{reg} cells in cancer is still unclear, high numbers of T_{reg} cells have been associated with cancer progression. Since our results were contrary to this trend, we questioned

the function of T_{reg} cells within the dormant BCL1 tumor microenvironment. It was possible that T_{reg} cells were rendered non-functional in mice harboring dormant BCL1 tumor cells by the active anti-tumor immune responses. Therefore, we examined the function of T_{reg} cells from mice bearing both dormant and non-dormant BCL1 tumor cells in order to perhaps find an explanation for our contradictory findings. These studies may also provide insight into how the tumor microenvironment affects the recruitment and/or development of T_{reg} cells *in situ*.

B. The *in vitro* T_{reg} cell suppression assay

1. The key features of the T_{reg} cell suppression assay

The standard experimental assay for assessing T_{reg} cell function is the T_{reg} suppression assay, which measures the ability and potency of T_{reg} cells to suppress the proliferation of conventional T cells. This assay is performed *in vitro*, and measures the direct effect of T_{reg} cells on the proliferation of "responder" T cells. A schematic of the T_{reg} suppression assay is illustrated in Figure 10. Graded doses of purified $CD4^+CD25^+$ T_{reg} cells isolated from the spleen of a mouse harboring dormant or non-dormant BCL1 tumor cells were co-cultured with $CD4^+$ or $CD8^+$ T cells purified from the spleen of normal BALB/c mice. To induce responder T

cell proliferation, irradiated splenocytes (as the source of antigen-presenting cells) and soluble anti-CD3 are added to the culture. Following a 3-day culture period, [^{3}H]thymidine is added to the cultures for uptake by the proliferating T cells in the final 17 hours of the assay. Cells are harvested from wells and [^{3}H]thymidine counts are measured as an indicator of active T cell proliferation.

Alternatively, responder T cells can be labeled with CFSE, a cell-permeating fluorescent dye, prior to culture with T_{reg} cells. The molecule enters the cell and the diacetate groups are cleaved by cytoplasmic esterases to convert the molecule to the fluorescent diester CFSE. The succinimidyl group forms covalent bonds with intracellular molecules allowing the molecule to remain stable within the cell. This property allows for analysis of cell proliferation because as cells divide into daughter cells, the dye is diluted through each division and can be tracked by flow cytometry. Moreover, the number of divisions that the labeled cells undergo can be quantified.

2. Examining T_{reg} cell-mediated suppression in the BCL1 tumor dormancy model

The purity of the T_{reg} cells and the responder cells is very critical in the T_{reg} cell suppression assay since it measures the direct effects of the T_{reg} cells on the responder T cell subsets. To ensure the purity of T_{reg} cells, donor spleen cells were pre-enriched for $CD4^+$ cells using magnetic beads. The enriched cells were then stained with antibodies against CD4, CD25, and a cocktail of lineage markers (B220 and CD11b). T_{reg} cells were sorted on the basis of being $Lineage^-$ $CD4^+$ $CD25^{hi}$. As a control cell subset for the T_{reg} cells, $CD4^+$ $CD25^-$ cells were sorted simultaneously with the T_{reg} cells. The responder cells, either $CD4^+$ or $CD8^+$ T cells, were purified by magnetic beads from spleens cells of normal BALB/c mice. Following cell enrichment, the purity of all subsets was confirmed by FACS analysis as follows: 99-100% (T_{reg} cells), > 95% ($CD4^+$ T cells), and > 85% ($CD8^+$ T cells).

The difference in T_{reg} cell numbers between the dormant and non-dormant groups, as observed initially by phenotype analysis, was also clearly evident during their FACS purification process. The frequency and percentage of T_{reg} cells was higher in mice with dormant BCL1 tumor cells than those with non-dormant tumors. As a result, consistently higher numbers of T_{reg} cells were purified from mice with dormant BCL1 tumor

cells. Furthermore, T_{reg} cells from the BCL1 cell control group could not be purified since their frequency (0.8%, Figure 6) was too low to purify them in sufficient numbers for examination.

The cellular targets for suppression by T_{reg} cells include $CD4^+$ and $CD8^+$ T cells, dendritic cells, NK cells, NKT cells, B cells, and macrophages (66). Although the induction of T_{reg} cells is described to be antigen-specific (138), activated T_{reg} cells can impart their suppressive effects in a broad or bystander manner. Examining the suppressive function of T_{reg} cells is a critical step in defining their role as suppressor cells. The *in vitro* T_{reg} cell suppression assay has proven to be a reliable, albeit not perfect, system to analyze T_{reg} cell function. There are several key factors inherent in the *in vitro* T_{reg} cell suppression assay that investigators must consider when interpreting their results. First, T_{reg} cells can target both responder T cells and the APCs for suppression. Several mechanisms by which T_{reg} cells can directly suppress responder T cells include: competition for IL-2 (65), secretion of suppressor cytokines (55, 59) and granzyme-mediated apoptosis (69). Although T_{reg} cells can target APCs for suppression through multiple pathways, this did not confound the interpretation of our assays since we first irradiated the APCs, thus eliminating their ability to proliferate. This also left the responder T cells as the only population capable of incorporating [^{3}H]thymidine. Although

T_{reg} cells can proliferate *in vitro*, they do so only in the presence of TCR stimulation and high levels of IL-2 (66). Moreover, in our T_{reg} cell suppression assays, T_{reg} cells cultured in the presence of irradiated APCs and anti-CD3 showed negligible proliferation.

In our suppression assays, T_{reg} cells and responder T cells were co-cultured in different ratios: 1:1, equal numbers of T_{reg} cells and responder T cells ($CD4^+$ or $CD8^+$), 0.5:1, one-half the number of T_{reg} cells, and 0.25:1, one-quarter the number of T_{reg} cells. As a proliferation control, responder T cells were plated with irradiated spleen cells and anti-CD3, but with no T_{reg} cells added (0:1). The proliferation of T cell groups that were co-cultured with T_{reg} cells was calculated as the percentage their total counts/ T cell only (0:1) counts x 100 and reported as "% T cell proliferation".

C. T_{reg} cells from the spleens of mice bearing dormant or non-dormant BCL1 tumor cells equally suppress the proliferation of $CD4^+$ T cells

At the highest T_{reg} cell-to-$CD4^+$ T cell ratio (1:1), T_{reg} cells from both dormant and non-dormant groups were able to completely suppress $CD4^+$ T cell proliferation to 2% and 1%, respectively (Figure 11A). T_{reg} cells

from both groups were still very potent even when their numbers were reduced by one-half (0.5:1). The only difference in suppressive capacity between T_{reg} cells from dormant and non-dormant groups was observed at the lowest T_{reg} cell-to-$CD4^+$ T cell ratio (0.25:1). T_{reg} cells from the non-dormant group were significantly more potent suppressors of responder $CD4^+$ T cells than their counterparts from the dormant group (88% vs. 24%, respectively; p = 0.028). The difference in potency may be due to the microenvironments from which the T_{reg} cells were purified. The non-dormant tumor microenvironment is more conducive to tumor growth than the dormant tumor microenvironment and may induce more potent T_{reg} cells. Analysis of the average total numbers of T_{reg} cells and $CD4^+$ T cells in the spleen (Figures 5 and 6) showed that mice bearing dormant and non-dormant BCL1 tumor cells had a T_{reg} cell-to-$CD4^+$ T cell ratio of 0.95:1 and 0.11:1, respectively. These average *in situ* T_{reg} cell-to-$CD4^+$ T cell ratios may provide one explanation for the difference between the dormant and non-dormant tumor microenvironments.

Standard T_{reg} cell suppression assays examine the suppression of the responder $CD4^+$ or $CD8^+$ T cells obtained from normal mice. However, there are marked differences in the physiology and the molecular environments between healthy and tumor-bearing tissues. The presence of tumor cells alters the cytokine and cellular profile of tissues,

which can influence the function of anti-tumor immune responses. Therefore, T cell subsets, including T_{reg} cells and $CD4^+$ T cells, present in tumor cell-infiltrated tissues may function differently than those in healthy tissues. We had speculated that a contributing factor to whether mice develop and maintain tumor dormancy would be the difference in T_{reg} cell function between the dormant and non-dormant tumor microenvironments. Perhaps, the prevalence of anti-tumor immunity in the dormant tumor microenvironment may diminish T_{reg} cell function whereas active BCL1 tumor cell proliferation in the non-dormant tumor microenvironment may enhance T_{reg} cell function. Moreover, $CD4^+$ T cells in the dormant tumor microenvironment may have superior anti-BCL1 tumor immunity than those in the non-dormant tumor microenvironment. To examine more accurately the T_{reg} cell-$CD4^+$ T cell interactions in the tumor microenvironments of the BCL1 dormancy model, we performed T_{reg} cell suppression assays by co-culturing both the T_{reg} cells and the "responder" $CD4^+$ T cells isolated from the same spleens of mice from the dormant or non-dormant groups.

The results of the suppression assays showed that T_{reg} cells from the dormant and non-dormant groups were equally efficient in inhibiting the proliferation of $CD4^+$ T cells isolated from their respective tumor microenvironments (Figure 11B). Moreover, T_{reg} cells were very effective

even at low numbers in suppressing $CD4^+$ T cells (0.25:1). Therefore, the differences associated with dormant and non-dormant tumor microenvironments did not affect the suppressive function of T_{reg} cells.

D. T_{reg} cells exert suppression through contact inhibition

We next examined the mechanism by which T_{reg} cells from mice bearing dormant BCL1 tumor cells suppress the proliferation of $CD4^+$ T cells in our *in vitro* assays. T_{reg} cell-mediated suppression occurs through the secretion of suppressive cytokines such as IL-10, TGF-β, and IL-35, and by direct cell-cell contact (66, 139). However, the examination of T_{reg} cell-mediated suppression by *in vitro* assays has failed to demonstrate a role by soluble factors alone (140, 141), even though neutralization of suppressive cytokines using blocking antibodies has been demonstrated *in vivo* (61, 142). This discrepancy between *in vitro* and *in vivo* studies of suppressive cytokines remains unresolved.

The tissue culture plate cannot replicate the complex environment of organ systems. Therefore, the difference may be related to the limitations inherent within *in vitro* assays and should not minimize the role that suppressive soluble factors have in the tumor microenvironment. Nevertheless, we wanted to examine whether soluble IL-10 was a factor in

our *in vitro* T_{reg} cells suppression assays. Addition of IL-10 neutralizing antibody (10 μg/ml) to co-cultures of T_{reg} cells and $CD4^+$ T cells did not abolish or lessen the suppression of $CD4^+$ T cells by the T_{reg} cells from mice bearing dormant BCL1 tumor cells. Therefore, our *in vitro* assay could not demonstrate a suppressive role for soluble factors as well. We next tested whether adding exogenous IL-10 directly to our cultures would inhibit the *in vitro* proliferation of $CD4^+$ T cells. Recombinant IL-10 (rIL-10), ranging from 5-500 ng/ml, was added to CFSE-labeled $CD4^+$ T cell cultures and their proliferation following a 5-day culture period was assessed by flow cytometry. Our results showed that addition of rIL-10 at any concentration did not suppress $CD4^+$ T cell proliferation (Figure 12). All cultures containing rIL-10 underwent a proliferation at a rate similar to the control samples where no rIL-10 was added. Overall, our results were in agreement with published reports describing the failure to demonstrate IL-10-mediated suppression using *in vitro* assays.

We next examined T_{reg} cell-mediated suppression through direct cell-cell contact. Contact inhibition has been clearly demonstrated by *in vitro* assays as a mechanism used by T_{reg} cells to suppress the proliferation of T cells (62, 141). We tested this by using a Transwell tissue culture system where T_{reg} cells are physically separated from the $CD4^+$ T cells by a semi-permeable barrier (0.4 μm). The Transwell insert

allows the free-flow of soluble factors but prevents the T_{reg} cells from establishing a direct contact with responder cells. For this assay, CFSE-labeled $CD4^+$ T cells, irradiated splenocytes, and soluble anti-CD3 were plated into wells of a 24-well tissue culture plate. The Transwell insert was fitted into the wells and T_{reg} cells (1:1) were deposited into the inserts. After a 5-day culture period, the Transwell inserts were removed and the cells were harvested from each well and analyzed by flow cytometry. Our results showed that preventing contact between T_{reg} cells and $CD4^+$ T cells completely abrogated the suppressive effects of T_{reg} cells **(**Figure 13). The significance of cell contact- mediated suppression was evident because the proliferation rate of the $CD4^+$ T cells in the Transwell cultures was similar to the control cultures where no T_{reg} cells were added (0:1). These results clearly show that T_{reg} cells isolated from the spleens of mice bearing dormant BCL1 tumor cells suppress naïve $CD4^+$ T cells through contact inhibition. However, we cannot rule out inhibitory soluble cytokines as co-factors for suppression mediated by T_{reg} cells.

E. T_{reg} cells from mice bearing dormant tumor cells suppress $CD8^+$ T cell proliferation

Since T_{reg} cells from the mice bearing dormant BCL1 tumor cells were fully functional in suppressing $CD4^+$ T cells, we next examined their ability to suppress $CD8^+$ T cells. Our phenotype analysis had shown that the $CD8^+$ T cell subset was completely abolished in mice injected with BCL1 tumor cells only, but maintained in mice bearing dormant BCL1 tumor cells (Figures 7 and 8). Therefore, we wanted to examine the capacity of T_{reg} cells isolated from mice bearing dormant BCL1 tumor cells to suppress $CD8^+$ T cells. We employed the standard T_{reg} suppression assay, except we used $CD8^+$ T cells from normal BALB/c mice as the responder cell population. CFSE-labeled $CD8^+$ T cells from normal BALB/c mice were co-cultured with graded doses of T_{reg} cells isolated from mice harboring dormant BCL1 tumor cells. Following a 5-day culture period, cells were harvested and analyzed for CFSE dilution by flow cytometry. $CD8^+$ T cells alone underwent substantial proliferation within the culture period since on average, 62 ± 7.6% of the population underwent at least one round of division as measured by CFSE dilution (Figure 14, 0:1). The addition of T_{reg} cells reduced the proliferation of $CD8^+$ T cells in a dose-dependent manner. $CD8^+$ T cell proliferation was reduced by 2-fold, 3.3-fold and 5-fold when co-cultured with increasing

numbers of T_{reg} cells (0.25:1, 0.5:1, and 1:1, respectively) (Figure 14). Therefore, T_{reg} cells within the spleens of mice harboring dormant tumor cells maintained their functional capacity to suppress the proliferation of $CD8^+$ T cells. These data suggest that the persistence of $CD8^+$ T cells in mice bearing dormant BCL1 tumor cells is not due to T_{reg} cell dysfunction.

F. T_{reg} cells do not inhibit BCL1 tumor cell proliferation and their secretion of Id-IgM antibody

There appeared to be no functional differences between T_{reg} cells from either the dormant or non-dormant groups with regard to suppressing T cell subsets. In the context of tumor immunology, T_{reg} cells have been studied for their capacity to suppress anti-tumor immune responses. Although T_{reg} cell activation is antigen-specific, they can impart their suppression in a bystander manner. In our BCL1 tumor dormancy model, the presence of high numbers of T_{reg} cells in the dormant group and relatively low numbers in mice with more advanced disease (non-dormant and non-immunized) led us to think about the possible interactions between T_{reg} cells and tumor cells. We hypothesized that perhaps T_{reg} cells are able to suppress the BCL1 tumor cells through direct or in-direct pathways.

The tumor microenvironment may also impart a bi-modal function to T_{reg} cells with respect to their interaction with infiltrating anti-tumor T cell subsets and the BCL1 tumor cells. For example, the strength and duration of the anti-tumor immune responses in the dormant microenvironment may induce T_{reg} cells to direct their suppression of the BCL1 tumor cells. However, in the non-dormant tumor microenvironment, where the tumor cells are more aggressively expanding, the T_{reg} cells may serve to suppress anti-tumor responses. Therefore, we tested the capacity of T_{reg} cells from both dormant and non-dormant tumor microenvironments to suppress BCL1 tumor cells. To do this, we examined BCL1 cell proliferation, and secretion of Id-IgM, following a co-culture period with T_{reg} cells.

To test BCL1 tumor cell proliferation, we adapted the T_{reg} cell suppression assay and used BCL1.3B3 tumor cells as the “responder” cell population. The BCL1.3B3 cell line is the *in vitro*-adapted cell line of BCL1 cells (143). We chose to use the BCL1.3B3 cells in our *in vitro* assays because isolation of BCL1 tumor cells from tissues did not yield a pure population or sufficient numbers of cells for examination. The BCL1.3B3 tumor cells were co-cultured for 3 days with graded doses of T_{reg} cells isolated from the spleens of mice bearing dormant or non-dormant BCL1 tumor cells. [^{3}H]thymidine was added to the cultures for the final 17 h of

the assay, and the sample counts were measured by a β-counter. The baseline proliferation of BCL1 tumor cells in culture (no T_{reg} cells added) was measured and used to determine the relative proliferation of BCL1 tumor cells that were co-cultured with T_{reg} cells. The results showed that contrary to our hypothesis, T_{reg} cells did not suppress the proliferation of BCL1 tumor cells (Figure 15A).

The addition of T_{reg} cells to cultures of BCL1 tumor cells, in fact, caused an increase in [^{3}H]thymidine incorporation over the control where no T_{reg} cells were added. On average, the addition of T_{reg} cells from mice with dormant BCL1 tumor cells induced a 1.5-fold expansion of the BCL1 tumor cells, although the expansion was not significantly greater than the expansion of BCL1 tumor cells alone (p = 0.86). Moreover, the addition of T_{reg} cells from mice bearing nom-dormant BCL1 tumor cells also did not significantly enhance tumor cell proliferation (1.2-fold, p = 0.98). Overall, T_{reg} cells did not appear to affect the proliferation of BCL1 tumor cells.

Since the T_{reg} cells did not negatively affect the proliferation of BCL1 tumor cells, we tested their ability to suppress or reduce the secretion of Id-IgM by BCL1.3B3 tumor cells. BCL1 tumor cells, when stimulated *in vitro* with LPS, secrete high amounts of Id-IgM (144). Recently, it has been shown that T_{reg} cells can directly suppress normal B cell activity by preventing their secretion of immunoglobulin (145). We

hypothesized that T_{reg} cells may negatively affect BCL1 tumor cells by preventing their secretion of Id-IgM. To test this, BCL1.3B3 tumor cells were cultured alone or with graded doses of T_{reg} cells for 3 days in the presence of LPS (10 μg/ml). At the end of the culture period, the media was collected and assayed by ELISA for soluble Id-IgM. The average concentration of soluble Id-IgM from the culture of BCL1.3B3 cells alone was 454 ± 88 ng/ml (Figure 15B). The addition of T_{reg} cells from mice bearing either dormant or non-dormant BCL1 tumor cells did not significantly reduce or enhance Id-IgM secretion by the cultured tumor cells (p = 0.86 and p = 0.37, respectively). Overall, T_{reg} cells did not suppress the proliferation of BCL1 tumor cells, or inhibit their secretion of Id-IgM. Therefore, T_{reg} cells from the tumor microenvironment of mice bearing dormant or non-dormant BCL1 tumor cells did not appear to negatively affect the BCL1 tumor cells.

G. Summary

We hypothesized that a contributing factor to success or failure in establishing BCL1 tumor dormancy may be due to differences in T_{reg} cell function in the dormant vs. non-dormant tumor microenvironments.

Moreover, we wanted to determine which subsets of cells in the tumor microenvironment were subject to suppression by T_{reg} cells.

In assessing T_{reg} cell function, we utilized the standard *in vitro* T_{reg} cell suppression assay. We purified T_{reg} cells from mice bearing dormant and non-dormant BCL1 tumor cells and tested the capacity of each to suppress T cell subsets (both $CD4^+$ and $CD8^+$) from normal mice and from the same tumor microenvironment as the corresponding T_{reg} cells. Moreover, we examined the possible suppressive effects that T_{reg} cells may impart on BCL1 tumor cells.

Our collective results show that T_{reg} cells from both the dormant and non-dormant groups were equally functional in their capacity to suppress both naive $CD4^+$ and $CD8^+$ cells. Interestingly, T_{reg} cells appeared to be more potent in suppressing $CD4^+$ T cells isolated from the tumor microenvironments, suggesting an antigen-specific activation and enhancement of T_{reg} cell function in the tumor microenvironment. Moreover, T_{reg} cell suppression was clearly mediated through cell contact as prevention of contact by T_{reg} cells completely abrogated their suppression of $CD4^+$ T cells.

T_{reg} cells, however, did not appear to negatively affect the BCL1 tumor cells. There was no significant suppression of tumor cell proliferation when co-cultured with T_{reg} cells. Moreover, the ability of

BCL1 tumor cells to secrete Id-IgM was not suppressed by T_{reg} cells. Therefore, T_{reg} cells from both the dormant and non-dormant tumor microenvironments suppressed T cell subsets but not the BCL1 tumor cells.

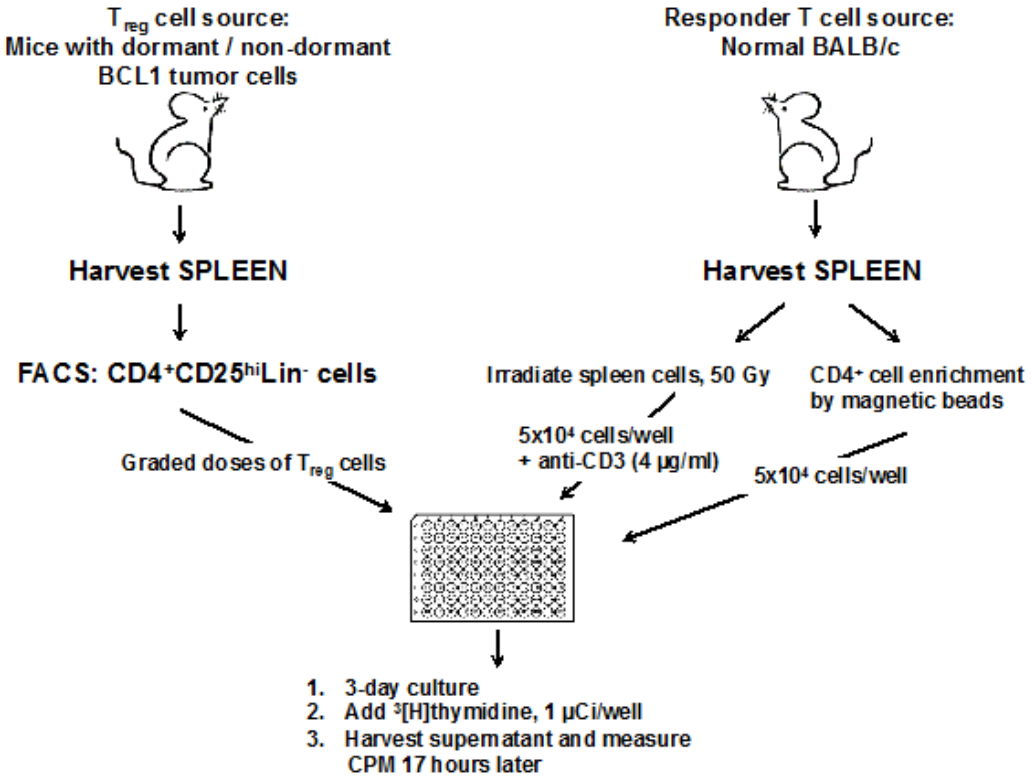

Figure 10. Outline of the T_{reg} cell suppression assay on $CD4^+$ T cell proliferation.

This *in vitro* assay examined the suppression potential of T_{reg} cells on the proliferation of normal $CD4^+$ T cells. T_{reg} cells from the spleens of donor mice (dormant or non-dormant group) were FACS-purified and co-cultured in graded doses with $CD4^+$ T cells isolated from normal mice by magnetic bead enrichment. Irradiated spleen cells and anti-CD3 were added to induce the proliferation of the $CD4^+$ T cells. All samples were plated in triplicates. After a 3-day culture period, ^{3}[H]thymidine was added for the final 17 hours in culture. Finally, the cells from each well were harvested and the incorporation of ^{3}[H]thymidine was measured using a liquid scintillation counter.

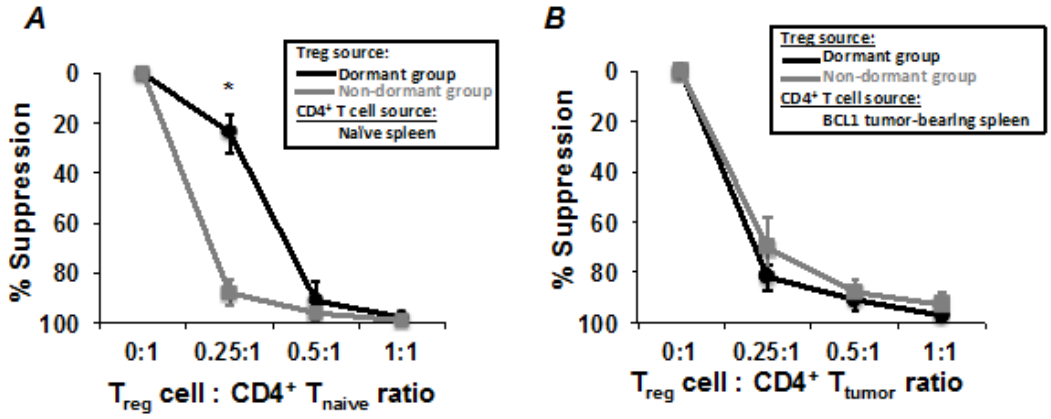

Figure 11. T_{reg} cells from BCL1 tumor-challenged mice suppress the proliferation of both naive and antigen-specific $CD4^+$ T cells.
A, T_{reg} cells ($CD4^+CD25^+$) were FACS-sorted from mice bearing dormant or non-dormant tumor cells and co-cultured in graded doses with purified "responder" $CD4^+$ T cells ($5x10^5$ cells/well) from spleens of normal mice in the presence of anti-CD3 (4 µg/ml) and irradiated spleen cells ($1x10^5$ cells/well). ^{3}[H]thymidine (1 µCi/well) was added for the final 17 hours of the culture period followed by harvest and measurement of thymidine incorporation. Suppression by T_{reg} cells was calculated relative to the samples containing $CD4^+$T cells only (0:1). The data are shown as mean ± SEM from three experiments (* $P < 0.05$, Student's t test.) *B*, T_{reg} cell isolation was the same as in *A.* The "responder" $CD4^+$ T cells ($CD4^+CD25^-$) were sorted from the same donors as the T_{reg} cells and cultured using conditions described in *A*. The data is shown as mean ± SEM from 2-3 experiments.

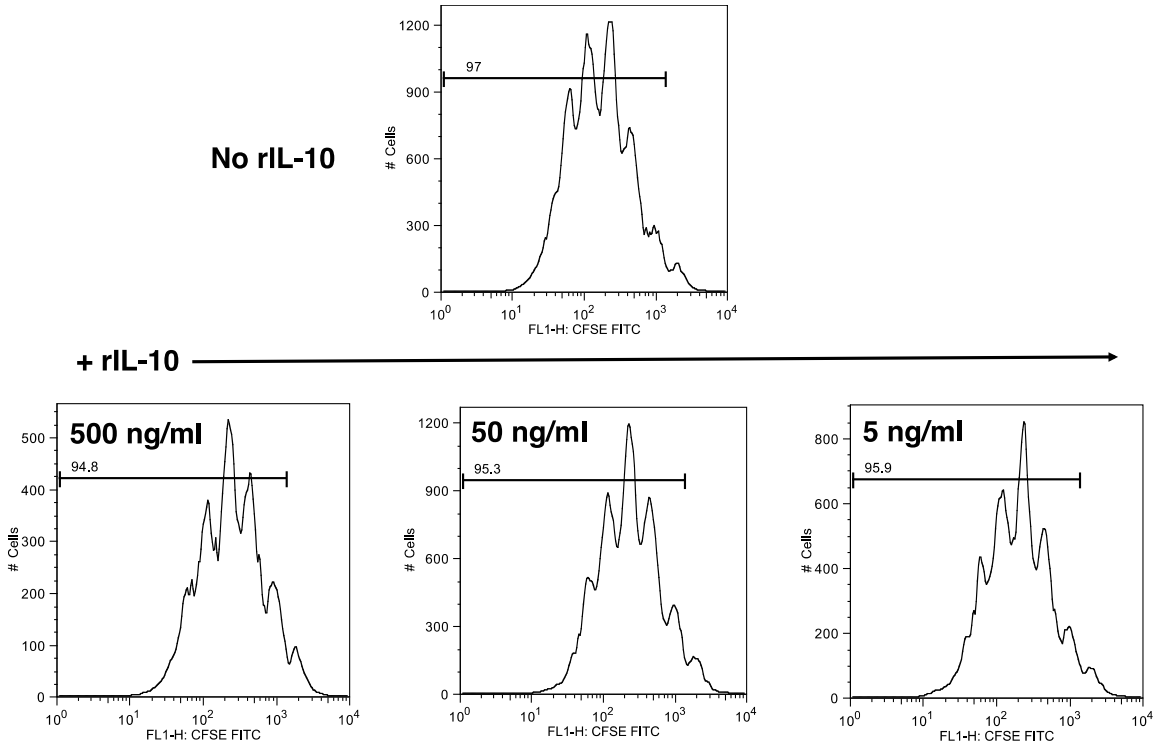

Figure 12. Soluble rIL-10 does not exhibit suppressive effects in *in vitro* T_{reg} cell suppression assays.
$CD4^+$ T cells were purified from healthy mice, labeled with CFSE (5 μM) and cultured ($5x10^5$ cells/well) with irradiated spleen cells ($1x10^6$ cells/well) and anti-CD3 (4 μg/ml). Different doses of rIL-10 were added to the culture wells and the cells were harvested after 5 days and stained with anti-CD4 and anti-CD25. Proliferation of $CD4^+$ T cells was determined by using CFSE analysis by flow cytometry. These data are representative of two independent experiments yielding similar results.

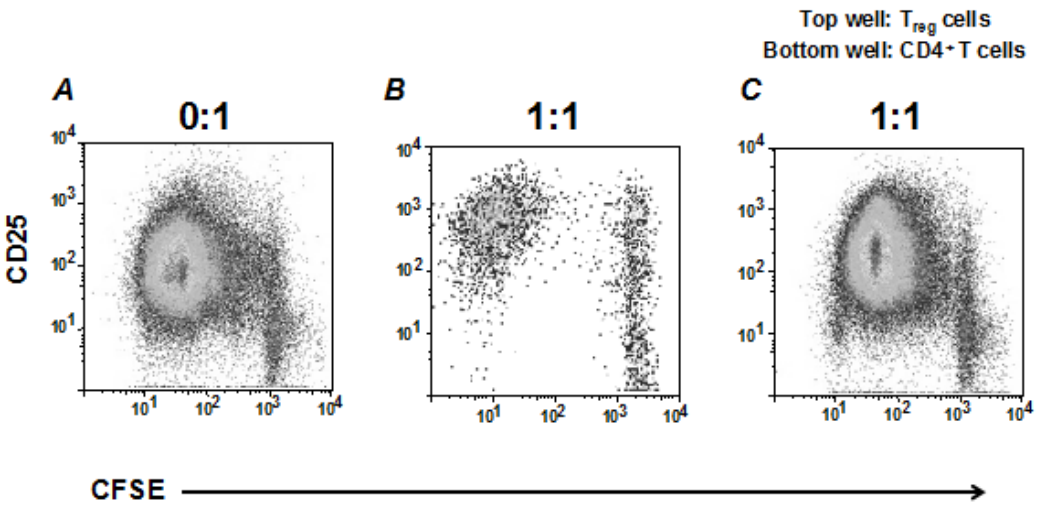

Figure 13. T_{reg} cells suppress the proliferation of $CD4^+$ T cells primarily through cell contact.
A, CFSE-labeled $CD4^+$ T cells ($5x10^5$ cells/well) were co-cultured with irradiated whole spleen cells ($1x10^6$ cells/well), and anti-CD3 (4 µg/ml). *B*, Equal numbers of FACS-purified T_{reg} cells ($5x10^5$ cells/well) were added to the cultures containing $CD4^+$ T cells.. The $CFSE^-CD25^+$ cells represent the T_{reg} cells. *C,* FACS-purified T_{reg} cells ($5x10^5$ cells/well) were added onto a Transwell insert (4 µm) that was placed into wells containing the $CD4^+$ T cells, irradiated spleen cells, and anti-CD3 MAb.. The insert membrane allowed the free flow of soluble factors but prevented direct cell contact. After 5 days in culture, cells were harvested and stained for CD4 and CD25 and analyzed for $CD4^+$ T cell proliferation using CFSE. These data are representative of four independent experiments yielding similar results.

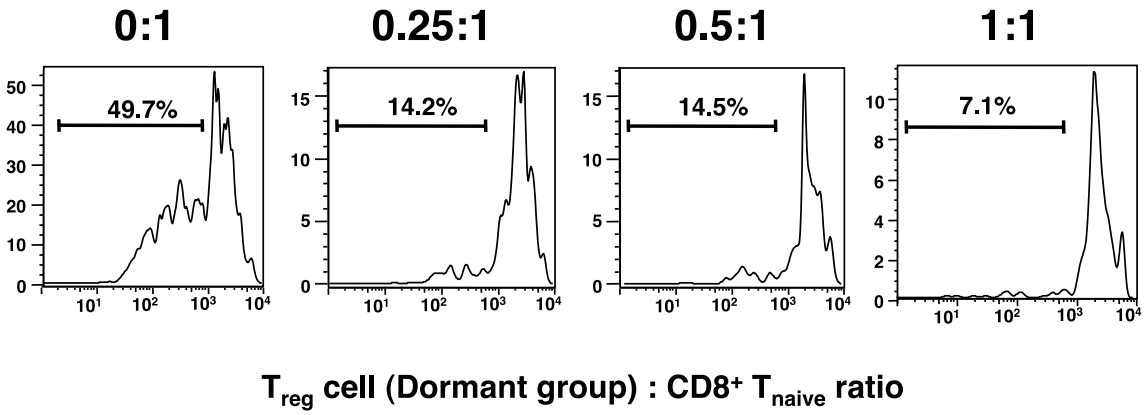

Figure 14. T_{reg} cells from BCL1 tumor-bearing mice suppress the proliferation of $CD8^+$ T cells.

T_{reg} cells were FACS-purified from the spleen cells of mice bearing dormant tumor cells were added in graded doses to cultured containing purified "responder" $CD8^+$ T cells from normal mice labeled with CFSE and anti-CD3 (4 μg/ml) and irradiated spleen cells. After 5 days in culture cells were harvested and stained with antibodies against CD8 and CD25 and using propidium iodide to exclude dead cells. Histogram plots are gated on $CD8^+$ cells and the values represent the percent CFSE dilution. These data are representative of three independent experiments yielding similar results.

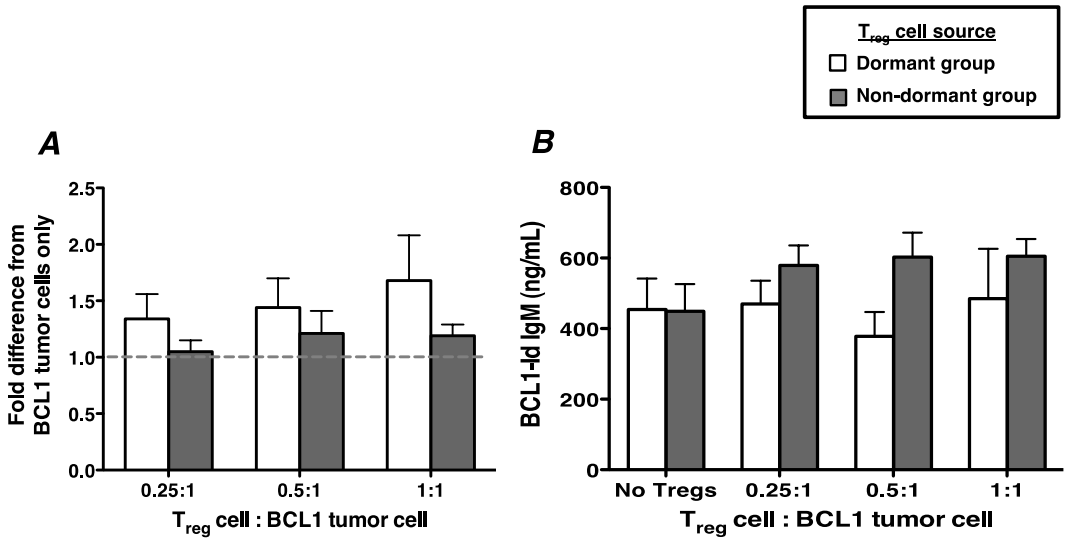

Figure 15. T_{reg} cells do not suppress the proliferation of BCL1 tumor cells nor inhibit their secretion of IgM.
A, T_{reg} cells from mice bearing dormant or non-dormant BCL1 tumor cells were sorted and co-cultured in graded doses with BCL1.3B3 tumor cells in the presence of anti-CD3 and irradiated spleen cells for 72 hours. [^{3}H]Thymidine was added in the final 17 hours of the culture period followed by harvest and measurement of thymidine incorporation. The thymidine uptake by BCL1.3B3 cells in the absence of T_{reg} cells was used as baseline proliferation. All other samples were calculated as the ratio of their thymidine uptake to the baseline samples. The data represent the average of 3 experiments. *B*, The BCL1.3B3 proliferation assay was set up as described in *A.* At the end of the 72-hour culture period supernatants from all wells were harvested and analyzed by ELISA for soluble BCL1 Id-IgM. The data represent the average of 4 experiments.

CHAPTER III: THE REGULATORY B CELL-TYPE FEATURES OF BCL1 TUMOR CELLS

A. Objective

The tumor microenvironment can negatively regulate cellular immune responses through multiple pathways. This microenvironment is composed of diverse populations of cells including the malignant, immune, and stromal cells. It is also highly vascularized and contains pro-angiogenic factors, cytokines, chemokine's, and other growth factors. Tumor cells thrive by constant manipulation of this environment, creating conditions that favor tumor expansion and render anti-tumor immune responses ineffective.

T_{reg} cells have been described to promote immune tolerance in the tumor microenvironment. However, in a diverse cellular environment such as in a tumor setting, multiple regulatory-type cells can participate in the negative regulation of immune responses. It is well known that tumor cells secrete immunosuppressive cytokines such as IL-10 and TGF-β and therefore contribute to immune dysregulation. More recently, a regulatory B cell (B_{reg} cell) subset with a distinct phenotype ($CD19^{+}CD1d^{hi}CD5^{+}$) that

secretes high levels of IL-10 was described (100). Similar in function to T_{reg} cells, this B cell subset can suppress autoreactive cells and thus contribute to the maintenance of immunological homeostasis.

In light of the characterization of B_{reg} cells, we hypothesized that since BCL1 tumor cells are malignant B cells and can evade immune responses, they may share features with B_{reg} cells. Therefore, we examined the BCL1 tumor cells for B_{reg} cell characteristics including phenotype, cytokine profile, and immunosuppression.

B. BCL1 tumor cells exhibit a regulatory B cell phenotype

Early studies on BCL1 tumor cells concluded that they were immature B cells that had become malignant (146). BCL1 tumor cells express cell surface markers such as CD19, MHC II molecules (I-A), CD5, and high levels of surface IgM but low levels of surface IgD (124, 125, 147-149), suggesting that they have a B-1a B cell origin (147). To date, multiple mouse B cell subsets have been described as promoting suppression in autoimmune disease models (reviewed in DiLillo *et al.* (109)). Most of these "regulatory B cell subsets" reside predominantly in the peritoneal cavity and spleen, secrete high levels of IL-10, and are $CD5^+$. More specifically, the B10 subset of B_{reg} cells has a phenotype

($CD19^+CD1d^{hi}CD5^+$) which overlaps with $CD5^+$ B-1a cells as well as marginal zone (MZ) and transitional-2 (T2)-MZ precursor B cell subsets (100, 106). Since BCL1 tumor cells also reside primarily in the spleen and appear to have an ontogeny similar to the B10 B_{reg} cell subset, we investigated further possible associations between these B cell subsets.

First, we assessed whether BCL1 tumor cells share surface marker expression with B10 B_{reg} cells. We isolated spleen cells from mice bearing dormant BCL1 tumor cells as well as non-immunized mice challenged with BCL1 tumor cells. Cells were co-stained with fluorescent-labeled anti-BCL1 Id-IgM, anti-CD1d, and anti-CD5 antibodies and analyzed by flow cytometry. This staining combination yielded 3 distinct subsets of spleen cells in both groups (Figure 17). The largest subset of the cells in both groups did not express either marker ($CD1d^-CD5^-$). In mice bearing dormant BCL1 tumor cells, 38% of the cells expressed this phenotype, whereas 56% of the spleen cells from non-immunized mice were $CD1d^-$ $CD5^-$. This subset did not contain BCL1 tumor cells since co-staining with anti-BCL1 Id-IgM antibody showed no expression of the BCL1-specific surface Id-IgM.

A second distinct subset was characterized as $CD1d^{lo}CD5^+$ and constituted 20-28% of the cells. This subset also did not co-stain with anti-Id antibody, therefore these cells were not BCL1 tumor cells (Figure

17). However, a third subset was evident that displayed the phenotype corresponding to B10 B_{reg} cells ($CD1d^{hi}CD5^{+}$). This subset constituted 34% of the spleen cells from the non-immunized group and 24% of the spleen cells from mice bearing dormant BCL1 tumor cells. Interestingly, in both groups, the cells expressing the B_{reg} cell appeared to be BCL1 tumor cells since they co-expressed the BCL1 tumor-specific marker Id-IgM. The difference in the intensity of Id-IgM expression between the dormant and the non-immunized group is due to the pre-immunization of the dormant group as has been described in an earlier study (40). Immunization induced the production of anti-BCL1-Id antibody in the dormant group which competed with the labeled anti-BCL1-Id antibody used for phenotyping and thus reduced the intensity of detection by flow cytometry. Although mice in the BCL1 cell control group do generate anti-BCL1-Id antibody, the immune response is not as potent as the pre-immunized mice and therefore, there are much lower levels of circulating anti-BCL1-Id in this group. These results clearly showed that the BCL1 tumor cells homogeneously expressed the phenotype of B10 B_{reg} cells.

C. The regulatory B cell phenotype expressed by BCL1 tumor cells is not homogeneously expressed by other B cell lymphoma cell lines

The discovery that BCL1 tumor cells also expressed a homogeneous B10 B_{reg} cell phenotype prompted several important questions: 1) are BCL1 tumor cells B_{reg} cells that became malignant?; 2) when normal B cells undergo neoplastic transformation, do they acquire a B_{reg} cell phenotype and associated regulatory features, enhancing their ability to evade anti-tumor responses?; and 3) Is the B_{reg} cell phenotype a feature of all B cell lymphomas, or unique to BCL1 tumor cells? To answer this last question we first examined the tissue culture-adapted BCL1 tumor cells, the BCL1.3B3 cell line, to ensure that these cells also expressed the B10 B_{reg} cell phenotype. BCL1.3B3 cells were stained with anti-CD1d and anti-CD5 antibodies and analyzed by flow cytometry. The results showed that this BCL1 cell line was uniformly $CD1d^{hi}CD5^{+}$, and therefore reflected the B_{reg} cell phenotype observed for the *in vivo* BCL1 tumor cells (Figure 18A). The BCL1 tumor cells had maintained the B_{reg} cell-type features through the *in vitro* adaptation process, therefore the BCL1.3B3 cells could be used to further examine the B_{reg} cell-type features of BCL1 tumor cells in a tissue culture setting.

Next, we wanted to determine if other B cell lymphoma cell lines shared the B_{reg} cell phenotype observed for the BCL1 tumor cells. We

examined two B cell lymphoma cell lines with similar characteristics to the BCL1.3B3 cells; the WEHI 231 (150) and WEHI 279 cell lines (151). Both are murine lymphomas and are described as being immature B cell lines, bearing surface IgM and IgD. However, unlike BCL1.3B3 cells, which constitutively secrete IgM, the WEHI 231 cell line secretes IgM only following LPS stimulation. The WEHI 279 cell line does not secrete IgM. We found that all three B cell lymphoma cell lines expressed similar levels of CD19 and CD5, but differed in their surface expression of CD1d. Only 63% of the WEHI 231 cells and 3% of the WEHI 279 cells were $CD1d^{hi}$ (Figure 18, B-C). Therefore, not all B cell lymphoma cell lines homogeneously express the B10 B_{reg} cell phenotype.

D. BCL1 tumor cells produce high levels of IL-10, a defining feature of regulatory B cells

Negative regulation of immune responses by B_{reg} cells is described to be mediated through IL-10 (98, 152) (109). Moreover, the B10 subset of B_{reg} cells appear to produce only IL-10 (107). Since the BCL1 tumor cells exhibited the phenotype of B10 B_{reg} cells, we sought to determine if

they also had a similar cytokine secretion profile. To examine cytokine secretion by the tumor cells, we cultured BCL1.3B3 cells for three days with or without LPS (10 μg/ml). Following the culture period, we collected the supernatants to examine soluble cytokines by ELISA. The "baseline" concentration of IL-10 from supernatants of un-manipulated tumor cells was 250 pg/ml. LPS stimulation induced a significant increase in IL-10 secretion (> 12,000 pg/ml) although it was also associated with increased cell loss. LPS secretion also induced the secretion of IL-6 (1,002 pg/ml) but not IL-1β, TGF-β, IFN-γ, or TNF-α (Figure 19). Therefore, BCL1 tumor cells, like B10 B_{reg} cells, produced high levels of IL-10. Since BCL1 tumor cells are malignant B cells, they may have characteristics beyond those that strictly define B_{reg} cells, in this case, the production of IL-6.

E. BCL1 tumor cells possess functional features of regulatory B cells such as their capacity to suppress $CD4^+$ T cells

Similar to T_{reg} cells, B_{reg} cells have been described to contribute to the maintenance of peripheral tissue tolerance by suppressing immune responses (96). To determine if BCL1 tumor cells had B_{reg} cell-like characteristics beyond their phenotype and cytokine secretion profile, we examined their functional capacity to suppress the proliferation of $CD4^+$ T

cells. For these experiments, we adapted the T_{reg} suppression assay by using the BCL1 tumor cells as the suppressor cells in a "B_{reg} cell suppression assay". To ensure the purity of the suppressor cells in the assay, we utilized the BCL1.3B3 cell line. For the responder cells we purified $CD4^+$ T cells from the spleens of normal BALB/c mice, labeled them with CFSE and cultured them with irradiated BALB/c splenocytes and anti-CD3.

When cultured for 6 days, the $CD4^+$ T cells proliferated extensively, as indicated by their CFSE dilution (Figure 19A, 0:1 ratio). The $CD4^+$ T cells also proliferated when co-cultured with low numbers of BCL1.3B3 cells (0.2:1). However, the addition of greater numbers of BCL1.3B3 tumor cells (0.5:1 and 1:1) appeared to kill the $CD4^+$ T cells, as indicated by the paucity of $CD4^+$ cells. Upon further analysis of $CD4^+$ T cell viability, the results showed no statistical differences between $CD4^+$ T cells cultured alone or with low numbers of BCL1.3B3 cells [14.3 ± 7% vs. 11.2 ± 2.1%, p = 0.49] (Figure 19B). In contrast, $CD4^+$ T cell viability was significantly reduced when increasing numbers of BCL1.3B3 cells were added to the cultures. At the 0.5:1 ratio, $CD4^+$ T cell viability was only 2.1 ± 2.6% (p = 0.014), which was 5-fold less than when $CD4^+$ T cells were cultured alone. Furthermore, $CD4^+$ T cell viability was 18-fold lower when co-cultured with equal numbers of BCL1.3B3 cells (0.8 ± 0.5%, p = 0.007).

These results show that when BCL1.3B3 cell numbers expand beyond a certain threshold, they induce the killing of $CD4^+$ T cells.

F. BCL1 tumor cells induce $CD4^+$ T cell apoptosis by activation of the caspase-3 pathway

Apoptosis, or programmed cell death, occurs by multiple mechanisms that include both the extracellular triggering of “death” signals and intracellular changes in response to stress. Extracellular signals are transmitted through two defined pathways designated by their cell surface receptors: 1) the tumor necrosis factor receptor (TNFR) family, which includes TNF receptor 1 (TNFR1), and 2) TNF-related apoptosis-inducing ligand receptors (TRAIL-1 and -2) and CD95 (APO-1, Fas) (153-156). Binding of CD95L (Fas-Ligand) to CD95 on cells leads to the formation of the death-inducing signaling complex (DISC), which transmits signals from the extracellular death receptor (157). The caspase family of intracellular proteins plays a key role in this process. Caspases are a family of highly conserved cysteine-dependent aspartate-specific proteases that are divided into two classes: initiator caspases, (caspase-2, 8, 9, and 10), and effector caspases (caspase-3, 6, and 7) (158). Activation of the initiator caspases leads to their cleavage of the effector procaspase zymogens.

Once activated, the effector caspases proteolytically degrade intracellular proteins, which ultimately leads to cell death.

T cell apoptosis triggered through the CD95-CD95L pathway is a key regulator of T cell homeostasis in the periphery (159). This mechanism is most apparent in the contraction phase following the clonal expansion of T cells in response to foreign antigen. In this phase, T cells upregulate CD95L and can induce both T cell "suicide" and "fratricide" as a way to restore peripheral homeostasis (157). Interestingly, studies have also reported CD95L expression by tumor cells (160-162) (163). They demonstrated that tumor cells expressed CD95L and killed infiltrating (CD95-expressing) lymphocytes. These findings suggested a new mechanism by which tumor cells evade the immune system.

Since we observed $CD4^+$ T cell killing by the BCL1 tumor cells, we sought to determine the mechanism by which this occurred. First, we explored the CD95-CD95L pathway of apoptosis because we had observed that BCL1.3B3 tumor cells express CD95L on their cell surface. To investigate the mechanism of T cell death we examined activated caspase-3 levels in $CD4^+$ T cells as an indicator of the apoptosis pathway leading to cell death. $CD4^+$ T cells were co-cultured with graded doses of BCL1.3B3 tumor cells for both 5 hours and 48 hours *in vitro*. Cells were then harvested, stained for intracellular caspase-3, and analyzed by flow

cytometry. The caspase-3 levels in $CD4^+$ T cells co-cultured with BCL1.3B3 tumor cells were compared with those from $CD4^+$ T cells cultured alone. Following a 5-hour co-culture period, there was no increase in activated caspase-3 levels in $CD4^+$ T cells when co-cultured with BCL1 tumor cells.

Using flow cytometry, we also observed that BCL1 cells express programmed death 1 ligand (PD-L1). PD-L1 is expressed by malignant cells and is an important immune modulator capable of suppressing host immunity (164). PD-L1 binds with high affinity to its receptor, PD-1, expressed by T cells,. After binding, it delivers a signal that inhibits T cell proliferation. Inhibition occurs through the recruitment of protein tyrosine phosphatases such as SHP-1 and SHP-2, and results in the inhibition of signaling events downstream of the TCR (165). We hypothesized that BCL1 tumor cells may utilize negative signaling by PD-L1 to inhibit T cell proliferation. Therefore, we attempted to block PD-L1 signaling by adding anti-PD-L1 antibody (10 μg/ml) to co-cultures of BCL1.3B3 cells and naïve $CD4^+$ T cells. We observed no differences in T cell proliferation following the addition of neutralizing anti-PD-L1 antibody.

Increased activated caspase-3 levels were observed following a 48-hour co-culture period with BCL1 tumor cells in a dose-dependent manner (Figure 21A). Low numbers of BCL1 tumor cells did not enhance

caspase-3 activation beyond the control samples ($CD4^+$ T cells only). However, greater numbers of BCL1 tumor cells (5:1 and 10:1) induced an increase in activated caspase-3 levels in the $CD4^+$ T cells. Although not statistically significant (p = 0.055 for the 10:1 samples), the levels of activated caspase-3 in $CD4^+$ T cells co-cultured with BCL1 tumor cells were as much as 4-fold higher than the control samples ($CD4^+$ T cells only). These results showed that increasing numbers of BCL1 tumor cells enhanced the killing of $CD4^+$ T cells through the caspase-3-mediated apoptosis pathway.

Next, we attempted to neutralize the CD95L signal transduction pathway. We added anti-CD95L antibody (FasL; 20 µg/ml) to block the binding of CD95L to its receptor CD95. Our results showed that, at least *in vitro*, blocking CD95L did not reverse caspase-3 activation in the $CD4^+$ T cells. These results suggest that, the CD95-CD95L pathway does not primarily mediate either T cell apoptosis, or that the examination of this pathway cannot be demonstrated using an *in vitro* system. However, we cannot ignore the possibility of T cell apoptosis through the other signaling receptors such as TNFR and TRAIL.

G. BCL1 tumor cells induce $CD8^+$ T cell apoptosis by activation of the caspase-3 pathway

$CD8^+$ cytotoxic T lymphocytes (CTLs) also infiltrate the tumor microenvironment as part of anti-tumor immune responses. Therefore, we examined caspase-3 activation in CTLs following a co-culture period with BCL1.3B3 tumor cells as we had done for the $CD4^+$ T cells. The CTL results revealed a trend similar to the $CD4^+$ T cell results (Figure 21B). Co-culture of CTLs with low numbers of BCL1.3B3 tumor cells (0.5:1 and 1:1) did not increase active caspase-3 levels beyond the controls (CTLs alone). However, high numbers of BCL1.3B3 tumor cells induced a 7-fold increase in active caspase-3 levels in the CTL. These results show that as BCL1 tumor cells expand they reach a threshold levels where they can trigger apoptosis in T cell subsets.

H. BCL1 tumor cell-mediated suppression of $CD4^+$ T cells is not primarily dependent on soluble factors

One way by which regulatory cells can impart suppression is through the production of inhibitory cytokines, notably IL-10 (109, 166). More specifically, the B10 subset of B_{reg} cells produces high levels of IL-10 but not TGF-β (107). Although TGF-β is also an inhibitory cytokine, its

pivotal role is in the maintenance and expansion of regulatory cell subsets (167). To assess the role of these inhibitory cytokines in BCL1 tumor cell suppression of $CD4^+$ T cells *in vitro*, we added anti-IL-10 and anti-TGF-β antibodies (10 μg/ml, each) to co-cultures of BCL1 tumor cells and CFSE-labeled $CD4^+$ T cells. Following a 6-day culture period, cells were harvested and analyzed by flow cytometry. The results showed that $CD4^+$ T cell viability was < 1% in samples containing equal numbers of BCL1.3B3 tumor cells and $CD4^+$ T cells (1:1). Even when tumor cell numbers were reduced to one-half (0.5:1) $CD4^+$ T cell viability was still below 1% (Figure 22). The FACS profile of the viable $CD4^+$ T cells showed a single cluster of $CFSE^{hi}$ cells indicating that the surviving $CD4^+$ T cells failed to undergo cellular division and thus had not diluted the intracellular CFSE to their daughter cells. Therefore, neutralizing the inhibitory cytokines IL-10 and TGF-β did not reverse the tumor cell-mediated suppression of the $CD4^+$ T cells (Figure 22). These results suggest that, at least *in vitro*, BCL1.3B3 tumor cells do not rely on inhibitory cytokines such as IL-10 and TGF-β to suppress $CD4^+$ T cells.

I. BCL1 tumor cells mediate T cell suppression through contact inhibition

Since IL-10 and TGF-β did not contribute to BCL1-mediated suppression of $CD4^+$ T cells *in vitro*, we next tested whether BCL1 tumor cells could suppress $CD4^+$ T cell proliferation through contact inhibition. As in the T_{reg} cell suppression assay, we added BCL1.3B3 cells into 0.4 μm Transwell inserts to prevent their contact with the $CD4^+$ T cells, yet allow the free-flow of soluble factors during the culture period. When cell contact was prevented, the $CD4^+$ T cells proliferated at a rate similar to cultures where no tumor cells were added (Figure 23A and B). These results showed that BCL1 tumor cells suppress $CD4^+$ T cells primarily through cell contact.

Although contact inhibition appeared to be the principal mechanism of suppression by tumor cells, we investigated additional pathways that may contribute to BCL1 tumor cell suppression of $CD4^+$ T cells. Since BCL1 tumor cells generate and secrete inhibitory cytokines, we attempted to neutralize this mechanism of suppression. Therefore, in addition to the Transwell feature, we added blocking antibodies to IL-10 and TGF-β(10 μg/ml, each). The results showed that blocking the inhibitory cytokines enhanced the proliferation rate of $CD4^+$ T cells by 12-16% over preventing contact alone (Figure 23C). Therefore, inhibitory soluble factors are not

the prime inhibitors of $CD4^+$ T cells but appear to have an accessory role in their suppression.

J. Summary

The goal of this study was to examine whether the BCL1 tumor cells exhibited the phenotype, cytokine secretion profile, and function of B_{reg} cells.

We examined the phenotype of spleen cells freshly isolated from mice that had been injected with BCL1 tumor cells. Moreover, we also examined the BCL1.3B3 cell line that had been generated by adapting BCL1 spleen cells in tissue culture over multiple passages. Our results showed that both fresh BCL1 spleen cells and the BCL1.3B3 cell line homogeneously expressed the phenotype of the B10 subset of mouse B_{reg} cells ($CD1d^{hi}CD5^+$). Interestingly, examination of other B cell lymphoma cell lines showed variable expression of the B_{reg} cell phenotype, suggesting that the B_{reg} cell phenotype may be unique to BCL1 tumor cells.

After determining that BCL1 tumor cells expressed the phenotype of B10 B_{reg} cells, we next examined the cytokine secretion profile of BCL1 tumor cells. It has been shown that B_{reg} cells secrete high levels of IL-10,

a potent immunosuppressive cytokine. In fact, one subset of B_{reg} cells has been identified as "B10" cells for their ability to strictly secrete IL-10. To examine IL-10 secretion by BCL1 tumor cells, we performed ELISA assays on tissue culture supernatants with or without LPS stimulation. The results showed that BCL1 tumor cells did produce high levels of IL-10, but not TGF-β, another potent inhibitory cytokine. Therefore, the cytokine profile of BCL1 tumor cells appears to be identical to the B10 subset of B_{reg} cells.

Lastly, we tested the functional capacity of BCL1 tumor cells to suppress T cell subsets. Our "B_{reg} cell suppression assay" showed that co-culture of BCL1 tumor cells with $CD4^+$ or $CD8^+$ T cells resulted in the killing of the T cells. Furthermore, the killing was in part due to the activation of the caspase-3 pathway of apoptosis in both $CD4^+$ and $CD8^+$ T cell subsets.

Further study of the suppressive nature of the BCL1 tumor cells revealed that they suppress T cells primarily through cell-cell contact. Preventing cell contact between the BCL1 tumor cells and $CD4^+$ T cells completely abrogated their suppressive capacity. Interestingly, although the suppressor cytokines IL-10 and TGF-β enhanced the capacity of BCL1 tumor cells to suppress the proliferation of $CD4^+$ T cells, their primary mode of suppression was clearly through contact inhibition. Overall, these

results showed that BCL1 tumor cells uniquely express the phenotype and function of B_{reg} cells. This novel characterization may help devise or improve current anti-tumor approaches for clinical therapy.

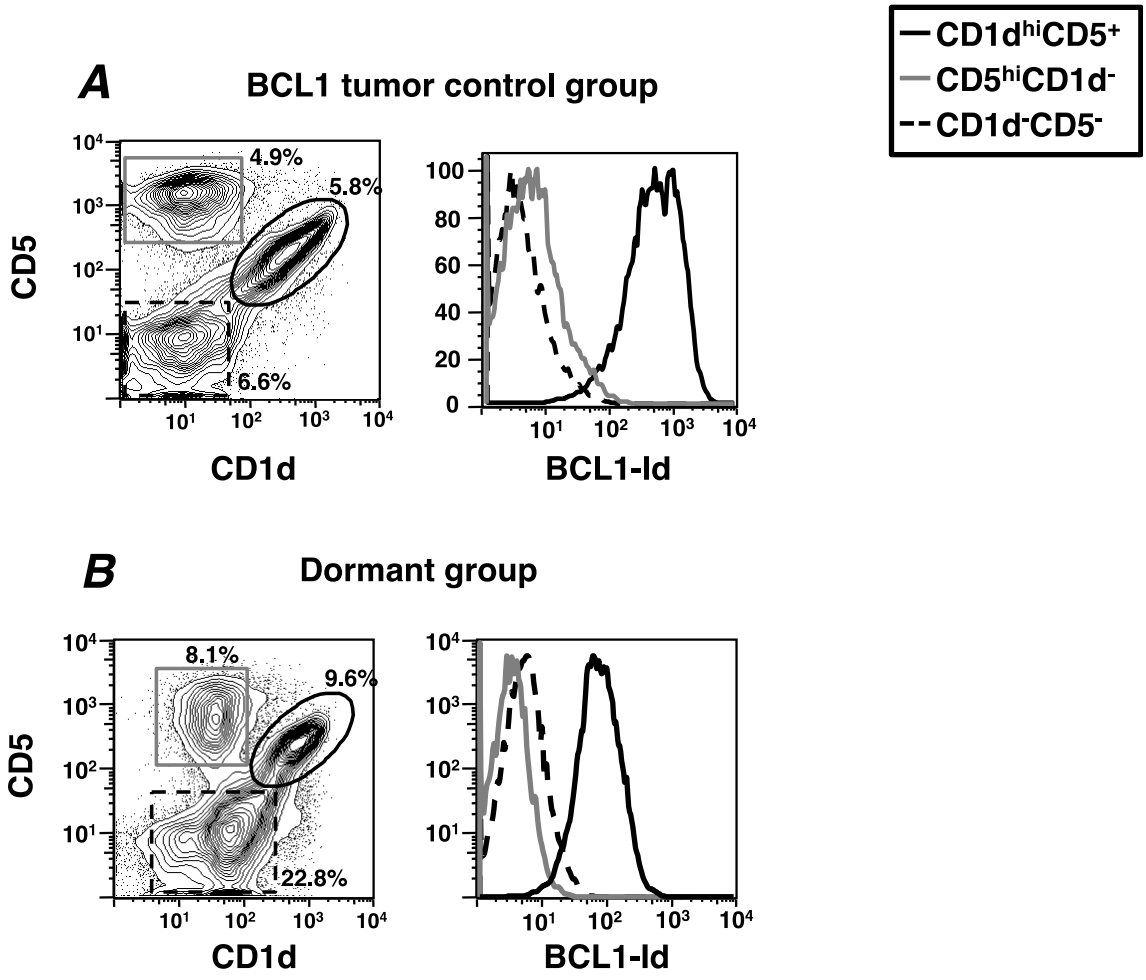

Figure 16. BCL1 tumor cells share the phenotype of the B10 subset of B_{reg} cells.

Spleens from mice injected with BCL1 tumor cells only (BCL1 tumor control group) and mice pre-immunized against BCL1 Id-IgM and challenged with BCL1 tumor cells (dormant group) were harvested on D+60 post tumor cell injections. Spleen cells were stained with antibodies against CD5, CD1d, and the BCL1 tumor specific marker, BCL1 Id-IgM, and analyzed by flow cytometry. *A*, Representative FACS of the BCL1 tumor control group. The CD1d *vs.* CD5 profile shows three distinct subsets of which the $CD1d^{hi}CD5^{+}$ subset is characteristic of the B10 subset of regulatory B cells. Co-staining with anti-BCL1-Id shows reveals the $CD1d^{hi}CD5^{+}$ subset to be BCL1 tumor cells (histogram, BCL1-Id^{+}). *B*, Analysis of spleen cells from mice with dormant BCL1 tumor cells for the co-expression of CD1d and CD5. These plots are representative of two

(dormant) and three (BCL1 tumor control group) analyses yielding similar results.

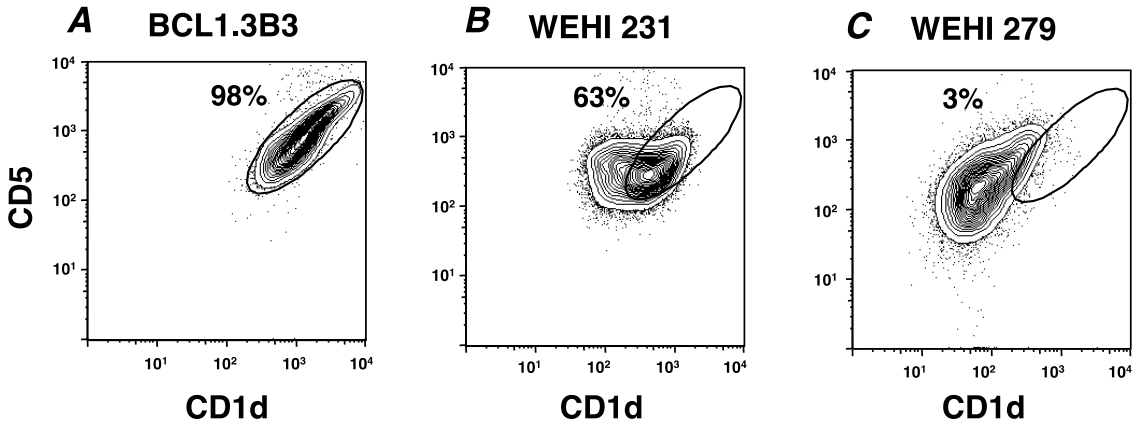

Figure 17. A comparison of the B10 B_{reg} cell subset phenotype to various B cell lymphoma cell lines.
Representative FACS plots showing the analysis of BCL1.3B3 cells which are the tissue culture-adapted BCL1 tumor cells and two other B cell lymphoma cell lines (WEHI 231 and WEHI 279) all of which are uniformly express CD19. Cells were compared for their expression of the B10 B_{reg} phenotype. These plots are representative of two (WEHI cell lines) and three (BCL1.3B3) analyses yielding similar results.

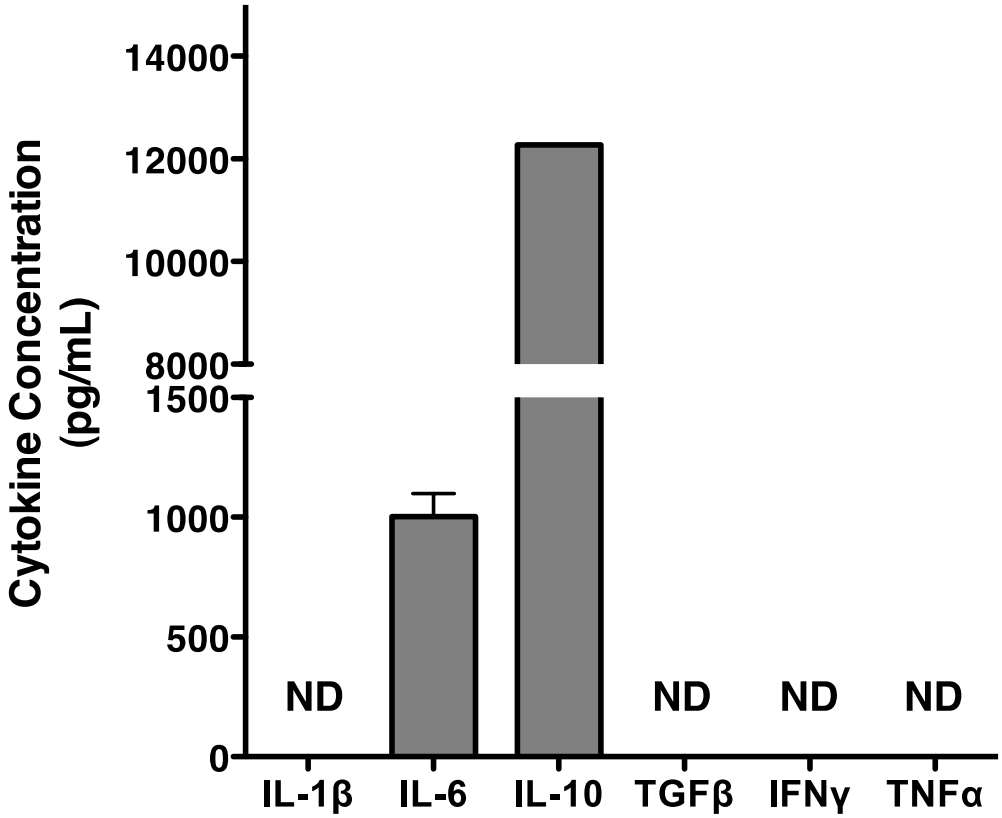

Figure 18. The cytokine profile of the *in vitro* BCL1.3B3 tumor cells. BCL1.3B3 tumor cells were cultured for 72 hours in RPMI medium supplemented with fetal bovine serum (10%), non-essential amino acids, sodium pyruvate, β-mercaptoethanol, and L-glutamine. The supernatants were harvested and examined for soluble cytokines by ELISA. Total values are shown as mean ± SEM from 2-3 experiments. ND, below detection limits.

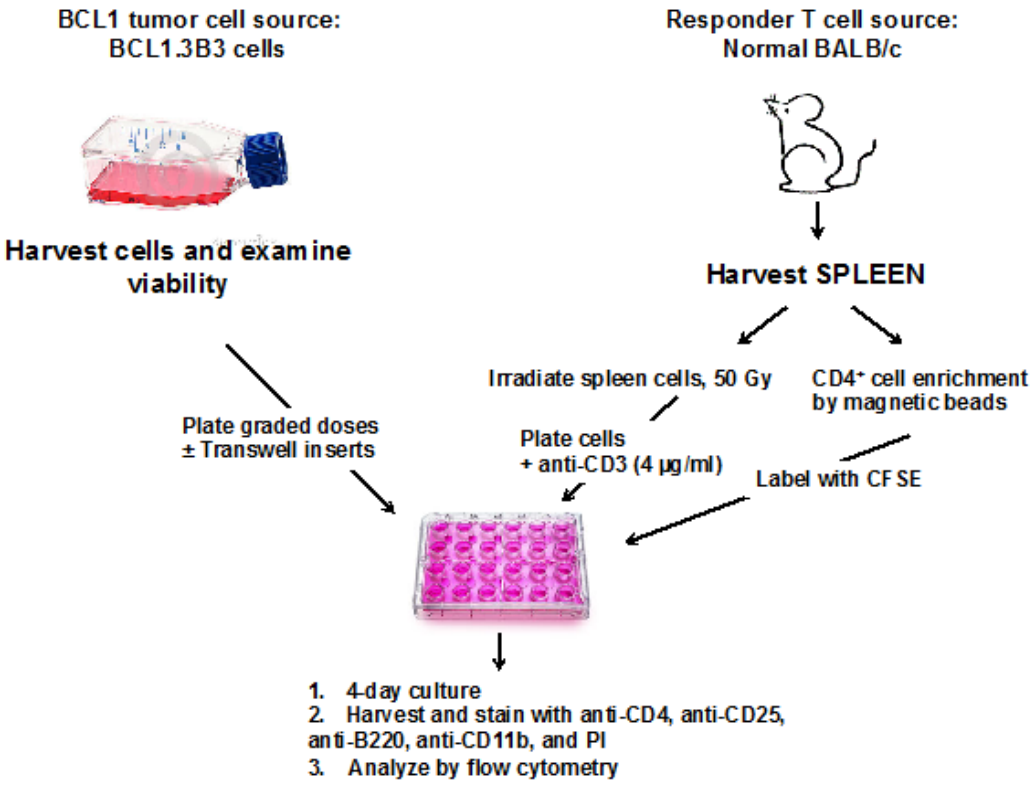

Figure 19. Outline of the B_{reg} cell suppression assay on $CD4^+$ T cell proliferation.
This *in vitro* assay examined the suppression of $CD4^+$ T cells by BCL1 tumor cells. BCL1.3B3 cells were expanded in culture and co-cultured in graded doses with $CD4^+$ T cells isolated from normal mice by magnetic bead enrichment. They were then labeled with CFSE. Irradiated spleen cells and anti-CD3 were added to induce the proliferation of the $CD4^+$ T cells. After a 4-day culture period, cells were harvested from each well and stained with antibodies against CD4, CD25, B220, and CD11b and analyzed by flow cytometry. Propidium iodide was used to exclude dead cells.

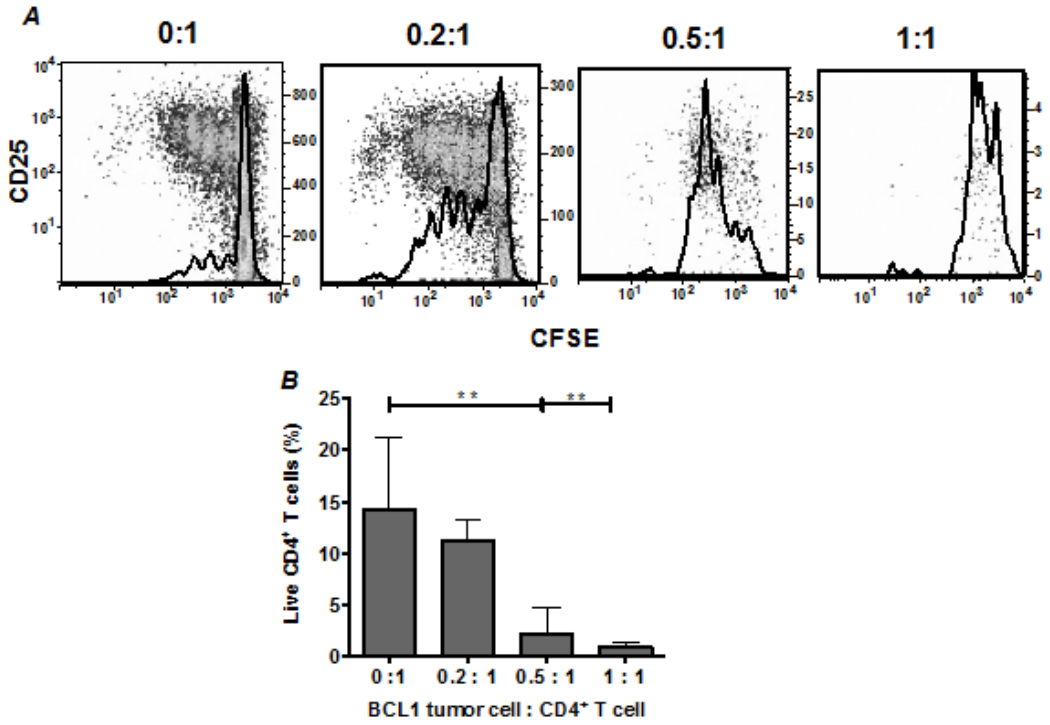

Figure 20. BCL1 tumor cells suppress the proliferation of $CD4^+$ T cells.

$CD4^+$ T cells were purified by magnetic beads from healthy BALB/c mice and labeled with CFSE. The cells were cultured in 24-well plates in the presence of anti-CD3 (4 µg/ml) and irradiated BALB/c spleen cells. Graded doses of BCL1 tumor cells were co-cultured with the T cells for 5 days, then harvested and stained with anti-CD4, anti-CD25, anti-B220, and anti-CD11b. Samples were analyzed for CFSE dilution by flow cytometry. *A*, Analysis plots showing the overlay of a histogram and dot plots of the BCL1 tumor cell co-culture with CFSE-labeled $CD4^+$ T cells. At high tumor cell numbers (1:1 and 0.5:1), very few $CD4^+$ T cells cells were viable at the time of analysis as indicated by the paucity of cells in the dot plots. A representative plot of 3 experiments is shown. *B*, Percentage of viable $CD4^+$ T cells following co-culture with BCL1.3B3 tumor cells as described in *A* is examined among the different culture conditions. Data are shown as mean ± SEM (** $P < 0.005$; Student's t-test).

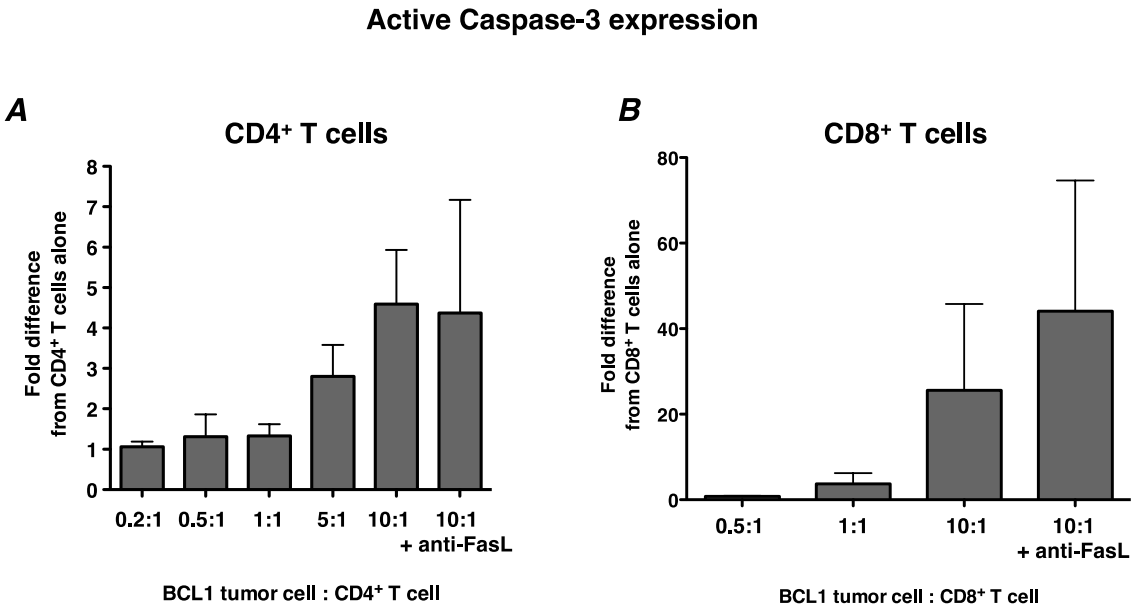

Figure 21. BCL1 tumor cells kill $CD4^+$ and $CD8^+$ T cells partially through the caspase-3 pathway.

CD4+ and CD8+ T cells were purified from normal BALB/c mice and co-cultured with graded doses of BCL1.3B3 tumor cells for 48 hours in the presence of irradiated splenocytes and anti-CD3 (4 μg/ml). Cells were harvested and analyzed for intracellular activate caspase-3 by flow cytometry. Caspase-3 levels by the T cell subsets co-cultured with BCL1.3B3 cells are presented as the ratio over T cells cultured alone. Where indicated, anti-FasL (20 μg/ml) was added to the cultures. Data represent the mean ± SEM of two to four experiments.

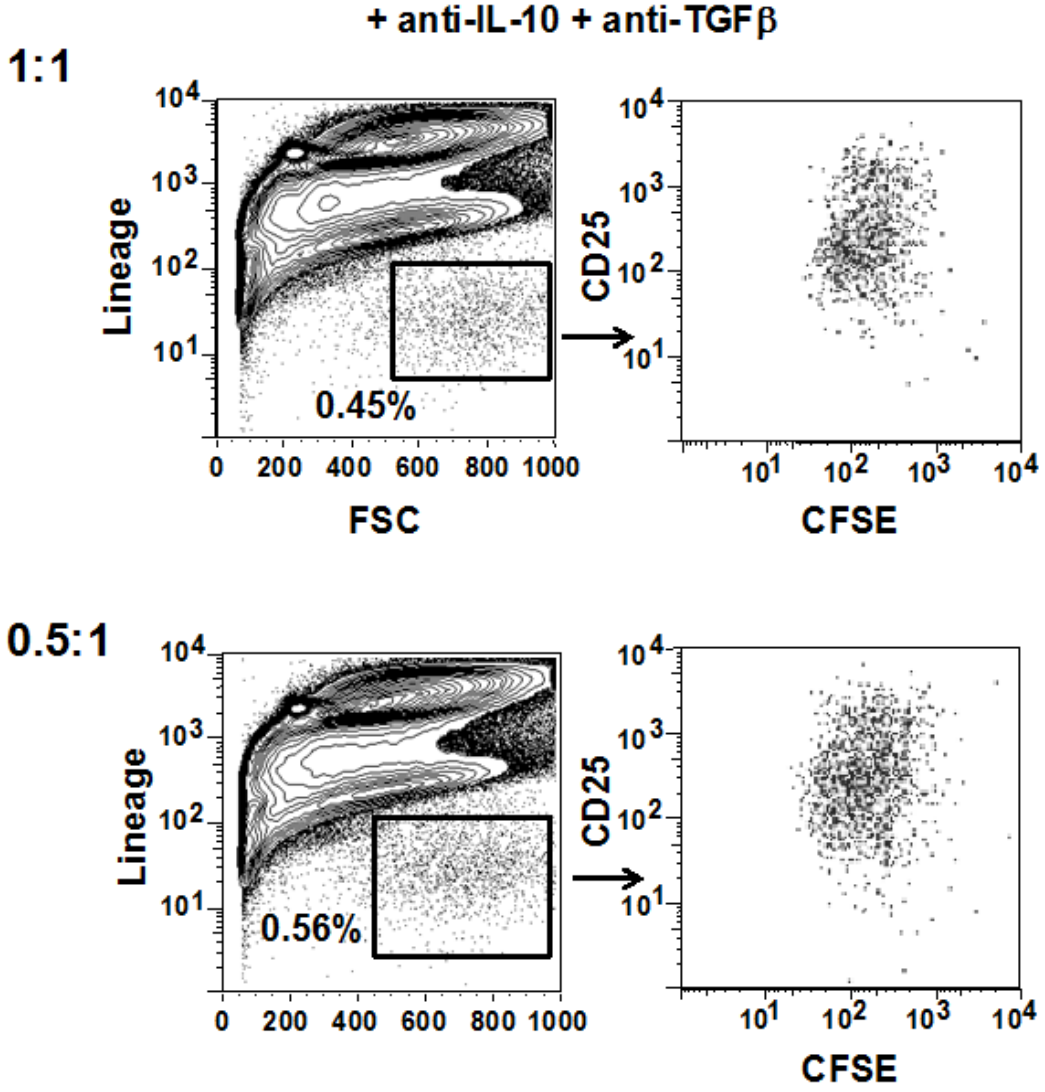

Figure 22. Neutralizing inhibitory cytokines does not overcome the BCL1-mediated suppression of CD4$^+$ T cells.
Co-cultures of graded doses of BCL1 tumor cells with CFSE-labeled naïve CD4$^+$ T cells were supplemented with blocking antibodies against IL-10 and TGF-β (10 μg/ml) and cultured for 4 days. Cells were harvested and stained with antibodies against CD4, CD25, and lineage markers (anti-B220, anti-CD11b, anti-Ter119). Dead cells were excluded by propidium iodide staining. The analysis shows that < 1% of the CD4$^+$ T cells were

viable after the culture period. Furthermore, the remaining viable $CD4^+$ T cells did not proliferate as indicated by their lack of CFSE dilution. Plots from 2 experiments are shown.

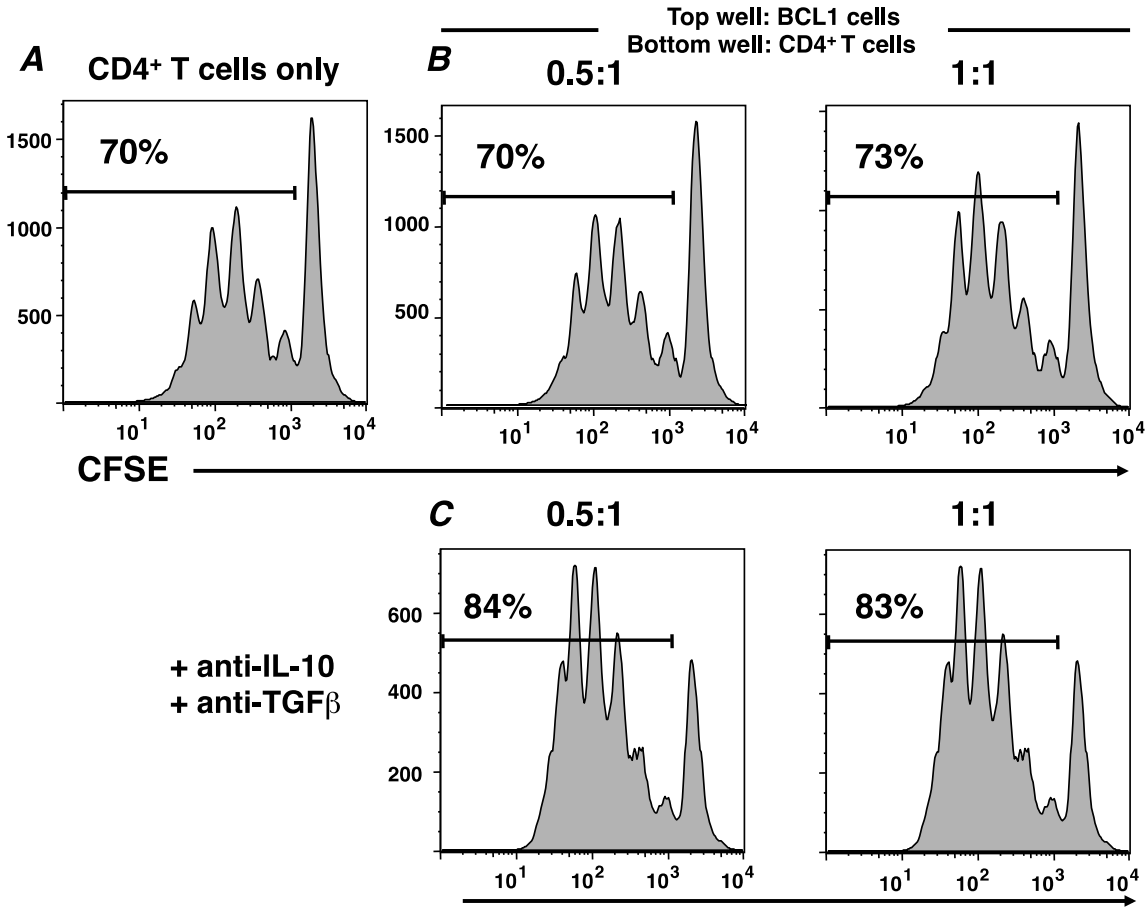

Figure 23. BCL1 cell-mediated suppression of CD4$^+$ T cells is primarily through cell contact but augmented by neutralizing soluble inhibitory cytokines.

A, CD4$^+$ T cells were purified by magnetic beads from healthy BALB/c mice and labeled with CFSE. The cells were cultured in 24-well plates in the presence of anti-CD3 (4 μg/ml) and irradiated BALB/c spleen cells. The number represents the percentage of CD4$^+$ T cells undergoing proliferation as indicated by CFSE dilution. *B*, Graded doses of BCL1 tumor cells (0.5 and 1) were added onto a Transwell insert that was placed into wells containing the CD4$^+$ T cells to prevent cell-cell contact. *C,* Neutralizing antibodies against IL-10 and TGF-β were added to cultures as described in *B*. Representative plot of 2-4 experiments is shown.

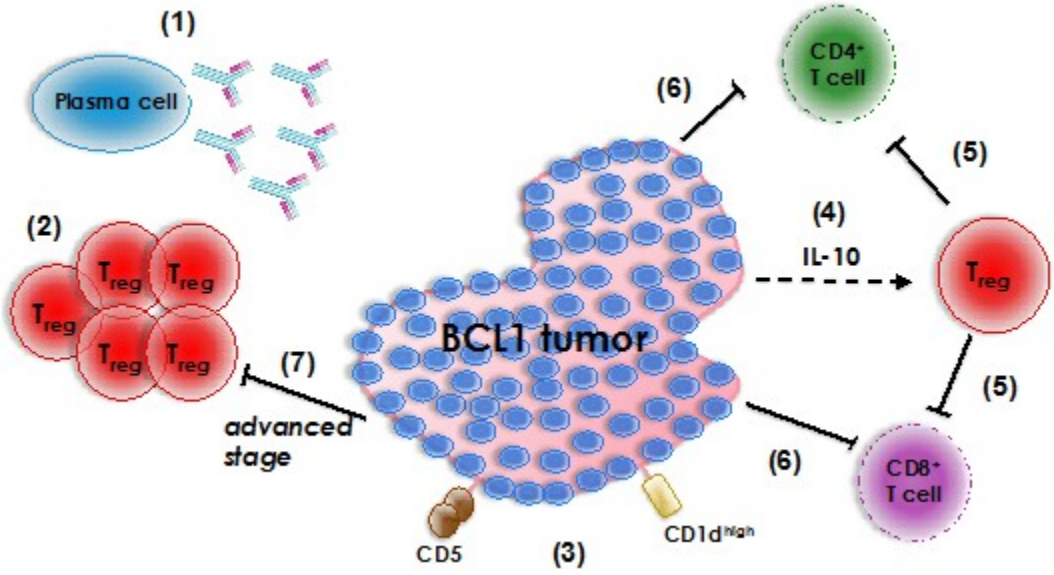

Figure 24. A model of the interaction between T cells and tumor cells in the tumor microenvironment.
The BCL1 tumor microenvironment is proposed to undergo dynamic changes as tumor cells seed the spleen and undergo rapid proliferation. (1) Immunization with BCL1-Id + CFA prior to tumor cell injection induces a strong and durable anti-BCL1-Id antibody response. (2) Immunization alone also induces the accumulation of T_{reg} cells in the tumor microenvironment. In the absence of anti-BCL1 immunity, the tumor cells begin to expand and restructure their environment. (3) BCL1 tumor cells exhibit characteristics of B_{reg} cells, *i.e*. they are $CD1d^{hi}CD5^{+}$ and they secrete high levels of IL-10. (4) In their initial phase of expansion, tumor cells may induce T_{reg} cells because they secrete IL-10. (5) The T_{reg} cells, in turn, are able to suppress infiltrating $CD4^{+}$ and $CD8^{+}$ T cells. (6) As the tumor cells continue to expand, they may also directly kill anti-tumor T lymphocytes. (7) However, in the late stages, as the numbers of tumor cells constitute more than 50% of the cells in the tumor microenvironment, they negatively affect the resident T_{reg} cells in addition to the $CD8^{+}$ T cells.

DISCUSSION

A. Study Objectives and Major Findings

Our examination of the role of T_{reg} cells in the BCL1 tumor dormancy model began at the time when T_{reg} cells had re-emerged as a bona fide subset of T cells that suppressed immune responses and promoted immune tolerance. At that time, T_{reg} cells had been examined primarily in the context of autoimmune diseases; their role in cancer had not been clearly defined. Most reports that did examine T_{reg} cells in the context of cancer focused on the quantification of T_{reg} cells in cancer patients, with attempts to correlate increased T_{reg} cell numbers with cancer progression. Therefore, although T_{reg} cells were proposed to contribute to the development of cancer, their exact role remained unclear.

Previous studies from our laboratory had described the role of both antibody-mediated and $CD8^+$ T cell responses in the establishment and maintenance of tumor dormancy. The most recent study by Pop *et al.* was the first to investigate the role of T_{reg} cells in the BCL1 tumor dormancy model. Pop *et al.* depleted T_{reg} cells using an anti-CD25 antibody, to determine whether this would prolong dormancy. Despite excellent, albeit incomplete, depletion dormancy was not prolonged. It was, however, not possible to determine whether the remaining T_{reg} cells were still active or

whether the anti-CD25 antibody also depleted the effector T cells. In the present study, we continued to investigate the role of T_{reg} cells in BCL1 tumor dormancy. To this end, we investigated the differences in both the numbers and function of T_{reg} cells from mice harboring dormant or non-dormant BCL1 tumor cells.

At the time we started these studies, the first reports describing "regulatory B cells" that were also suppressive were published, extending the concept of peripheral immune tolerance to B cells as well. Like B_{reg} cells, BCL1 tumor cells were also immunosuppressive suggesting that they might represent malignant B_{reg} cells.

The major findings to emerge from these studies are as follows: 1) T_{reg} cells were most abundant in the spleens of the mice with dormant tumors and least abundant in the spleens of mice with the largest tumor burdens; 2) T_{reg} cells from mice bearing both dormant and non-dormant tumors suppressed the proliferation of $CD4^+$ and $CD8^+$ T cells; 3) T_{reg} cells did not directly suppress the proliferation of BCL1 tumor cells or inhibit their secretion of IgM *in vitro*; 4) the BCL1 tumor cells were $CD1d^{hi}CD5^+$, which correlates with the phenotype of the B10 subset of B_{reg} cells, and they also secreted high levels of the suppressive cytokine IL-10 and the pro-inflammatory cytokine IL-6; 5) BCL1 tumor cells inhibited the proliferation of $CD4^+$ T cells in a cell contact-dependent manner. These

last two findings strongly suggest that BCL1 tumor cells exhibit features of B_{reg} cells.

B. Quantification of tumor cells and T cell subsets in the BCL1 tumor dormancy model

1. BCL1 tumor cell proliferation in mice with or without prior immunization with BCL1 Id-IgM

The injection of just one BCL1 tumor cell into a normal BALB/c mouse eventually results in splenomegaly (22). The injection of $5x10^4$ BCL1 tumor cells results in palpable splenomegaly 30 days later (40, 44). By 60 days, spleens contain approximately 10^8 cells (39). When dormancy is induced by immunizing the mice with the BCL1 Id-IgM, the BCL1 tumor cell numbers average 10^6 cells/spleen throughout the lifetime of the mice (22, 44).

In this study, we first confirmed the results of previous reports by quantifying BCL1 tumor cells in the spleens of non-immunized mice, as well as the immunized mice harboring dormant and non-dormant tumor cells 60 days after challenge with BCL1 tumor cells. As expected, the spleens of non-immunized mice had significantly larger numbers of tumor

cells than the immunized mice. Moreover, immunized mice bearing non-dormant BCL1 tumor cells had significantly higher numbers of tumor cells in their spleens than mice with dormant tumor cells. In the present study, the average number of tumor cells in the spleens of mice bearing non-dormant BCL1 tumor cells was 10^7 cells/spleen, a number approximately 10-fold higher than reported previously. This discrepancy may be due to different FACS-staining methodology and reagents used, however it does not alter the overall interpretations that mice bearing dormant BCL1 tumor cells had the lowest average number of tumor cells in their spleens.

2. Examination of T_{reg} cells in the BCL1 tumor dormancy model

T_{reg} cells have been proposed to play a key role in the maintenance of self-tolerance by suppressing infiltrating anti-tumor lymphocytes (74, 75, 135). Moreover`, tumor cells appear to recruit T_{reg} cells (129, 168, 169) and also convert infiltrating $CD4^+CD25^-$ T cells to T_{reg} cells in the tumor microenvironment (81, 170). These studies and many others have argued that T_{reg} cells contribute to cancer progression. Furthermore, many studies have concluded that increased numbers of circulating or tumor-infiltrating T_{reg} cells are predictive of poor survival in cancer patients (77, 171, 172).

In the BCL1 tumor dormancy model, a role for T cells has not been established and indeed anti-Id passively administered to SCID mice induced dormancy (45). However, Farrar *et al.* showed that $CD8^+$ T cells along with IFN-γ clearly prolonged tumor dormancy (46). Interestingly, this study also suggested that $CD4^+$ cells could be suppressing the activity of $CD8^+$ cells. This study was performed at a time when T_{reg} cells had not been well defined, but in retrospect alluded to the possibility that T_{reg} cells might be involved. A few years later, Pop *et al.* examined the possible role of T_{reg} cells in BCL1 tumor dormancy (47). An anti-CD25 antibody was administered to mice to deplete the T_{reg} cells in an effort to determine if this would prolong dormancy. It was hypothesized that the depletion of T_{reg} cells would unleash tumor-specific effector T cells and thus prolong tumor dormancy. However, this did not occur. Unfortunately, effector T cells also express CD25, therefore no definitive conclusions could thus be drawn regarding the role of T_{reg} cells in BCL1 tumor dormancy.

In the present study, we aimed to clarify the role of T_{reg} cells in tumor dormancy. Based on the accumulating evidence that T_{reg} cells contribute to cancer development and progression, we developed several hypotheses with regard to the BCL1 tumor dormancy model. Although it was clear that anti-Id antibody played the major role in inducing and maintaining dormancy, we speculated that T_{reg} cells might also suppress

the T cell-mediated anti-BCL1 tumor immune responses, thus allowing the expansion of BCL1 tumor cells in anti-Id-immunized mice. Our hypotheses were:

1) High numbers of T_{reg} cells in the primary tumor site (the spleen) would be predictive of high tumor burden and disease progression.

2) The number of T_{reg} cells would be relatively lower in mice bearing dormant BCL1 tumor cells.

3) The breakdown of dormancy, or relapse, would be due in part to increasing numbers of T_{reg} cells in the primary tumor site.

Therefore, we set out to quantify T_{reg} cell numbers in the four main experimental groups in the BCL1 tumor dormancy model: 1) the immunization control group (no challenge with BCL1 tumor cells, 2) the dormant group, 3) the non-dormant group, and, 4) the BCL1 challenge control group (no immunization).

We isolated the spleen and draining lymph node cells (inguinal and mesenteric) 60 days after challenge with BCL1 tumor cells, which is defined as the time-point when dormancy is established, and quantified T_{reg} cells, $CD4^+$ and $CD8^+$ T cells, and BCL1 tumor cells by flow cytometry. We defined T_{reg} cells as being $CD4^+CD25^+FoxP3^+$. Although many other markers have been associated with T_{reg} cells, such as GITR

(glucocorticoid inducted TNFR-related protein), and CTLA-4, the $CD4^+CD25^+FoxP3^+$ combination specifically identifies mouse T_{reg} cells.

Upon examination of the absolute numbers of T_{reg} cells, our results showed that the numbers of T_{reg} cells were highest in mice that were immunized only and in mice bearing dormant tumor cells. In contrast, the number of T_{reg} cells was lowest in the mice with progressively higher tumor burden (non-dormant group and non-immunized group). These results were contrary to our hypothesis and showed an increase in the number of BCL1 tumor cells correlated with a decrease in the total number of T_{reg} cells.

Mice that were immunized only had the largest numbers of T_{reg} cells in their spleens. Our immunization protocol included the use of CFA to enhance immune activation. Since T_{reg} cell numbers increased following immunization, it suggested that the adjuvant might have also induced T_{reg} cells. Other studies have reported an increase in T_{reg} cell numbers due to CFA (129) and aluminum hydroxide (130). The increases in T_{reg} cell numbers may be due to the recruitment or induction of tolerogenic dendritic cells, which in turn induces an increase in T_{reg} cells. Hence, adjuvants induce local inflammation at the site of injection. The response by the immune system to this protracted “insult” may be the generation and/or recruitment of T_{reg} cells and other anti-inflammatory

leucocyte subsets such as tolerogenic dendritic cells and myeloid suppressor cells in order to re-establish immune homeostasis. Therefore, one reason why T_{reg} cell numbers increased following immunization was likely due to the presence CFA.

Conversely, active proliferation of BCL1 tumor cells may have contributed to the decrease in the numbers of T_{reg} cells in the spleens of mice that harbored non-dormant tumor cells. Our data show a direct correlation between increasing BCL1 tumor cells numbers and lower T_{reg} cell numbers. Mice that were not immunized, but challenged with BCL1 tumor cells, had the lowest number of T_{reg} cells. In contrast to the situation in immunized mice, the T_{reg} cells in the non-immunized mice would not have the benefit of a possible CFA-induced boost in numbers, in contrast to immunized mice. Furthermore, the aggressive proliferation of tumor cells may have: 1) directly killed the T_{reg} cells, 2) out-competed the relatively rare T_{reg} cells for space, and, 3) created an environment that may be too toxic for the T_{reg} cells to endure.

The inverse relationship between numbers of BCL1 tumor cells and T_{reg} cells was also observed in the spleens of mice with dormant and non-dormant tumors. Although both groups of mice experienced a boost in the numbers of T_{reg} cells following immunization, a subsequent challenge with BCL1 tumor cells reduced the total number of T_{reg} cells. However, the

loss of T_{reg} cells in both the dormant and non-dormant mice was less pronounced when compared to their loss in non-immunized mice. This is most likely because immunization suppressed the proliferation of BCL1 tumor cells, albeit to varying degrees, as compared to non-immunized mice.

Overall, both immunization and the number of BCL1 tumor cells affected the numbers of T_{reg} cells in the spleen. Whereas immunization boosted the numbers T_{reg} cells, the active proliferation of the BCL1 tumor cells resulted in the reduction of T_{reg} cells in the spleen.

The prevailing theory concerning the association of T_{reg} cells and cancer progression suggests that tumor cells can recruit T_{reg} cells and also convert $CD4^+$ T cells to T_{reg} cells, all in an effort to suppress the anti-tumor immune responses by the host. This theory is largely based on the observation that patients with advanced cancer have high numbers of circulating T_{reg} cells. However, in our model, mice bearing dormant BCL1 tumor cells had the largest numbers of T_{reg} cells in their spleens. Therefore, in the BCL1 tumor dormancy model, T_{reg} cells did not appear to prevent the establishment of tumor dormancy by suppressing the anti-BCL1 immunity.

There are a few key differences between our studies and others that may explain the discrepancy in these results. In the BCL1 tumor

dormancy model, mice were immunized with the BCL1 Id prior to tumor cell challenge. Therefore, all immunized mice were exposed to the tumor-specific protein and generated antibody and perhaps T cell responses against the tumor cells. Current studies quantify T_{reg} cells in mice that have only been inoculated with tumor cells. This includes clinical studies where immunization is not an option. We have observed that immunization clearly changes the dynamics of the mobilization and recruitment T_{reg} cells. Therefore, our studies introduce a new variable that might have an impact on the analysis of T_{reg} cells and tumor cell proliferation. Moreover, CFA in the immunization process clearly boosted the numbers of T_{reg} cells. This is a significant variable when comparing T_{reg} cells from mice immunized using CFA to mice that were inoculated with tumor cells without prior immunization.

T_{reg} cells present in the spleens of mice bearing dormant BCL1 tumor cells may have also been rendered ineffective due to the presence of high titers of anti-Id antibody. The robust anti-Id antibody-mediated immunity against BCL1 tumor cells is reflected by the generation of plasma cells. Immunized mice that successfully developed anti-tumor immunity were able to induce their BCL1 tumor cells to enter a dormant state. Moreover, these mice have higher titers of circulating anti-Id antibody than those with non-dormant tumor cells (44). As discussed,

these mice also had large numbers of T_{reg} cells. It is possible that the titers of anti-Id antibody were above a threshold where T_{reg} cells were relevant. Moreover, T_{reg} cells may not have been capable of suppressing anti-Id-secreting memory plasma cells, therefore were ineffective in suppressing the humoral anti-tumor response. It has been reported that T_{reg} cells prevent the long-term development of plasma cells (173). However, this study utilized T_{reg} cell-deficient mice and observed plasma cell accumulation in their bone marrow. Therefore, this is an indirect assertion regarding the role of T_{reg} cells and plasma cells. Based on our results, the high number of T_{reg} cells was not sufficient to overcome the anti-tumor suppression induced by BCL1-Id immunization.

3. Examination of $CD4^+$ and $CD8^+$ T cells in the BCL1 tumor dormancy model

In addition to the effects on T_{reg} cell numbers present in the spleen, we also examined the effects of immunization and subsequent BCL1 tumor cell injection on both $CD4^+$ and $CD8^+$ T cells to gain a better understanding of the dynamics of all T cell subsets in the BCL1 tumor dormancy model. Immunization of mice with BCL1 Id alone resulted in an increase in the total numbers of both $CD4^+$ and $CD8^+$ T cells in the spleen.

However, their percentage was unchanged in comparison to control mice that were not immunized. Immunization resulted in an increase in the total numbers of spleen cells. Therefore, the elevated numbers of $CD4^+$ and $CD8^+$ T cells were likely due to the overall increase in the numbers of spleen cells, and not the specific expansion of these T cells per se.

Although total numbers of $CD4^+$ T cells were increased, the total percentage of T cell subsets did not significantly increase. Likewise, $CD8^+$ T cells did not increase following immunization.

Challenging mice with BCL1 tumor cells resulted in a decrease in the number of T cells. Mice with the largest tumor burdens had the greatest reduction of T cells in their spleens. The $CD8^+$ T cells were completely ablated in the non-immunized mice. While the $CD4^+$ T cells also decreased, this decrease was less striking. Overall, the quantification of the T_{reg} cells, and $CD4^+$ and $CD8^+$ T cells in the BCL1 tumor dormancy model shows that T_{reg} cells and $CD8^+$ were most severely affected by the expansion of the tumor cells. Since the T_{reg} cells and $CD8^+$ T cells represented a smaller percentage of the total T cells (< 10%), it is possible that they were more rapidly eliminated than the $CD4^+$ T cells.

C. Examination of the function of T_{reg} cells isolated from mice harboring dormant and non-dormant BCL1 tumor cells

1. T_{reg} cells from mice bearing dormant or non-dormant BCL1 tumor cells can suppress the proliferation of $CD4^+$ T cells

We had hypothesized that T_{reg} cells could aid tumor progression by contributing to the prevention of or relapse from dormancy. Despite their presence in similar numbers, it was possible that T_{reg} cells from mice bearing non-dormant BCL1 tumor cells might exhibit a greater capacity to suppress T cells compared to T_{reg} cells from mice with dormant BCL1 tumor cells. Therefore, we next assessed functional differences between T_{reg} cells from mice bearing dormant or non-dormant BCL1 tumor cells by examining the ability of their T_{reg} cells to suppress the proliferation of $CD4^+$ and $CD8^+$ T cells.

An *in vitro* T_{reg} cell-mediated suppression assay was developed to examine the suppressive function of T_{reg} cells (140, 141). This assay is a convenient and reliable test for measuring T_{reg} cell function. Although the tissue culture plate does not completely recapitulate the *in vivo* environment, this assay can be used to directly examine the interactions between T_{reg} cells and responder T cells.

First, we examined the capacity of T_{reg} cells from mice bearing dormant or non-dormant BCL1 tumor cells to suppress the proliferation of naive $CD4^+$ T cells. When equivalent numbers of T_{reg} cells were co-cultured with naïve $CD4^+$ T cells (1:1), we observed almost total inhibition of $CD4^+$ T cell proliferation, regardless of whether the T_{reg} cells were isolated from mice bearing dormant or non-dormant BCL1 tumor cells. However, T_{reg} cells from mice bearing non-dormant BCL1 tumor cells were significantly more potent than those from the dormant group at lower numbers [0.25:1] (88% vs. 24% suppression, $p = 0.028$).

While we did not investigate the mechanisms underlying this increased potency, it could be due to differences in the soluble and cell surface molecules that contribute to T_{reg} cell function as influenced by the dormant versus non-dormant BCL1 tumor microenvironment. Several key molecules that contribute to the suppressive function of T_{reg} cells include CD25, FoxP3, and CTLA-4 (174). FoxP3 controls the expression of CD25 and CTLA-4 (174). IL-2 signaling in T_{reg} cells by high-affinity IL-2 receptors was described to be critical for the maintenance and function of T_{reg} cells (175). Likewise, CTLA-4, which transmits negative signals to T cells, appears to be a key regulator used by T_{reg} cells since blockade of CTLA-4 abrogates the suppressive activity of T_{reg} cells (70). Since we FACS-sorted T_{reg} cells based on CD25 expression, it is likely that T_{reg} cells

from mice bearing dormant and non-dormant BCL1 tumor cells had equivalent levels of CD25 expression. However, the difference in potency could have been due to differential CTLA-4 expression. The tumor environment in the spleens from the non-dormant mice might be more enriched in inflammatory factors due to the active proliferation of the BCL1 tumor cells. These conditions may induce greater surface expression of CTLA-4 on T_{reg} cells. Therefore, at lower numbers, T_{reg} cells from the non-dormant BCL1 tumor microenvironment may be more potent in promoting immunosuppression than the T_{reg} cells from the mice bearing dormant BCL1 tumor cells.

In contrast to T_{reg} cell suppression of naïve $CD4^+$ T cells, when T_{reg} cells were co-cultured with $CD4^+$ T cells from their respective tumor microenvironments, we observed no difference in the suppressive capacity between T_{reg} cells from the dormant and non-dormant tumor microenvironments. The distinguishing factor between these sets of experiments is the source of the $CD4^+$ T cells. Tumor cells try to subvert anti-tumor immunity, and one way they achieve this is by actively downregulating anti-tumor immune responses that include infiltrating T cells (1). Therefore, it is likely that the $CD4^+$ T cells isolated from the spleens bearing dormant or non-dormant BCL1 tumor cells were more

susceptible to T_{reg} cell-induced suppression because they were exposed to the milieu of the tumor environment.

2. T_{reg} cells from mice bearing dormant BCL1 tumor cells suppress the proliferation of $CD8^+$ T cells

Tumor-infiltrating cells include $CD8^+$ CTLs that can kill tumors in the absence of T_{reg} cells (88). To fully examine the function of T_{reg} cells from the spleens of mice with dormant tumors, we also assessed their capacity to suppress $CD8^+$ T cells since they have been shown to contribute to BCL1 tumor dormancy (46). Our suppression assays demonstrated that T_{reg} cells from mice bearing dormant tumor cells suppressed naïve $CD8^+$ T cell proliferation even at the lowest T_{reg} cell : CD8+ T cell ratio (0.25:1). An examination of spleens from mice bearing dormant BCL1 tumor cells showed that by day 60, the ratio of T_{reg} cells to $CD8^+$ T cells was 0.5:1. Therefore, the ratio of suppressor to responder cells we used in our T_{reg} cell suppression assays reflected the *in vivo* setting. These results demonstrated that T_{reg} cells in the dormant microenvironment maintained a functional suppressive capacity by suppressing $CD8^+$ T cells as well as $CD4^+$ T cells. Therefore, the differences in anti-BCL1 tumor immunity in the spleens from the mice bearing dormant versus non-dormant BCL1

tumors was not likely due to the lack of T_{reg} cell function in the spleens from the mice with dormant tumor cells.

3. T_{reg} cell-mediated suppression is primarily contact-mediated

Since T_{reg} cells from both the dormant and non-dormant BCL1 tumor microenvironments were functional due to their capacity to suppress T cell proliferation, we examined their possible mechanism(s) of suppression. T_{reg} cells impart their suppression by secreting soluble inhibitory cytokines such as IL-10, TGF-β, and IL-35 as well as through direct cell contact (59, 66, 176). The examination of both mechanisms of suppression has resulted in conflicting opinions. While some investigators believe that the inhibitory cytokines are directly involved in T_{reg} cell-mediated suppression, others contend that cell contact is the primary mode (58, 177, 178).

These discrepancies may be related to the manner T_{reg} cell function is examined. It has been clearly shown that IL-10 is essential for T_{reg} cell-mediated suppression *in vivo* (61, 142). However, *in vitro* studies using neutralizing anti-IL-10 antibody suggested that IL-10 is not required for T_{reg} cell-mediated suppression (140, 141). A recent study by Collison *et al.* attempted to resolve this issue, by concurrently preventing cell contact

using Transwell assays, as well as using anti-IL-10 neutralizing antibody. They concluded that inhibiting IL-10 alone has no effect in abrogating T_{reg} cell suppression. However, adding anti-IL-10 to the Transwell assay further reduced T_{reg} cell suppression (62).

Our results were similar to those of Collison *et al.* The addition of recombinant IL-10 to our *in vitro* assays did not prevent the proliferation of $CD4^+$ T cells. However, preventing contact between T_{reg} cells from mice bearing dormant BCL1 tumor cells and naïve $CD4^+$ T cells completely abrogated the suppressive effects of T_{reg} cells. Therefore, T_{reg} cells appeared to mediate their suppression primarily through cell contact. However, we cannot completely discount the effects of soluble inhibitory cytokines, as we did not examine them using *in vivo* assays.

4. T_{reg} cells do not directly inhibit BCL1 tumor cell function

The target cells suppressed by T_{reg} cells have been shown to extend beyond T cell subsets. T_{reg} cells have been described to also suppress myeloid cells (66, 174). T_{reg} cells can down-regulate the co-stimulatory molecules on dendritic cells to render them ineffective as T cell stimulators (179). Moreover, T_{reg} cells can suppress B cell function by inhibiting IgG secretion (145). Thus, we examined the effects of T_{reg} cells

on BCL1 tumor cells, which are malignant B cells. We hypothesized that if T_{reg} cells can suppress B cells, then T_{reg} cells may prevent the expansion of BCL1 tumor cells in the spleens of mice with dormant tumor cells. Therefore, we isolated T_{reg} cells from mice bearing dormant BCL1 tumor cells and assessed their capacity to suppress the proliferation of as well as IgM secretion by the BCL1 tumor cells.

Our results showed that the addition of T_{reg} cells to BCL1.3B3 tumor cell cultures neither suppressed their proliferation, nor inhibited their secretion of Id-IgM. Therefore, the T_{reg} cells do not directly suppress BCL1 tumor cells. It is important to recognize that our studies attempted to test the suppression of malignant, rather than normal B cells. The sustained proliferation of the BCL1 tumor cells may be too robust for T_{reg} cells to overcome. Moreover, we did not examine the effects of T_{reg} cells on dormant BCL1 tumor cells. It is possible that the state of tumor dormancy induced by immunization may render these tumor cells more susceptible to T_{reg} cell suppression than their malignant counterparts.

In summary, we attempted to uncover possible functional differences between T_{reg} cells present in the spleens of mice bearing dormant BCL1 tumor cells and those with non-dormant tumor cells. However, T_{reg} cells from both tumor environments proved to be equally functional in that they suppressed the proliferation of both naïve and

tumor-infiltrated $CD4^+$ T cells. Suppression by T_{reg} cells was primarily mediated through cell contact as observed in *in vitro* assays, which is in agreement with current studies of T_{reg} cell-mediated suppression.

T_{reg} cells did not have a negative impact on BCL1 tumor cells since they did not suppress their proliferation nor inhibit IgM secretion. Since T_{reg} cells did not assert their suppression on the BCL1 tumor cells, we next examined the impact of the BCL1 tumor cells on T lymphocytes.

D. BCL1 tumor cells exhibit characteristics of regulatory B cells

1. BCL1 tumor cells express the phenotype of B10 B_{reg} cells

The tumor microenvironment is created by the tumor and functions to down-regulate anti-tumor immune responses (1). Since tumor cells play an essential role in constructing the tumor milieu, we directly examined the BCL1 tumor cells in our dormancy model. Concurrently with our shift in focus from T_{reg} cells to the BCL1 tumor cells, a study was published reporting the nature and function of regulatory B cells that induce immune tolerance (100). This study described the existence of a subset of B_{reg} cells with a specific phenotype ($CD1d^{hi}CD5^+$) that produced high levels of IL-10, but not other cytokines, and suppressed T cell-

mediated inflammation. These cells were termed "B10 B_{reg} cells" due to their high levels of IL-10 production.

The existence of "regulatory B cells" was proposed over 40 years ago, and over time multiple studies described how B cell subsets contributed to immune tolerance (reviewed in (96)). However, the study by Yanaba *et al.* was able to associate a subset of these B_{reg} cells with a distinct phenotype. Since tumor cells can influence the negative regulation of immune responses, and the BCL1 tumor cells were of a B cell origin, we assessed whether the BCL1 tumor cells expressed the phenotype and cytokine secretion profile of the B10 B_{reg} cells.

We first examined the phenotype of BCL1 tumor cells isolated from the dormant tumor environment as well as from non-immunized mice. We found that the BCL1 tumor cells from both tumor environments homogeneously expressed the phenotype of B10 B_{reg} cells ($CD1d^{hi}CD5^{+}$). Furthermore, examination of the in vitro-adapted BCL1 tumor cell line, BCL1.3B3, gave the same results as the *in vivo* tumor cells. Next, we examined other B cell lymphoma cell lines to determine if the regulatory phenotype was a general feature of malignant B cells or limited to the BCL1 tumor cells. The adoption of regulatory cell-type characteristics by neoplastic cells may confer a survival advantage to them against anti-tumor immune responses. However, analysis of the WEHI 231 cells and

WEHI 279 cell lines, which like BCL1 cells are of B cell origin, showed that only 63% and 3% of the cells expressed the B10 B_{reg} cell phenotype, respectively. These results suggested that the B10 B_{reg} cell phenotype is not a common feature of all malignant B cells.

The differential expression of the B10 B_{reg} cell phenotype among the three malignant B cell lines may provide more insight into the ontogeny of the BCL1 tumor cells. Following their initial characterization, BCL1 tumor cells were described to derive from immature B cells (39, 120, 180). Since BCL1 cells also express CD5, they were also proposed to originate from B-1 cells (147). Moreover, the B10 B_{reg} cells were shown to share overlapping phenotypic features with B1a, marginal zone, and transitional-2 marginal zone precursor B cells (101, 106, 109). Therefore, considering the ontogeny and the phenotype of BCL1 tumor cells and B10 B_{reg} cells together suggests that the BCL1 tumor cells may be malignant B10 B_{reg} cells.

2. BCL1 tumor cells secrete high levels of IL-10, a feature of B10 B_{reg} cells

A defining characteristic of B10 B_{reg} cells is their capacity to secrete high levels of IL-10, but not other cytokines (100, 107). We, therefore,

examined the cytokine secretion profile of the BCL1 tumor cells to determine if they also shared this feature with B10 B_{reg} cells. Our analysis showed that BCL1 tumor cells indeed secrete very high levels of IL-10 (> 12 ng/ml), but not TGF-β, another key immunosuppressive cytokine. Moreover, BCL1 tumor cells did not secrete IL-1β, TNF-α, and IFN-γ.

Interestingly, we detected moderate levels of IL-6 (1 ng/ml), a cytokine not secreted by B10 B_{reg} cells. The production of IL-6 may be a feature unique to the BCL1 cells, and more generally B lymphoma cells. O'Garra *et al.* examined a panel of Ly-1^+ B cell lymphomas and observed that upon LPS stimulation, most produced IL-6 and IL-10 (98). IL-6 has also been reported to mediate the proliferation of $CD5^+IgM^+$ B cell lymphomas (181), suggesting that IL-6 may be utilized in an autocrine manner to expand malignant B cells. Therefore, the secretion of high levels of IL-10 by BCL1 tumor cells supports the hypothesis that they share characteristics with B10 B_{reg} cells. However, IL-6 production appears to be a feature of their malignant nature.

3. BCL1 tumor cells kill $CD4^+$ and $CD8^+$ T cells partially through the caspase-3 pathway

A defining feature of tumor cells is their ability to evade specific anti-tumor immune responses. Moreover, B_{reg} cells have been shown to inhibit autoreactive T cells in various models of autoimmunity (91, 94, 106, 182). Since BCL1 cells are malignant B cells and exhibit features of B_{reg} cells, we examined their potential capacity to suppress the proliferation of T cell subsets. For this, we modified the T_{reg} cell suppression assay by using the BCL1 tumor cells as the suppressor cells and $CD4^+$ T cells as the candidate "responder cells." Following a co-culture period with high doses of BCL1 tumor cells (1:1 and 0.5:1), only < 3% of the T cells were viable, which was significantly lower than when T cells were cultured alone (14%). It is important to note that when designing *in vitro* suppression assays, the selection of the numbers of cells to be plated is relatively arbitrary and often limited by technical considerations such as the number of cells available. Therefore, the "high" dose of BCL1 tumor cells here refers to a 1:1 ratio with the T cells. However, the relative ratio of tumor cells to T cells *in situ* is more likely higher. For example, in the BCL1 tumor dormancy model, the comparison of absolute BCL1 tumor cell and $CD4^+$ T cell numbers in the spleens of non-immunized mice 60 days after tumor cell challenge revealed a 25:1 ratio. This suggests that the *in vitro*

assay is a conservative measurement of the ability of tumor cells to exert suppression.

We next investigated the mechanism by which BCL1 tumor cells were killing the $CD4^+$ T cells. The primary factors for inducing T cell suppression are soluble inhibitory cytokines and cell-cell contact. Our attempt to neutralize the key inhibitory cytokines IL-10 and TGF-β by adding blocking antibodies to the co-cultures did not abrogate BCL1 tumor cell killing of T cells. Interestingly, the remaining viable T cells expressed a CFSE profile that suggested apoptosis or cell-cycle arrest. The viable T cells appeared in a cluster at about mid-point on the CFSE scale. This suggested that the T cells might have undergone one or more rounds of division before being arrested in their state.

There may be several explanations for this pattern observed in the $CD4^+$ T cells. First, the aggressive proliferation of the tumor cells in the tissue culture plate may have usurped the nutrients in the media and effectively deprived the T cells. Secondly, the T cells may have been surrounded by the rapidly proliferating tumor cells, which suppressed their proliferation through direct cell contact. Finally, the tumor cells surrounding the T cells may have prevented them from interacting with the APC's in the culture wells.

To test T cell suppression through cell-cell interaction, we prevented contact between the BCL1 tumor cells and $CD4^+$ T cells using a Transwell insert. This resulted in the total rescue of $CD4^+$ T cells from suppression and the T cell proliferation rates matched those wells where no BCL1 tumor cells were added. These results demonstrated that cell contact was the primary mechanism by which the BCL1 tumor cells killed the T cells. Moreover, these results showed that the BCL1 tumor cells did not deprive T cells of nutrients since the T cells were able to proliferate when co-cultured with the tumor cells in the Transwell assay. However, these results do not resolve the question of whether tumor cells directly suppressed the T cells or prevented their interactions with APC's. It is likely that T cell killing may have been the result of both proposed pathways but what is clear is that the BCL1 tumor cells, whether directly or indirectly, induced the killing of the $CD4^+$ T cells.

Although the BCL1 tumor cells killed the T cells by cell contact, soluble inhibitory factors appeared to contribute to this killing. The addition of anti-IL-10 and anti-TGF-β neutralizing antibodies to the Transwell assays enhanced T cell proliferation by at least 12%. These results suggest that although the primary model of suppression by the BCL1 tumor cells is by cell contact, inhibitory cytokines also contribute to the suppression of $CD4^+$ T cells.

We next assessed the killing of $CD4^+$ and $CD8^+$ T cells in co-culture with BCL1 tumor cells by examining active intracellular caspase-3 levels, a key component in the induction of apoptosis in T cells. High numbers of BCL1 tumor cells induced active caspase-3 levels in both $CD4^+$ and $CD8^+$ T cells confirming that BCL1 tumor cells can induce apoptosis in infiltrating T cells.

E. A model of the BCL1 tumor microenvironment

The evaluation of the BCL1 tumor dormancy model in the context of regulatory cells has led to the characterization of BCL1 tumor cells as malignant B_{reg} cells (Figure 24). The antibody-mediated anti-tumor a activity provided by immunization was initially described by Vitetta *et al.* (44). When effective, this protection induces tumor dormancy where BCL1 tumor cell expansion is tightly controlled for up to the lifetime of the mouse. Our studies have now shown that the process of immunization also enhances the numbers of T_{reg} cells in the tumor microenvironment. Therefore, while immunization produces anti-BCL1 immunity, it may also induce T_{reg} cells to control the deleterious effects of prolonged inflammation. In addition, tumor cells may also contribute to the accumulation of T_{reg} cells by producing IL-10, which enhances the

numbers of T_{reg} cells. The T_{reg} cells may, in turn, suppress tumor-infiltrating $CD4^+$ and $CD8^+$ T cells in the tumor microenvironment.

However, in the absence of antibody -mediated anti-BCL1 immunity, the tumor cells continue to expand. The increasing numbers of BCL1 tumor cells causes a rapid transformation of the spleen into an "inhibitory" tumor microenvironment due to high levels of IL-10 secreted by the BCL1 tumor cells. In addition, the tumor cells appear to directly kill infiltrating T cells, further enhancing their survival and expansion. Although, BCL1 tumor cells may enhance the accumulation of T_{reg} cells early on, as the BCL1 tumor cells expand, they may be toxic to thee T_{reg} cells, which suffer the same fate of other infiltrating T cells.

F. Summary

This study shows that BCL1 tumor cells have the phenotype and cytokine profile of B10 B_{reg} cells. To our knowledge, this is the first report demonstrating a link between B_{reg} cells and malignant B cells. Tumor cells in general have thought to be endowed with immune-inhibitory characteristics that are now ascribed to regulatory cells. Whereas B_{reg} cells can subdue autoreactive immune cells, tumor cells would utilize these inhibitory characteristics to subvert anti-tumor immune responses.

Therefore, it is possible that BCL1 cells may adopt these B_{reg} cell-like features to evade immune responses.

The exact ontogeny of BCL1 tumor cells is unknown. It is thought however, that they were derived from an "immature B cell" or have a B1a B cell lineage. B10 B_{reg} cells are also derived from B1a precursor cells. Since the B10 B_{reg} cells and BCL1 tumor cells appear to have overlapping precursor cell characteristics, it is possible that the BCL1 tumor cells are the malignant form of B10 B_{reg} cells.

The most interesting and clinically relevant application from this study is shared characteristics of BCL1 tumor cells and B10 B_{reg} cells. It is possible that adoption of B_{reg}-like characteristics is important for tumor cell proliferation, and specifically the prevention of or relapse from dormancy. While further studies are needed to investigate the timing, mechanism, and role of B cell lymphoma expression of B_{reg} characteristics, future therapies for B cell lymphomas could be directed to exploit these features.

G. Future directions

This project explored the role of T_{reg} cells and the BCL1 tumor cells as negative regulators in the tumor microenvironment of the BCL1 tumor dormancy model. Our results have answered the questions we initially

asked, but also led us to formulate new ones that remain to be addressed in the future. In our study we quantified the numbers of T_{reg} cells in mice 60 days after challenge with BCL1 tumor cells since this was the time-point when tumor dormancy is established. By this time, mice that were not immunized but challenged with BCL1 tumor cells had the lowest numbers of T_{reg} cells, suggesting that the expansion of the tumor cells had a negative impact on the numbers of T_{reg} cells. Therefore, the quantification of T_{reg} cells soon after tumor challenge and weekly up to day 60 may provide new insight into the effects of BCL1 tumor cell expansion on the proliferation or reduction of T_{reg} cells.

Our results also showed that immunization alone resulted in an increase in the numbers of T_{reg} cells. Future studies should examine the mechanisms by which this occurs. The direct effects of immunization using CFA or other adjuvants on the expansion of T_{reg} cells would be a critical undertaking in terms of vaccine research, since the goal is to boost the immune system, not accumulate negative regulatory cells.

Mice bearing dormant tumor cells had relatively high numbers of T_{reg} cells in their spleens. The T_{reg} cells were proven to be functional *ex vivo* yet it was not determined why such high numbers accumulated in the spleens of these clinically healthy mice. The depletion of T_{reg} cells may provide insight into their effects in the dormant tumor microenvironment.

Furthermore, “remodeling” the BCL1 tumor dormancy using T_{reg} cell-deficient mice will also shed light on the role of T_{reg} cells in the establishment of tumor dormancy.

The direct effect of BCL1 tumor cells on T_{reg} cells needs to be further investigated. Since BCL1 cells are a rich source of IL-10, why are there fewer T_{reg} cells in the spleens with high tumor burdens? It is possible that BCL1 tumor cells have a bimodal effect on T_{reg} cells. As the tumor cells begin to proliferate in the spleen, they may induce T_{reg} cell accumulation through direct recruitment or by conversion of effector $CD4^+$ T cells. However, the growth of the BCL1 tumor may reach a threshold beyond which their continued expansion may negatively affect T_{reg} cells.

BCL1 tumor cells killed $CD4^+$ and $CD8^+$ T cells partly by activating the caspase-3 pathway. The mechanism of BCL1 killing must be further explored to examine other killing pathways by which BCL1 tumor cells undertake for killing target cells. BCL1 tumor cells express Fas-ligand and PD-L1, which are essential for inducing apoptosis in target cells. Investigation into these apoptosis-inducing pathways, as well as others such as TRAIL, may lead to a better understanding of the mechanisms by which BCL1 tumor cells employ to kill target cells.

Both BCL1 tumor cells and T_{reg} cells secrete IL-10, which is a potent inhibitory cytokine. Our studies showed that neutralizing IL-10 did

not overcome the suppression of T cells *in vitro*. However, the effects of IL-10 *in vivo* were not investigated. Attempts to neutralize IL-10 in the tumor site may be more effective than doing so *in vitro*. Therefore, administration of anti-IL-10 to mice that are immunized and/or challenged with BCL1 tumor cells may reduce the accumulation of T_{reg} cells and attenuate the expansion of BCL1 tumor cells.

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